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Exploring the Astrocytic Neuroprotective Functions in a Chronic Mild Stress Model of Depression

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Your vision of where and who you want to be is the greatest asset you have. Without having a goal is difficult to score.

- Paul Arden -

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ABSTRACT

Depression is a multidimensional psychiatric disorder that affects millions of people worldwide. In order to unveil the pathophysiology of this disorder, increasing significance is being given to the study of the abnormal neurotransmission in brain areas affected in depression. Particularly, glutamatergic excitotoxicity has been suggested as one of the possible process underlying the installation of the disease. Previous studies have suggested that glutamate release is increased in depressed subjects. Additionally, the β-lactamic antibiotic ceftriaxone (CEF) was described to increase glutamate uptake by the glutamate transporter GLT-1 (expressed mainly in astrocytes), preventing cellular damage.

Taking these findings into account, the main goal of this work was to understand whether CEF-triggered enhancement of glutamate uptake might be used to prevent or reverse the deleterious effects of glutamatergic excitotoxicity in an animal model of depression, the unpredictable chronic mild stress (uCMS). For this, animals were subdivided in two different main sets - Prevention and Treatment. Different groups of animals were administered either with CEF and/or two antidepressants (ADs; fluoxetine and imipramine).

CEF administration, at an early stage, prevented the installation of depressive-like behavior, yet it failed to reverse the installed depressive phenotype induced by uCMS exposure. CEF seems to reverse partially the cognitive deficits caused by uCMS exposure. Analysis of the collected brain tissue revealed that the prevention of the depressive-like behavior was correlated with an increased GLT-1 gene expression in the ventral hippocampus and with an increased expression of GLT-1 transporter in the dorsal dentate gyrus (DG) of the hippocampus. Morphological analyses disclosed neuronal atrophy of the DG granule neurons in the dorsal DG after uCMS exposure, which was prevented by CEF administration.

These results suggest that CEF administration promotes GLT-1 transporter up-regulation, which may prevent excitotoxicity processes at glutamatergic synapses in the hippocampus, thus preventing the installation of the depressive-like behaviors triggered by uCMS. These observations elucidate the potential use of CEF, or similar drugs, in the prevention of depressive behavior, paving the way for the development of new therapeutic strategies.

RESUMO

A depressão é uma doença psiquiátrica que afeta milhões de pessoas em todo o mundo. Na tentativa de compreender os mecanismos fisiopatológicos da doença, a comunidade científica tem-se dedicado ao estudo de defeitos na neurotransmissão em áreas cerebrais afetadas na doença. Em particular, a excitotoxicidade glutamatérgica tem sido sugerida como um processo subjacente à instalação da doença. Estudos anteriores verificaram que a libertação de glutamato está aumentada em indivíduos deprimidos. Neste contexto, foi também descrito que o antibiótico β-lactâmico ceftriaxona (CEF) aumenta a captação de glutamato pelo transportador GLT-1 (expresso principalmente em astrócitos), prevenindo os danos celulares.

Considerando estas observações, o objetivo deste trabalho foi compreender se o aumento da captação de glutamato desencadeado pela CEF poderá prevenir ou reverter os efeitos deletérios da excitotoxicidade glutamatérgica num modelo animal de depressão, de exposição crónica ao stress (uCMS). De acordo com o objectivo, os animais foram divididos em dois grupos principais – Prevenção e Tratamento. A diferentes subgrupos de animais foram administrados CEF e/ou dois antidepressivos (fluoxetina e imipramina).

A administração de CEF preveniu o desenvolvimento do comportamento depressivo, no entanto foi ineficaz na reversão deste fenótipo já instalado após exposição crónica a stress. Adicionalmente, a CEF parece reverter parcialmente os défices de cognição induzidos pelo uCMS. Análises do tecido cerebral dos animais revelaram uma correlação entre a prevenção do comportamento depressivo e a expressão do gene GLT-1 no hipocampo ventral e do transportador GLT-1 no girus denteado (GD) dorsal. A avaliação da morfologia dos neurónios granulares do GD revelou que a CEF foi capaz de prevenir a atrofia dendrítica no GD dorsal provocada pela exposição ao stress.

Estes resultados sugerem que a administração de CEF estimula a expressão de GLT-1, prevenindo os eventos excitotóxicos nas sinapses glutamatérgicas no hipocampo e o consequente desenvolvimento do comportamento depressivo causado pela exposição ao stress crónico. Estas observações indicam um potencial efeito da CEF ou de outros fármacos que promovam a expressão do GLT-1, na prevenção do desenvolvimento de comportamento depressivo, abrindo caminho para o desenvolvimento de novas possibilidades terapêuticas.

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ABBREVIATIONS LIST

%	Percentage
µ	Micrometer
AD	Alzheimer's disease
ADs	Antidepressants
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNL	Brain-derived neurotrophic factor
CA	Cornus ammonis
cAMP	Cyclic adenosine monophosphate
CEF	Cetriaxone
CMS	Chronic Mild Stress
cm	Centimeters
CNS	Central nervous system
CREB	Clean renewable energy bonds
CT	Control
CUS	Chronic Unpredictable Stress
DAB	3,3'-Diaminobenzidine
DG	Dentate gyrus
dlPFC	Dorsolateral Prefrontal Cortex
EAAC1	Excitatory amino acid carrier 1
EAAT	Excitatory amino acid transporter
EAE	Experimental autoimmune encephalomyelitis
EPM	Elevated Plus Maze
FLX	Fluoxetine
FST	Forced swimming test
GABA	Gama-aminobutyric acid
GFAP	Glial Fribilary Acidic Protein
GLAST	Glumate-aspartate Transport
Gln	Glutamine

GLT-1	Glutamate transporter 1
GS	Glutamine Synthetase
Glu	Glutamate
HPA	Hypothalamic-pituitary-adrene
IMIP	Imipramine
IR	Immunireactive
LB	Laemmi Buffer
MDD	Major depressive disorder
min	Minutes
MWM	Morris Water Maze
NDMA	Nmethy-D-aspartate
nm	Nanometers
NOR	Novel object recognition
NSC	Neutral Steam Cell
OB	Olfactory Bulb
OF	Open field
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
RMS	Rotral Migratory Stream
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Real time polymerase chain reaction
SAL	Saline
SDS	Dicarboxylate Symporter Family
SDT	Sweet Drive Test
SEM	Standard error of the mean
SEZ	Subependymal zone
SGZ	Subgranular zone
SPT	Sucrose preference test
SSRI	Selective serotonin reuptake inhibitors
TBS	Tris-buffered saline
uCMS	Unpredictable Chronic Mild Stress

WB	Western Blot

I. INTRODUCTION

1. INTRODUCTION

Major depressive disorder (MDD) is one of the world leading causes of morbidity, often associated to suicidal attempts. Despite of the importance of this disease in modern societies and the large investment of resources in its study, the processes underlying its pathophysiology remain poorly understood. The importance of glial cells, namely astrocytes in brain processes has been rising in the past years. Astrocytic actions may confer protection to vicinal neurons and may be used in the context of brain disorders as well. The use of astrocytic protective functions in the context of depression urges to be addressed.

1.1 DEPRESSION

1.1.1 State of the art

Depression is a highly prevalent mood disorder affecting more than 120 millions of people and is projected to be one of the major causes of worldwide burden by 2030 (Belmaker and Agam, 2008; WHO, 2008; Willner et al., 2013). Patients suffering from this disorder usually present loss of interest for experiencing pleasurable activities (anhedonia), changes in sleep and appetite, sadness, suicidal ideation and anergia. Furthermore, rather than low self-esteem, depressive patients present a deeply negative view of the world and the future, and display deficits of attention, interpretation and memory (Mathews and MacLeod, 2005). Evaluation reports on cognitive responses state a decreased control for processing negative information, which contributed to high levels of negative automatic thoughts and pathological rumination in these patients (Gotlib et al., 2008). Together with the observed inability to anticipate aversive events or rewards (Chase et al., 2010; McFarland and Klein, 2009; Pizzagalli et al., 2008), these facts provide a cognitive explanation for the core symptom of depression, namely anhedonia (Willner et al., 2013).

Depression and anxiety disorders are often comorbid with each other, since its symptoms are related. In fact, mood disorders such as depression and anxiety are frequently associated to patients suffering from other disorders such as chronic pain (Holley et al., 2013), inflammation (Slavich and Irwin, 2014), cardiovascular disorders (Van der Kooy et al., 2007), stroke

(Ramasubbu and Patten, 2003), Alzheimer's disease (AD) (Green et al., 2003), epilepsy (Hesdorffer et al., 2000), diabetes (Mezuk et al., 2008) and cancer (Rooney et al., 2013). Furthermore, the emotional disruption presented in depression happens in parallel to cognitive impairments, namely in memory processes, which can be the cause of the onset and recurrence of depressive episodes. For all these reasons, depression is further characterized as a multidisciplinary disorder that affects three different behavioral dimensions: mood, anxiety and cognition (Bessa et al., 2009a). Even though there is no knowledge of a real cause for the precipitation of MDD, vulnerability or predisposition to become depressed may occur in several ways and throughout different life stages. It is accepted that early life experiences, particularly inadequate familial relations, increase the risk for precipitating depression (Slavich and Irwin, 2014; Willner et al., 2013). Also, it was previously identified some genetic predisposition to inherit this disorder in a range of 31 to 42% (Kendler et al., 2002, 2006; Sullivan et al., 2000). It is believed that there are "stress-provoking" genes passing on through generations and providing a stressful family environment (Slavich and Irwin, 2014). On the majority of the population only the interaction between multiple risk genes and environmental factors (e.g. stress-related factors or parental negligence) are sufficient to cause depression, specially at early childhood (Kendler et al., 2001; Widom et al., 2007). Personality characteristics intervening in social interaction and autonomy may also contribute to the vulnerability of oneself to express depressive symptoms. Actually, two types of depression named as "endogenous" or "reactive" are described to characterize autonomous or social-dependent people, respectively. Endogenous depression is associated with an interpersonal distance, feelings of failure, anhedonia, hopelessness, and blame, which minimize possible environmental precipitants (Willner et al., 2013). Interestingly, people suffering from a reactive depression tend to be socially dependent, relying on the satisfaction for approval of others. On the other hand, endogenous depressed people present an interpersonal sensitivity, guilt, anxiety and rumination, with temporary mood improvements but a constant need for attention within an exacerbated depressive state (Willner et al., 2013).

1.1.2 Pathophysiology and animal models

Although the neurobiological causes of this disorder are yet to be fully understood, depressive subjects present cell atrophy and loss in the brain (neurons and glial cells) which can be reverted by antidepressant (AD) treatment (Banasr et al., 2011; Bessa et al., 2009b; Martin et al., 2013).

Pathological changes in size of specific brain regions and alterations in neuronal morphology, neurochemical and signaling molecules; plus alterations in gene expression and epigenetic regulation are factors known to be affected in depression (Mateus-Pinheiro et al., 2011; Tsankova et al., 2007). Contributing to the etiopathological knowledge of depression are studies employing animal models of this disorder. The validity of an animal model for formulation of hypotheses and for the development of novel therapeutic strategies encompasses: use known etiological factors (etiological validity) to mimic the behavioral and neurological symptoms observed in human disease (face validity) and importantly, responsiveness to clinically effective treatments (predicted validity) (Berton et al., 2012; Bessa et al., 2009a; Patrício et al., 2013). There are several animal models of depression described in the literature: chronic unpredictable stress (CUS), chronic mild stress (CMS), social stress, early life stress, learned helplessness, fear conditioning and olfactory bulbectomy (Duman, 2010; Nestler et al., 2002). Nonetheless, these models do not mimic and recapitulate completely the complexity and heterogeneity of the human disease. The unpredictable Chronic Mild Stress (uCMS) protocol, based in the principles of the CMS and CUS protocols was proven to be a robust approach to study the human depression at our lab. In this model, it was observed that after the exposure to stress animals presented depressive-like symptoms such as anhedonia, anxiety and cognitive deficits (Bessa et al., 2009a; Mateus-Pinheiro et al., 2013a), showing therefore alterations in the three behavioral domains known to be affected in humans with depression (Figure 1.1). Additionally, a variety of behavioral tests were designed not only for validation of the depressive-like phenotype in animal models of depression, but also to validate ADs efficacies (Bessa et al., 2009b). Regarding the emerging knowledge given by the use of animal models and from *post-mortem* studies of depressive patients, the neurobiological causes of depression remains not fully understood.

Currently, several theories have been proposed to explain the causes of depression at a neuronal and molecular level. One of the classical theories of depression is the monoamine hypothesis, which is based on altered neurotransmitter pathways related to serotonin and norepinephrine (Hirschfeld, 2000). Giving the low availability of these two neurotransmitters in a context of depression, classical treatment with antidepressants (ADs) was developed to increase the serotonin and norepinephrine levels. Within the clinics, different classes of ADs are used, namely the serotonine-selective reuptake inhibitors (SSRIs; e.g.fluoxetine), norepinephrine-selective reuptake inhibitors, the tricyclic agents (e.g. imipramine) and other atypical (e.g. tianeptine and agomelatine). Despite its effectiveness for reverting impairments caused by

depressive episodes, around 50% of patients do not present total remission after ADs treatment (Nestler et al., 2002). Besides, the conventional ADs present undesired side effects such as sedation, increase of weight and sexual dysfunction, which may promote a low commitment to treatment, resulting in a break-up and further recurrence of depression (Keller et al., 2002; Lang and Borgwardt, 2013). Therefore, the urging need to prevent the depressive episodes by focusing on different possible physiological mechanisms and the treatment with alternative strategies beyond the monoamine hypothesis might help to obtain more effective therapies regarding this problem.

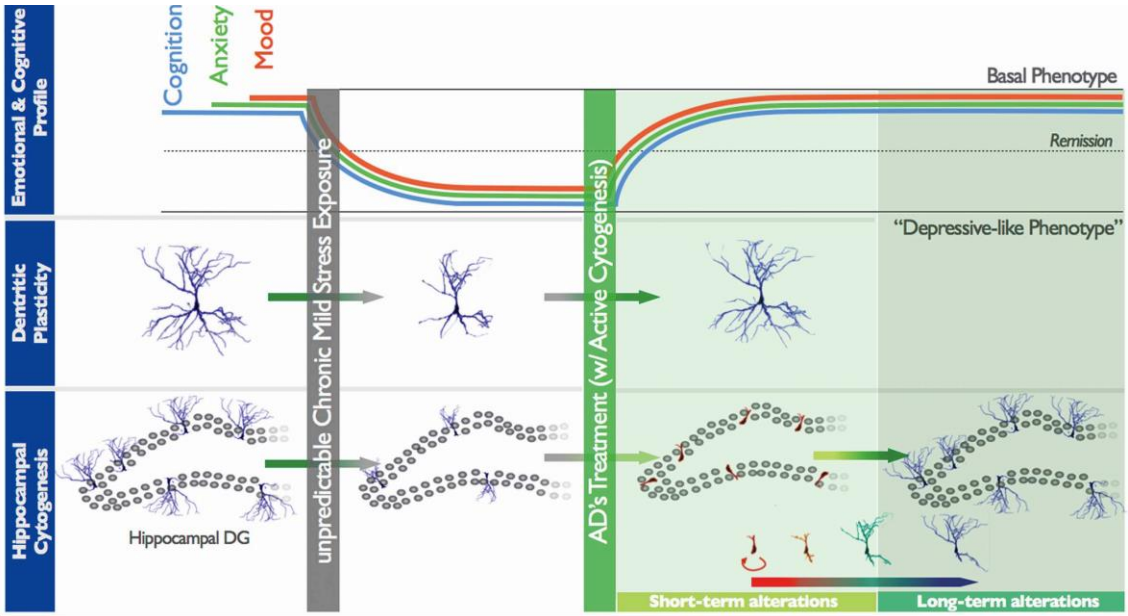

Figure 1.1 The effects of stress as a trigger factor of depression. In animal models of depression stress exposure leads to neuroplastic alterations such as neuronal dendritic atrophy of hippocampal and pre-frontal cortex and impaired cytogenesis. These alterations correlate to behavioral deficits in mood, anxiety and cognitive dimensions. Antidepressant's (AD's) treatment restore the deleterious effects produced by unpredictable Chronic Mild Stress in emotional and cognitive profile, and reestablish neuronal dendritic morphology and cytogenesis in the hippocampal dentate gyrus (DG) which is associated to remission from depressive-like behavior and sustained long-term recovery (adapted from (Mateus-Pinheiro et al., 2013b).

Other relevant hypotheses have been proposed to explain the etiology of depression: the hypothalamic-pituitary-adrenal (HPA) axis dysfunction hypothesis that is based on hyperactivity and/or disruption of the axis leading to increased levels of glucocorticoids (Pariante and Lightman, 2008); the neurogenic hypothesis that encompasses decreased neurogenesis in the

hippocampus (Jacobs et al., 2000); the cytokines theory, that postulates an altered cytokine profile associated to depression (Miller et al., 2009; Schiepers et al., 2005); and the neurotrophin hypothesis, which is based on growth and trophic factors deficiencies, such as brain-derived neurotrophic factor (BDNF) reduction seen in the context of depression, that might contribute to neuroplastic and neurogenic alterations that enhance individuals vulnerability to depression (Duman, 2009; Hayley and Anisman, 2013). Many of these changes were also verified in animal models of depression, such as the uCMS model (Farooq et al., 2012; Gumuslu et al., 2014; Mateus-Pinheiro et al., 2013a).

Other urging theory is the glial hypothesis of depression. According to this hypothesis there are a series of molecular dysfunctions associated to glial neuroprotective functions, such as decreased flux through the glutamate/glutamine shuttle and consequently reduced glutamate reuptake at the synapse (Sanacora and Banasr, 2013). Additionally it was described that there is an impairment of the NMDA-receptor function, a disturbed neuronal metabolism for disruption of energy supply and reduced GABA synthesis (Choudary et al., 2005; Duman and Li, 2012). Glutamatergic dysfunctions in a context of mood disorders have already been described (Jun et al., 2014; Plitman et al., 2014). Moreover, blockage of glial glutamate uptake was able to induce behavioral alterations consistent with symptoms of mood disorders (Bechtholt-Gompf et al., 2010; John et al., 2012; Lee et al., 2007).

Although many theories have been put forward, none of these are mutually exclusive and may probably contribute together to the vast spectrum of depressive disorders.

1.2 NEUROPLASTICITY IN DEPRESSION

1.2.1 Remodeling of neuronal circuits

Since the first studies of Ramón y Cajal the brain is known to consist of neural cells (neurons and glia) and a fixed system of neuronal circuits (Ramón y Cajal, 1928). However, contrarily to what was then believed, in the recent years it is accepted that neuronal circuits and connections are dynamic and suffer modifications and reorganizations throughout life. This process of reorganization named neuroplasticity produces the generation of new cells and dendritic morphology changes in response to external and internal stimuli. Environmental alterations act as signals upon neuronal systems, brain nuclei, synapses and receptors promoting structural and

functional neuronal adaptation (Zilles, 1992). Basically, this process is responsible for development of new synapses and consequent retrograde of pre-existing ones due to continuous modifications such as axonal growth and collateral sprouting that will also change dendritic arborization and spines density, and therefore the number of post-synaptic sites (Carvalho et al., 2010; Serafini, 2012). Previous studies state that promoting the specific and multifaceted changes at the synapse may be the mossy fibers and hippocampal pyramidal neurons (Popov and Bocharova, 1992); also, chronic stress induced by corticosterone induced Cornus Ammonis (CA)3 pyramidal neurons dendritic shrinkage (Magariños et al., 1996; Sousa et al., 2000; Woolley et al., 1990) and reorganization of dendritic arbors in the medial prefrontal-cortex (Wellman, 2001).

Generally, neuroplasticity is a process of resiliency, meaning that encompasses the ability to adapt and react to environmental alterations that can also hold stressful life events. Several brain areas that are related to cognition or emotional responses can be involved in this process: pre-frontal cortex, hippocampus and amygdala. In fact, reduced hippocampal volume is commonly found in depressive patients and can be correlated to prolongation of the depressive episode (Lorenzetti et al., 2009; Sheline et al., 2003). This reduction is often associated to neural cell loss or compromised cytogenesis processes that involve somatodendritic, axonal and synaptic changes (Serafini, 2012). In animal models, this abnormal reduced hippocampal size was already related to: loss of dendritic spines, decreased number of synapses, loss of glia and impairment of neurogenesis (D'Sa and Duman, 2002; Duman, 2010; Pittenger and Duman, 2008). Loss in dendritic complexity and synaptic sites, which determine a reduction in connectivity, as well as loss in glial cells that have neuroprotective roles, and consequent reduction of neurotransmission are all putative changes contributing to neurogenesis impairments (Licznerski and Duman, 2013; Serafini, 2012). Furthermore, chronic stress is described to promote morphological alteration in the prefrontal cortex (PFC) of rats, namely dendritic retraction, loss of spines and decreased number and size of glial cells (D'Sa and Duman, 2002; Fuchs et al., 2004; Marsden, 2013). *Post-mortem* studies have shown reduced neuronal density and a relevant reduction of PFC thickness (Cotter et al., 2002; Rajkowska et al., 1999). Therefore, understanding neuroplasticity changes at a structural and functional level may help assessing the mechanisms accompanying such changes.

Many mechanisms may contribute to atrophy and loss of neurons, specifically failure of signaling cascades and target genes that control cell survival. Brain-derived neurotrophic factor

(BDNF) is critical for the survival and function of neurons during development and in the adult brain. BDNF has a role in processes of learning and memory long-term potentiation that may be associated with plasticity of the brain. In absence of this neurotrophic factor neurons undergo a process of programmed cell death or apoptosis (Duman et al., 2000), but the survival of the cell may also be due to the synaptic connections with other cells (neuroplasticity) (Goldberg and Barres, 2000). Signaling pathways as cAMP-CREB cascade when upregulated can inhibit cell death pathways and promote BDNF gene expression, leading to cell survival (Duman et al., 2000).

1.2.2 Neurogenesis as a process of neuroplasticity

Also contributing to neuroplasticity is the process of generating new neurons, neurogenesis. Often, in a context of depression adult neurogenesis is extensively studied and comparisons between pre-existing neurons and newly generated neurons are made. This process encompasses mitotic division of neural progenitors to produce new neurons in the adult brain, involving several steps such as, the commitment of the new cell to a neuronal phenotype, migration and maturation of the cells, and establishment of appropriate contacts that culminate with a full integration on pre-existent network (Patrício et al., 2013). It has already been described that adult neurogenesis happens in different brain regions, mainly in two restricted germinal zones: the subependymal zone (SEZ) of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Zhao et al., 2008). In both regions, astrocytes act as primary precursors of the newly generated neurons (Seri et al., 2001). The neuronal progenitor cell population arising from astroglial cells composes the narrow layer of three nuclei wide, the SGZ, where neurogenesis occurs. This multipotent cell population is constituted by the neural stem cells (NSCs), which express nestin and glial fribilary acidic protein (GFAP; a marker also presented in mature astrocytes). NSCs can be divided in two cell subtypes according to their orientation in the SGZ: radial astrocytes/NSCs and horizontal astrocytes/NSCs. In adult mammalian brain, neurons born in the SGZ migrate into the granular cell layer of the DG and differentiate into glutamatergic granule cells (Zhao et al., 2008). Otherwise, neuroblasts born in the SEZ migrate along the rostral migratory stream (RMS) becoming mostly mature GABAergic granule and periglomerular interneurons in the olfactory bulb (OB). Despite of the controversy around this topic, some reports describe generation of new neurons in other brain regions such

as cortex, amygdala, hypothalamus, striatum and substancia nigra (Bédard et al., 2006; Ehninger and Kempermann, 2003; Gonçalves et al., 2008; Kodama et al., 2004; Kokoeva et al., 2005; Yoshimi et al., 2005). Furthermore, this process of hippocampal neurogenesis has already been correlated to emotional and memory processes (Burghardt et al., 2012; Denny et al., 2012; Malberg et al., 2000; Mateus-Pinheiro et al., 2013a).

1.3 GLIAL CELLS

Glial cells are the most abundant cells within the central nervous system (CNS). There are four types of glia cells in the brain: astrocytes, oligodendrocytes, microglia and NG2-positive cells. Oligodendrocytes are responsible for production of myelin along the axons, helping in the conduction of electrical signals, whereas microglia are smaller cells with phagocytic functions. NG2-positive cells are known as oligodendrocytes progenitor cells but can also give rise to astrocytes. Astrocytes are the most abundant glial cells in the brain and play important roles in metabolic support of neurons, in the control the blood flow and modulation of the blood brain barrier by supplying energy and nutrients to neurons and maintaining homeostasis.

1.3.1 Astrocytes

These cells are classically defined by their star-shape morphology and expression of glial fibrils and for their extended numerous processes surrounding neighboring neurons and blood vessels (Wang and Bordey, 2008b). As they establish connections to the capillaries of the blood brain barrier (BBB) they may influence the microenvironment in this region (Fuchs et al., 2004; Wang and Bordey, 2008b). Astrocytes also present neurotransmitters' receptors that may lead to electrical and biochemical events inside the cell (Fuchs et al., 2004). Astrocytes express several membrane receptors, namely G-protein coupled receptors, ionotropic receptors and other receptors for growth factors, chemokines, steroids, and receptors involved in innate immunity such as Toll-like receptors (Abbracchio and Verderio, 2006; Franke and Illes, 2014; Heiman et al.; Wang and Bordey, 2008a). Moreover, these cells present a family of high affinity sodium-dependent glutamate transporters, which are responsible for the uptake of glutamate (Figure 1.3 a). Physiologically, there are three glutamate transporters in the rodent forebrain that provide glutamate clearance of the synaptic cleft during synaptic transmission: GLT-1 (a homolog of

EAAT2 in humans), glutamate-aspartate transporter (GLAST, homolog of EAAT1 in humans) that are expressed mainly in astrocytes (Rothstein et al., 1996; Wang and Bordey, 2008b); whereas, the excitatory amino acid carrier 1 (EAAC1, homolog of EAAT3) is expressed in neurons (Zink et al., 2010). Also part of these transporters family are the EAAT4 expressed in Purkinje cells (Huang et al., 2004) and EAAT5 which is widely expressed in peripheral tissues (Lee et al., 2013a). Interestingly, both variant 4 and 5 seem to act more like inhibitory glutamate receptors than glutamate transporters, mainly due to their high uncoupled anion conductance (Zhou and Danbolt, 2013). Nevertheless, together these transporters contribute to the balance of neurotransmitters at the synaptic cleft, preserving the responsiveness of glutamate receptors, and others such as Nmethyl-D-aspartate (NMDA) receptors and ATP receptors, and conferring neuroprotection against excitotoxicity (Lipski et al., 2007; Tzingounis and Wadiche, 2007).

GLT-1 and GLAST are highly efficient glutamate transporters and its inhibition with pharmacological blockers, antisense oligonucleotides or by transgenic knockout lead to increased levels of glutamate and consequent cell death (Izumi et al., 2002; Rothstein et al., 1996; Tanaka et al., 1997). Moreover, the use of pharmacological blockers cause Nmethyl-D-aspartate (NMDA) receptor-dependent cell death and enhances excitotoxicity induced by exogenous glutamate (Selkirk et al., 2005; Wroge et al., 2012), suggesting an interplay between this receptor and the astrocytic glutamate transporters.

1.3.2 Neuron-astrocyte interactions

The classical paradigm that brain information processing is exclusively the result of neuronal activity has been challenged by an emerging body of evidence (Araque et al., 2014; Kettenmann and Verkhratsky, 2008; Perea et al., 2009; Wang and Bordey, 2008a). Indeed, recent findings rather strongly support the concept of a “tripartite synapse” (Figure 1.2), in which a cross-talk between astrocytes and neurons complements and modulates the communication between pre- and post-synaptic structures (Araque et al., 2014). Upon an elevation of synaptically released neurotransmitters, astrocytes can increase the intracellular calcium ([Ca2+]i) resulting in the release of glutamate via regulated exocytosis (Rossi and 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 (Charles et al., 1991; Cornell-Bell et al., 1990; Sofroniew and Vinters, 2010). Indeed, one of main functions of astrocytes is the regulation of the

glutamate concentration in the synaptic cleft of glutamatergic neurons. Important for this astrocytic function is the glutamate-glutamine shuttle, which includes not only the uptake of extrasynaptic glutamate but also the production and release of glutamine from the astrocytes via glutamine synthetase (GS), which will be used by neuronal elements to refill glutamate supply (Chiang et al., 2007). In summary, the astrocytic features that favor this concept are: (i) the expression of functional neurotransmitter receptors (eg. for glutamate, ATP, GABA), which sense surrounding neuronal activity; (ii) the ability to process intracellular calcium signaling which may propagate to vicinal astrocytes; and (iii) the ability to release neuro- and vasoactive substances such as glutamate, d-serine, ATP, GABA, TNFα, prostaglandins, or peptides, which influence and regulate synaptic transmission, blood flow, the permeability of the blood brain barrier and metabolic support (Oliveira et al., 2011; Perea et al., 2009; Wang and

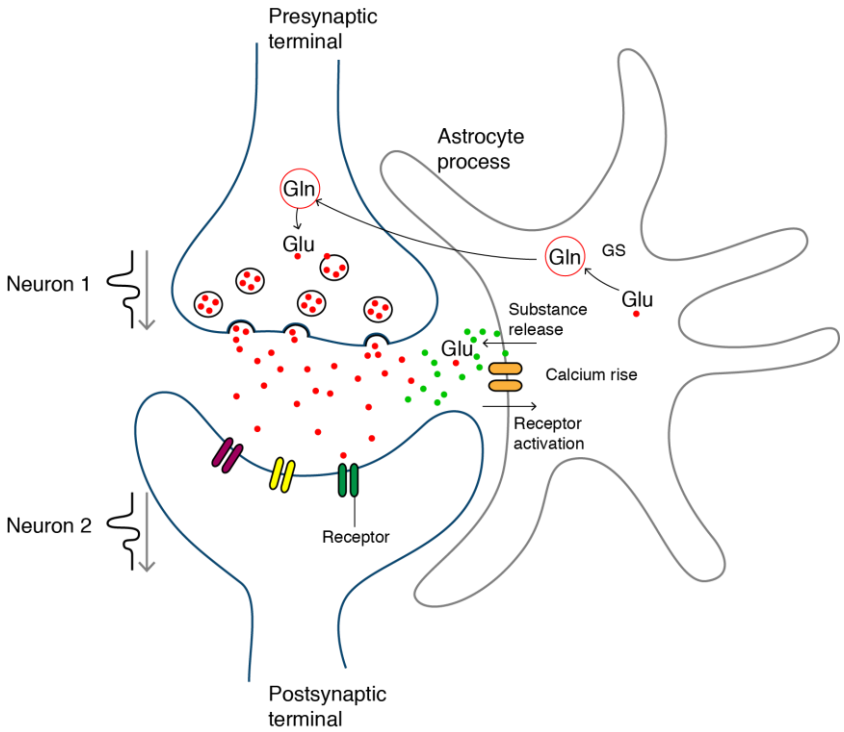

Figure 1.2 The tripartite synapse. As neurons, astrocytes express many receptors and transporters that are activated upon neurotransmitters release from the presynaptic terminal. This activation increases calcium ions inside the astrocyte and parallel release of substances such as ATP. This gliotransmission will counter-act on neurons to either inhibit or enhance neuronal activity. Astrocytes also play an important role in modulation of presynaptic functions and postsynaptic responses to neurotransmitters. One of the main functions of astrocytes is to provide glutamate clearance from the synaptic cleft, creating a neuron-astrocyte interaction through a glutamate-glutamine shuttle. Glutamate, Glu; glutamine, Gln; glutamine synthetase, GS; red dots, neurotransmitter; green dots, gliotransmitter.

Bordey, 2008a). Alterations of cellular cross-talk conceptualized as the tripartite synapse (Perea et al., 2009) have been extensively demonstrated both in brain slice preparations (Henneberger et al., 2010; Martineau et al., 2013; Perea and Araque, 2007; Woo et al., 2012; Yang et al., 2003) and *in vivo* (Chen et al., 2013; Halassa et al., 2009; Han et al., 2012; Navarrete et al., 2013; Takata et al., 2011) in rodents and, more recently, in humans (Navarrete et al., 2013). These features provide astrocytes with the mechanisms to modulate neuronal function (Araque et al., 2014), thereby influencing network regulation and computation of behavior responses. Despite of the extensive demonstration of cellular and molecular cross-talk pointed out, little is known about the impact of neuron-astrocyte interactions on the production of network outputs.

Back in 2010, our team decided to study this phenomenon and dissect a putative role of astrocytes in network computation. Using an L-α-aminoadipate animal model in which astrocytes were affected specifically in the PFC, a severe impairment in cognitive tasks that depend on this brain region was induced in those animals (Lima et al., 2014). The PFC is intimately related to the computation of complex cognitive functions such as information integration, learning, memory processing and behavior flexibility (Clark et al., 2004; Goldman-Rakic, 1995), known to be affected in the model of depression used in this study. Additionally, cognitive function was studied in the dnSNARE mice model of impaired gliotransmission (impaired exocytosis specifically in astrocytes). It was gathered data indicating a need for gliotransmitter release from astrocytes for the PFC to produce a correct cognitive processing (data under review). This observation is in agreement with previous demonstrations using the same dnSNARE model, in which absence of gliotransmission was responsible for the exacerbation of cognitive deficits associated with sleep loss (Fellin et al., 2009; Halassa et al., 2009). On one hand, these observations, together with data obtained from different animal models of astrocytic dysfunction (Han et al., 2012; Pannasch et al., 2014), suggests that neuron-astrocyte communication is critical for correct production of cognitive outputs. On the other hand, the engraftment of rodent forebrain brain with human glial progenitors that differentiate to human-like astrocytes (described as being about 20x larger and having more complex morphology), enhanced the cognitive abilities of these animals (Han et al., 2013).

1.3.3 Roles of astrocytes in brain disorders

Moreover, an increasing significance has been attributed to glutamatergic system in the pathophysiology of several mood disorders such as schizophrenia, bipolar disorder and depression, and also neurodegenerative disorders as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) (Kruminis-Kaszkiel et al., 2014; Verkhratsky et al., 2010; Webster et al., 2005). In fact, it has been reported that a dysfunction or reduced number of glial cells in patients suffering from major depressive disorder (MDD) can result in an increase of levels of glutamate in blood and cerebral spinal fluid (Hashimoto, 2011), causing a toxic accumulation of glutamate. An overabundance of glutamate accompanied by the failure of astrocytes to remove it, may lead to neuronal excitotoxicity resulting in neuronal loss, as seen for motor neurons in ALS (Potokar et al., 2013). In the context of AD it is known to occur a reactive astrogliosis promoting the neurodegenerative processes observed. Among the histopathological features is the presence of senile plaques and neurofibrillary tangles, peripheral loss of cholinergic and cholinoceptive neurons (in PFC and hippocampus) and the presence of activated macrophages and reactive astrocytes (Markiewicz and Lukomska, 2006). The senile plaques are deposits of β-amyloid protein and in turn, neurofibrillary tangles are intraneuronal stuctures composed of tau protein. β-amyloid can be a potent neurotoxic agent that promotes activation of astrocytes and cellular mechanisms exacerbation leading to neuronal damage. Furthermore, it is known that inflammation contributes to neuropathology associated to AD. Indeed, upon activation, astrocytes and microglia can release inflammation-promoting mediators potentially neurotoxic, since these glial cells are the brain representatives of the immune system. Although it is considered as a beneficial role in defense and repair, an escalating pathological glial activation may contribute to secondary nerve-cell damage (Markiewicz and Lukomska, 2006). In response to the extracellular β-amyloid deposits, microglia cells can produce TNF-α and other cytokines, and consequently promote the secretion of reactive oxygen species, further enhancing neuronal damage. Microglia cells by releasing pro-inflamatory citokines and reactive oxygen species can activate surrounding astrocytes which will internalize the debris that are being released by dying neurons being important for plaque degradation. In addition, astrocytes activated by β-amyloid will also release pro-inflamatory cytokines, oxygen reactive species and other inflammation-involved molecules that will attract microglia and further contribute to neuronal damage caused by and exarcebation of released pro-inflamatory cytokines (Markiewicz and Lukomska, 2006).

Astrocytes are also involved in many chronic neurological disorders, such as epilepsy, since these cells undergo morphological and functional remodeling in the epileptic brain. Proliferation of reactive astrocytes (astrogliosis) is a common feature of temporal lobe epilepsy, which is one of the most prevalent forms of localization-related epilepsies in humans (Coulter and Eid, 2012). Besides observed neuronal loss, astrogliosis in this pathology is correlated with an increased number of seizures and slower clearance of extracellular glutamate in the hippocampal formation (Cavus et al., 2005). Reactive astrocytes are widely correlated to the epileptogenesis via their effects on glutamatergic regulation, role in buffering potassium and interstitial volume control (Benarroch, 2009; Wetherington et al., 2008). The formation of the epileptic foci in the hippocampus has been associated to reactive astrocytes (Ortinski et al., 2010) and abnormal neuronal excitability in adult model systems (Gómez-Gonzalo et al., 2010). Since there are clues to an astrocytic dysfunction and a role of these cells in post-natal synaptogenesis has been described; it has been hypothesized whether this abnormal astrocyte development could be the cause of predisposition to epileptogenesis of the developing brain adjacent to alterations in the excitatory-inhibitory balance (Molofsky et al., 2012).

1.3.4 Gliopathology and depression

Of the main cells in the CNS, astrocytes are highly implicated in glial pathology in MDD. In several studies of *post-mortem* brain tissue of depressive subjects was seen a decrease in packing density and number of Nissl-stained population glial cells (Rajkowska and Stockmeier, 2013). This glia pathology was observed in several brain regions including dorsolateral prefrontal cortex (dlPFC) (Cotter et al., 2002; Rajkowska et al., 1999), ornitofrontral cortex (Rajkowska et al., 1999), subgenual cortex (Ongür et al., 1998), anterior cingulate cortex (Cotter et al., 2001a; Gittins and Harrison, 2011) and amygdala (Bowley et al., 2002). However, in elderly patients of MDD the alterations in glial density in the orbitofrontal and anterior cingulate cortex were not observed (Khundakar et al., 2011a, 2011b). Specifically, dlPFC is involved in executive function and emotional regulation (Davidson et al., 2000; Milham et al., 2001); its neuropathological abnormalities in glial cells and in GABAergic interneurons and pyramidal neurons are believed to contribute to the pathophysiology of MDD (Oh et al., 2012; Rajkowska and Miguel-Hidalgo, 2007; Sanacora and Saricicek, 2007). Moreover, in the dlPFC of depressive patients was observed atrophy and reduction of neuronal and glial density (Rajkowska et al., 1999), decreased GFAP

expression (Miguel-Hidalgo et al., 2000; Si et al., 2004) and decreased density and size of calbindin-immunoreactive (IR) GABAergic neurons (Rajkowska et al., 2007). Oh *et al*. (2012) have recently shown that glutamate changes negatively correlates to GFAP expression levels and to calretinin-IR GABAergic neuronal density in the PFC of MDD patients; calretinin-IR GABAergic neuronal alterations were positively correlated to changes in glial cells and pyramidal neurons markers in the dlPFC. These findings provided some insight on mechanistic basis for neuronal and glial excitotoxic damage promoted by glutamatergic transmission, in the dlPFC of MDD patients (Oh et al., 2012).

Within the basis of cytokines hypothesis of depression an interpretation of astrocytic hypertrophy as a reflection of local inflammation was made (Maes et al., 2009). Detailed analysis of Golgi stained astrocytes revealed cell bodies and processes hypertrophy in the white matter of the anterior cingulate cortex of depressed subjects dying by suicide (Torres-Platas et al., 2011). Recently, it has been shown in *post-mortem* studies that the pathology in the white matter of prefrontal brain is promoted by decreased oligodendrocyte density, reduction in the expression of genes related to cell functions as well as molecular changes in intercellular cell adhesion molecule expression levels and a possible mechanism of ischemia (Tham et al., 2011).

1.3.5 Astrocytic mechanisms in depression

There is an increasing evidence of astrocytic dysfunction in the context of depression (Choudary et al., 2005; Cotter et al., 2001b; Gosselin et al., 2009; Oh et al., 2012; Sanacora and Banasr, 2013). Contrarily to neurodegenerative disorders in MDD there is no prominent neuronal pathology, no astrogliosis processes and the expression of GFAP and other astrocytic markers is decreased (Rajkowska and Stockmeier, 2013). This astrocyte dysfunction and the neuronal impairments have been thought as two related consequences of tripartite synapse disturbance. In addition, in several *post-mortem* studies of depressive-brain no reductions in neuronal density or in total number were found (Cobb et al., 2013; Van Otterloo et al., 2009; Stockmeier et al., 2004). Instead, it has been reported a decrease in cell bodies size or reductions in dendritic branching (Chana et al., 2003; Hercher et al., 2010; Stockmeier et al., 2004). Together these findings suggest that in MDD is present a neuronal atrophy rather than neuronal loss. Furthermore, measure of marked enolase neurons in the serum levels displayed no changes in patients with MDD (Schroeter et al., 2008, 2010). Contrastingly, in the same study

it was observed an increase of serum levels of the astrocytic marker S100β. Also, other studies showed that mRNA for glial markers, such as glutamate transporters and glutamine synthethase, was significantly reduced in the locus coeruleus of depressive patients; the expression of mRNA for neuronal markers tested were not significantly changed for the same brain region in these patients (Bernard et al., 2011). Interestingly, glial toxic ablation rather than neuronal ablation lead to depressive-like behaviors (Banasr and Duman, 2008; Lee et al., 2013b). Regarding these previous studies, it appears that there is a selective cellular pathology for glial cells in the context of depression (Rajkowska and Stockmeier, 2013). Notably, the promotion of each type of cellular pathology seen in depression appears to be age-related. In fact, the glial cellular pathology appears to be related to younger depressive subjects (less than 60 years of age); contrasting with neuronal pathology that appears to relate to older subjects (more than 60 years of age) (Khundakar and Thomas, 2009; Miguel-Hidalgo et al., 2000; Si et al., 2004). In the older subjects, the neuronal pathology of depression seems to be cause of prominent reductions in the density of pyramidal glutamatergic neurons in the orbitofrontalcortex (Khundakar and Thomas, 2009; Rajkowska et al., 2005). In addition, unaltered astrocytes density and GFAP levels were seen in the elderly patients of depression (Davis et al., 2002; Miguel-Hidalgo et al., 2000; Si et al., 2004). Contributing to this neuronal pathology may be the excitotoxicity promoted by an excess of glutamate at the synapse cleft of glutamatergic neurons due to reduced number of astrocytes and astrocytic glutamate transporters (Rajkowska and Miguel-Hidalgo, 2007; Rajkowska and Stockmeier, 2013).

These thrilling findings, suggest that astrocytic modulation of neuronal activity affects the network activity and consequent output production, both on healthy and pathological processes. Importantly, astrocytes regulate synaptic maturation, transmission and maintenance providing a correct development of synapses (Slezak and Pfrieger, 2003). However, the dynamic mechanisms underlying this cross-talk between neurons and glia and their implications on cellular plasticity in depression are still underexplored.

1.4 GLUTAMATE TRANSPORTER 1: GLT-1

Solute carrier family 1, member 2 (Slc1a2; also known as glial high affinity glutamate transporter, GLT-1) is a glial glutamate transporter predominantly expressed in the rat

hippocampus; is mainly present in astrocytes plasma membrane and together with GLAST play a role in glutamate clearance during synaptic transmission (Rothstein et al., 1996; Wang and Bordey, 2008b) (Figure 1.3 a).

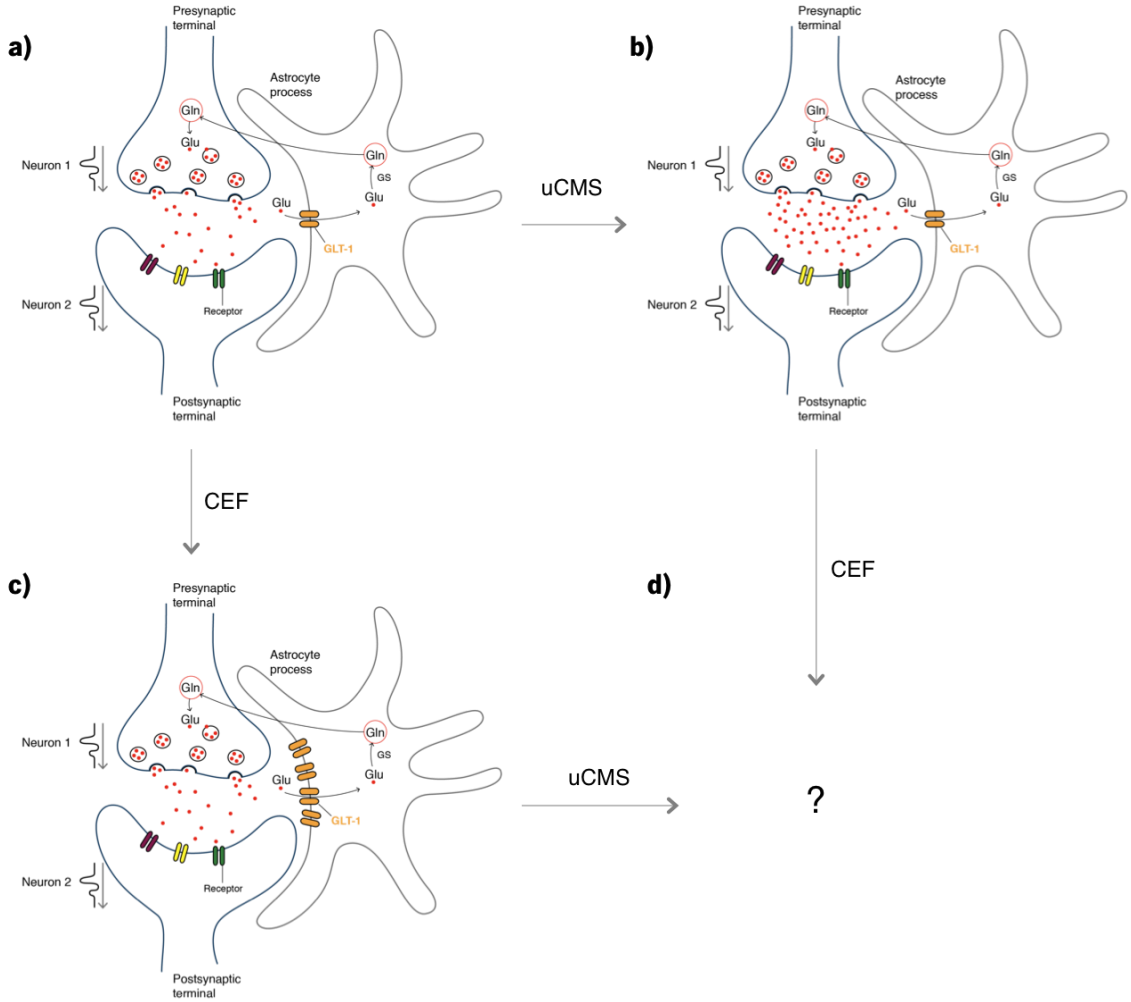

Figure1.3 Impact of stress exposure and ceftriaxone effect on glutamatergic transmission. Astrocytes uptake glutamate at the synaptic cleft of glutamatergic neurons through glutamate-glutamine shuttle, which includes not only the uptake of extrasynaptic glutamate (Glu) but also the production and release of glutamine (Gln) from the astrocytes via glutamine synthetase (GS) **(a)**. After stress exposure, astrocytic transporters such as GLT-1 help the synapse to cope with the increase of glutamate release, diminishing excitotoxicity. The chronic exposure to stressors (uCMS) may overcome the capacities of the existing GLT-1 and cause excitotoxic effects at the synapse **(b)**. Ceftriaxone (CEF) administration increases the expression of GLT-1 glutamate transporter in the astrocyte **(c)**. Further elucidation of CEF beneficial potential to prevent or revert the installation of the deleterious effects of uCMS exposure **(d)** will be te main goal of this study.

1.4.1 Structure and function

GLT-1 gene is located at locus 3q31 and encodes three transcript variants (1, 2 and 3) that differ both in 3' coding region and 5' terminal exon. These transcript variants encode three distinct isoforms (-a, -b and -c). Transcript variants 2 and 3 encode isoforms with distinct –C and –N terminus, respectively, compared to isoform –a. Transcript variant 1 encodes the longest isoform, isoform –a, which results in a protein with 573aa. The other two variants result in smaller proteins: isoform –b protein has 562aa and isoform –c has 570aa. These are glycosylated proteins that have a palmitoylation in the Cys-38, which seems to be important for its function in glutamate uptake. Interestingly, only isoforms –a and –b present a conservative domain Sodium-dicarboxylate symporter family (SDS) and are known to be transmembranar proteins (NCBI, 2014). It is also known that oligomerization is common feature of glutamate transporters and isoform –a and –b can interact and form hetero-oligomers in heterologous expression systems, in primary cultures from fetal rat and in the adult rat brain (González-González et al., 2009). However, this arrangement does not seem to interfere with the protein function and consecutively with glutamate transport, since its subunits present all the elements necessary for the translocation of glutamate (Grewer et al., 2005; Koch and Larsson, 2005). Furthermore, different expression was observed between post-natal and young-adult rats. With eight weeks of age, it was observed that GLT-1 isoform –a, represented 90% of total hippocampal GLT-1, but –b and –c represented only 1% each; however, at post-natal day 14, GLT-1 isoforms –b and –c were 1.7 and 2.5 times higher in relation to total GLT-1, respectively (Holmseth et al., 2009).

Although GLT-1 transporter was primarily detected in astrocytes, studies have also reported its presence also in neurons. The splice variant isoforms –a (GLT-1a) and –b (GLT-1b) are described to be widely expressed in astrocytes throughout different brain regions (Berger et al., 2005). Moreover, this splicing variation does not seem to alter transport characteristics of the isoforms. However, there has been descriptions that GLT-1a isoform is not exclusive for astrocytes and appears in CA3 neurons of hippocampus and in the olfactory nucleus of rat's brain (Berger et al., 2005; Chen et al., 2004). Nonetheless, previous studies have observed a noticeable variation in labeling intensity of GLT-1a mRNA expression in astrocytes of pyramidal and molecular CA1 layers of the hippocampus and other brain areas, but homogeneous expression of GLT-1b isoform in astrocytes throughout several brain areas (Berger et al., 2005). Immunoreactives of

the neocortical brain extracts have shown higher GLT-1a distribution in the hippocampus, compared to –b and –c isorforms, and similar distribution of the three variants in the cerebral cortex (Holmseth et al., 2009). Additionally, assessment of intracellular localization of GLT-1a and GLT-1b expression on the astrocyte was verified that GLT-1a mRNA was expressed primarily in astrocyte processes, whereas GLT-1b mRNA was more restricted to the astrocyte cell body (Berger et al., 2005; Holmseth et al., 2009). Together these findings suggest that there are different mechanisms regarding the activity and functional regulation of the GLT-1 isoforms.

1.4.2 GLT-1 regulation in mood disorders

It is known that this transporter has a reduced expression in the pathophysiological context of depression (Lee et al., 2007; Rajkowska and Stockmeier, 2013; Sanacora and Banasr, 2013; Zink et al., 2010). The glial hypothesis of depression admits a glutamine/glutamate shuttle malfunction, with reduced reuptake of glutamate, causing excitotoxicity at the synaptic cleft (Figure 1.3 b). It is believed that since astrocytes were shown to be affected in the context of brain disorders in humans and animal models of chronic stress, loss of GFAP seems to lead to a disturbance in the transfer of GLT-1 from intercellular space to cell surface (Hughes et al., 2004) and that stress exposure possibly triggers a reduction of GLT-1 in the periaqueductal gray matter (Imbe et al., 2012). Compromising the function of GLT-1 will putatively lead to neuronal death, suggesting that an increase in glutamate uptake may correct deficits caused by GLT-1 malfunction in depressive subjects. In fact, learned helplessness animal model of depression showed suppressed expression of GLT-1 in the hippocampus and cerebral cortex (Zink et al., 2010).

For these reasons, it is hypothesized that by targeting glutamate transporter-activity we may increase neuroprotection and therefore diminish excitotoxicity (Figure1.3 c).

1.5 GLT-1 REGULATION BY B-LACTAM ANTIBIOTICS - CEFTRIAXONE

β-lactam antibiotics are widely used within the clinics and present a variable spectrum of antimicrobial activity (Asbel and Levison, 2000). β-lactams include: penicillins, cephalosporins, cephamycins and carbapenems. Since there are patients allergic to penincillin and the bacterial resistance may be a problem to validity and efficiency of these drugs, other antibiotics have

continuously been designed to treat aerobic gram-negative infections. Non-penicillin β-lactams like cephalosporins and cephamycins have a wider spectrum of antimicrobial activity, higher resistance to beta-lactamase enzymes and can be used against enterobacteriecae family. Ceftriaxone (CEF), a type of cephalosporin, can be use to treat: sepsis, meningitis, Lyme borreliosis, abdominal infections such as peritonitis and gastrointestinal infections, respiratory tract infections such as pneumonia, bone infections and genital infections. Moreover, this antibiotic has already been described to cross the blood brain barrier (BBB) (Barichello et al., 2014; Nau et al., 2010). Rothstein *et al.* (2005), previously demonstrated that this β-lactam antibiotic could be involved in neuroprotection mechanisms by inducing overexpression of the GLT-1 transporter preventing neuronal loss induced by malfunction of GLT-1 transport (Figure 1.3 c). Also in this study, an ability of this antibiotic to prevent motorneuron loss, as well as a rescue of the loss of muscular strength and extended survival in a mice model of amyotrophic lateral sclerosis (ALS) was shown (Rothstein et al., 2005). Recently, immunohistochemistry findings have shown an increase of GLT-1 protein expression in CA1, CA3 and DG regions of the hippocampus after treatment with CEF, specially at a dosage of 200 mg/Kg (Karaman et al., 2013). Following these reports, curiosity for the effects of CEF in neuroprotection has been emerging. These effects may be of importance in the context of various disorders involving neuronal degeneration, astrocytic dysfunction and inflammation mechanisms. In fact, it has already been shown to be a promising target to treat chronic pain; a daily intrathecal treatment of rats with CEF upregulates GLT-1 expression in lumbar spinal cord and attenuates opiod-induced pain and prevented associated astrocyte activation; it could also revert established neuropathic pain and prevent the progression of paralysis in a rat model of multiple sclerosis (experimental autoimmune encephalomyelitis; EAE) (Ramos et al., 2010). Moreover, in a mice model of spinal muscular atrophy CEF was able to ameliorate neuromuscular phenotype by protecting neuromuscular units and increased survival (Nizzardo et al., 2011). Again, this effect is due to several mechanisms including the overexpression of GLT-1 transcripts and protein levels. Similar effects have been reported in animal models of Parkinson's disease, ischemia and in axotomy conditions (Chotibut et al., 2014; Inui et al., 2013; Soni et al., 2014; Yamada and Jinno, 2011). Remarkably, a study conducted in naïve mice has shown antidepressant-like effects of CEF in different behavioral domains (tail suspension test, forced swim test, and novelty-suppressed feeding test) (Mineur et al., 2007). These data suggests that enhancing neuroprotection by increasing the glutamate reuptake, may interfere with depressive-like behaviors.

Furthermore, studies carried out in primary human fetal astrocytes highlighted that the increased EAAT2 (GLT-1 homolog in humans) transcription levels by CEF is promoted by NF-κ B binding site at – 272 position (Lee et al., 2008). The authors further explain that CEF induces NF-κ B activation through degradation of Iκ Bα and induction of p65 nuclear translocation with further upregulation of its' downstream target EAAT2. Increase of glutamate uptake by overexpression of EAAT2 across the plasma membrane of astrocytes results as an effect of CEF.

In the future, it would be interesting to dissect the beneficial potential of CEF administration on depression would be to assess its neuroprotective effect in preventing or reverse the depressive symptoms. This may result in a novel approach and therapeutic target to treat depression.

1.6 RESEARCH OBJECTIVES

The mail goal of this work was to study the use of astrocytic neuroprotective functions in the prevention and reversion of the negative effects of chronic stress in a rat model of depression (Figure 1.3 d). Taking in consideration the effects of CEF, this goal was sub-divided in two objectives:

Objective 1,

To explore if treatment with CEF can increase the protective effect of astrocytes and therefore prevent the deficits induced by exposure to unpredictable chronic mild stress (uCMS; model of depression in rats);

Objective 2,

To explore if the protective feature of astrocytes conferred by CEF administration can revert the established negative effects of uCMS exposure similarly to ADs treatment.

These objectives were accomplished by evaluating the behavioral, cellular and molecular alterations of rats exposed to uCMS pre- or post-treated with CEF (alone or combined with ADs), and ADs-treated rats, comparing with the respective controls (non-treated with CEF).

II. MATERIAL AND METHODS

2. MATERIALS AND METHODS

In order to address the proposed objectives two set of animals were designed. One set of subjects was established to assess the effect of ceftriaxone (CEF) in the prevention of the effects of chronic stress. This set was named "Prevention". The second set of subjects was established to evaluate the potential of CEF to revert the deleterious effects of chronic stress exposure. This set was named "Treatment". Subjects from both sets were evaluated in order to measure behavioral, morphological and molecular alterations after exposure to chronic stress, treatment with CEF or antidepressants (ADs; alone or in combination), comparing with the respective controls (non-treated with CEF). Details on the subjects and treatments performed as given below.

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*.

Ninety-four animals were randomly assigned to eleven experimental groups (n=7-10) as described in Figure 2.1. The Prevention set comprised 4 experimental groups: two control groups and two chronically stressed, treated either with saline or CEF (Figure 2.1, a). The Treatment set comprised 7 experimental groups: one control group and six chronically stressed groups treated with saline, CEF and/or ADs – fluoxetine or imipramine (Figure 2.1, b). Details on the treatments carried out are given below.

For the sake of simplicity, animals from the Prevention set, treated in the first 3 weeks of the uCMS protocol will be, from now on, referred to as "**Prevention Groups**" (CT+SAL, CT+CEF, uCMS+SAL, uCMS+CEF; Figure 2.1, a). Groups belonging to the Treatment set, treated in the last two weeks of the uCMS protocol will be referred to as "**Treatment Groups**" (CT+SAL, uCMS+SAL, uCMS+CEF, uCMS+FLX, uCMS+IMIP, uCMS+CEF+FLX, uCMS+CEF+IMIP; Figure 2.1, b).

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

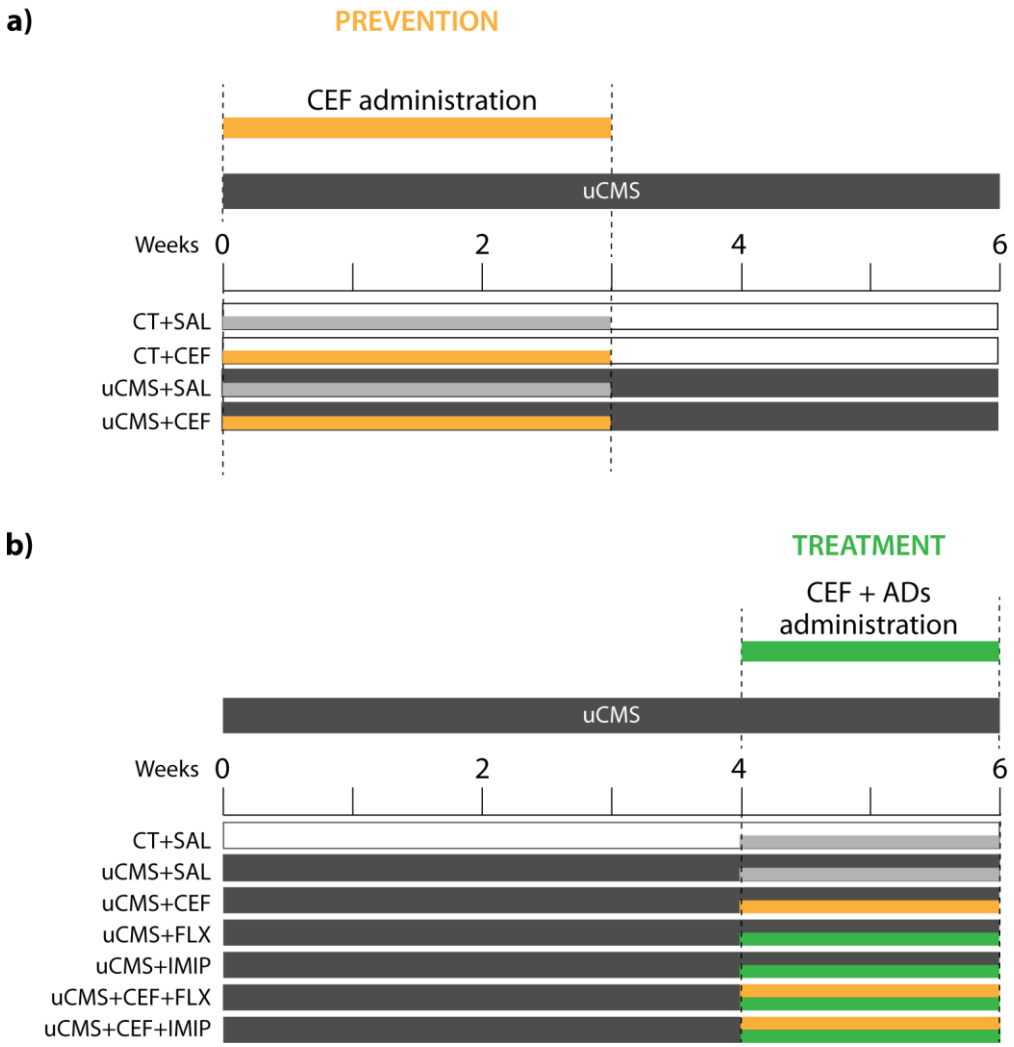

Figure 2.1 Schematic representation of the experimental groups. Ceftriaxone (CEF) was administered in two different time points. **a)** administration in the first 3 weeks of the uCMS protocol (Prevention); saline (SAL) and ceftriaxone (CEF) was administrated either to control (CT) and stressed (uCMS) animals. **b)** administration in the last 2 weeks of uCMS protocol (Treatment); ceftriaxone (CEF) was administrated either to control (CT) and stressed (uCMS) animals; antidepressants (ADs) imipramine (IMIP) and fluoxetine (FLX), were administered alone or in combination with CEF.

2.2 Unpredictable Chronic Mild Stress (uCMS) protocol

An unpredictable chronic mild stress (uCMS) protocol was applied for 6 weeks as previously described (Bessa et al., 2009b). Briefly, the uCMS protocol encompasses 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 4. Animals are random- and uninterruptedly exposed to these stressors during 6 weeks. Control animals were handled gently every week throughout the 6 weeks protocol.

2.3 Drug Treatment

Groups of stressed animals were administered with the different drugs and at distinct time points to address the different objectives proposed (Figure 2.1). In the Prevention set, ceftriaxone (CEF) was daily administered intraperitoneally (i.p.; 200 mg/Kg; Labesfal, Portugal) to non-stressed controls (CT) and stressed (uCMS) groups of animals during the first 3 weeks of uCMS exposure. In the Treatment set, CEF, as well as two antidepressants (ADs) from different classes, fluoxetine (FLX, selective serotonin reuptake inhibitor-SSRI; 10 mg/kg in ultra-pure water; Kemprotec, Middlesborough, UK) and imipramine (IMIP, Tricyclic antidepressant; 10 mg/kg in 0.9% saline solution; Sigma-Aldrich, St Louis, MO, USA) were daily administered, intraperitoneally, alone or in combination with CEF (200 mg/Kg; i.p.) to additional stressed groups of animals during the last 2 weeks of the uCMS protocol.

Non-stressed control (CT+SAL) and stress-exposed (uCMS+SAL) groups were administrated with saline (SAL), used as vehicle. The doses were chosen based on previous studies (Bessa et al., 2009a; Rothstein et al., 2005).

2.4 Behavior tests

Behavioral tests were performed at week 4 and at the end of week 6 of the uCMS protocol as depicted in Figure 2.2.

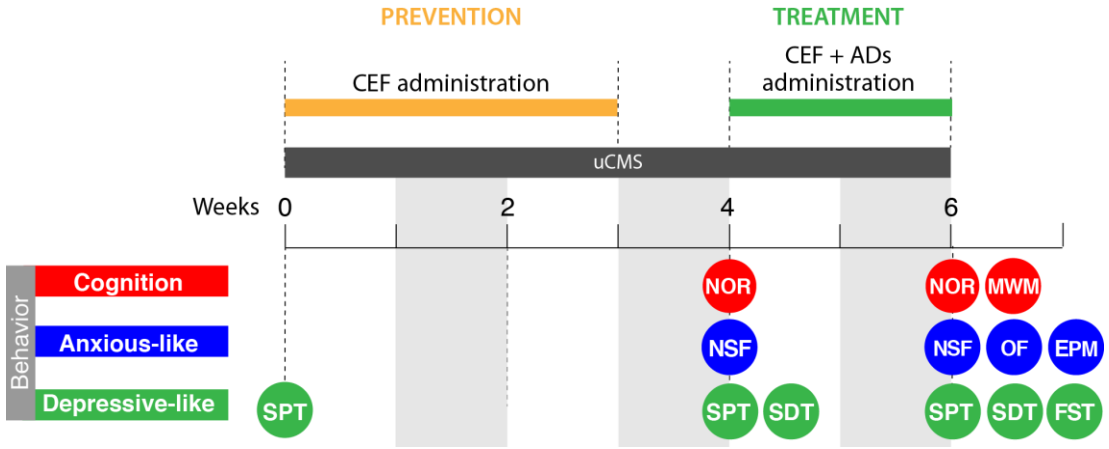

Figure 2.2. uCMS protocol and schematic representation of the behavioral analysis. Ceftriaxone (CEF) was administered in two different time points: during the first 3 weeks (Prevention) or in the last 2 weeks (Treatment) of uCMS protocol. Antidepressants (ADs) imipramine (IMIP) and fluoxetine (FLX), were administered in the last 2 weeks of uCMS protocol, alone or in combination with CEF. To establish baseline values for sucrose preference all animals have performed Sucrose Preference Test (SPT). The mood dimension of behavior was assessed with SPT, Sweet Drive Test (SDT) and Forced Swim Test (FST) at weeks 4 and 6 of the uCMS protocol. The anxiety-like signs were evaluated through Open field (OF) and Novelty suppressed feeding (NSF) tests also at the 4th and 6th weeks of the uCMS protocol. Novel Object Recognition (NOR) test evaluated the cognitive dimension at weeks 4 and 6; Morris Water Maze (MWM) test was also performed to evaluate cognitive function, at the last week of the uCMS protocol.

2.4.1 Sucrose preference test

The Sucrose Preference Test (SPT) was used to evaluate anhedonia at weeks 4 and 6 of the uCMS protocol, as previously described (Bessa et al., 2009a). For each assay, animals were food- and water- deprived for 12h during the non-active period (day period). The room was cleaned with ethanol 96% and the test was performed under dim illumination. Each animal was placed individually in a cage with two pre-weighted bottles placed in opposite sites of the cage: the one containing a 2% (m/v) sucrose solution in the food site and the one with tap water placed in its original site. Both bottles were placed simultaneously in the cage and consumption was measured for 1h. All animals performed this test and were allowed to habituate to the sucrose solution in a three-trial paradigm, one week before the uCMS protocol, to establish the baseline values for sucrose preference.

Sucrose preference was calculated by the following formula: sucrose preference=[(sucrose consumption / Total consumption) x 100] as previously described (Bessa et al., 2009a). Anhedonia was defined as a reduction in sucrose preference in relation to the baseline levels.

2.4.2 Sweet drive test

Sweet drive test (SDT) was also used as a measure of anhedonic behavior as previously described (Mateus-Pinheiro et al., 2014). Animals were habituated to sweet pellets (3.77 kcal/g; Honey Cheerios®; Nestlé Portugal S.A., Portugal) 4 weeks before testing and in the day before the first trial, overnight. Animals were submitted to 3 trials (1 trial every 48h) in the dark/active phase, under red light illumination. Before each trial, animals were food-deprived for 12h during the light/non-active period and the exposure to stressors was suspended. The test apparatus consisted of a black acrylic enclosed arena (82 cm x 44 cm x 30 cm) divided by transparent and perforated walls into 3 closed chambers and one pre-chamber in which the animal is initially placed. This pre-chamber is connected to a middle chamber by a trap door. Once the animal crossed the trap door, this door closes and the animal is allowed to explore de other 2 chambers, one on the left and one on the right side of the apparatus. Part of the apparatus was also a transparent acrylic lid to ensure surroundings noise-reduction (Mateus-Pinheiro et al., 2014). A total of 20 pellets of regular food (3.60 kcal/g; Certificate standard diet 4RF21; Mucedola, S.R.L., Italy) were positioned in the corner of the left chamber and 20 sweet pellets were placed in the corner of the right chamber. The animals were allowed to explore freely for 10 minutes (min) per trial. The number of pellets that were eaten by the animal in each chamber was counted to determine the consumption preference. Number of entrances in each chamber was used as exploratory parameters. This test was performed by the prevention groups performed this test at the 4th week, and by all groups at the 6th week.

2.4.3 Novelty suppressed feeding test

Novelty suppressed feeding test (NSF) was used to measure the anxiety-like behavior in the animals from Prevention groups, at week 4 of the uCMS protocol. Animals from Treatment

groups have performed this test at week 6. After 18h of food deprivation animals were placed (for 10 min) in an open-field arena covered with bedding, with a single food pellet on the center. When reaching the pellet, the animal was immediately placed individually in the home cage and allowed to feed a pre-weighted regular food pellet for 10 min. The latency to feed in the open-field arena provided a measure of anxiety-like behavior. The amount of food pellet consumed in the home cage was used as a measure of appetite drive (Bessa et al., 2009a; Mateus-Pinheiro et al., 2013a).

2.4.4 Elevated-plus maze

Anxious-like behavior was measured in the elevated plus-maze (EPM) test, in a 5 min trial as previously described (Bessa et al., 2009a). The EPM apparatus consisted of two opposite open arms and two enclosed arms elevated 72.4 cm above the floor with an intersection area (hub) of 100 cm^2. Animals were placed individually in the center of the maze, facing the edge of two arms. Behavioral activity was recorded by means of a video camera and analyzed using EthoVision XT 10.0 (Noldus Information Technology, Netherlands). After each trial the maze was cleaned with 10% ethanol. The time spent in the open arms was taken as a measure for anxiety-like behavior (Bessa et al., 2009a; Mateus-Pinheiro et al., 2013a). All animals have performed this test at week 6 of the uCMS protocol.

2.4.5 Open-field test

The open-field (OF) test was also used to assess anxiety-like and exploratory behavior as previously described (Prut and Belzung, 2003) in the 6th week of the uCMS protocol, only to the prevention groups. The OF test consists of a closed apparatus of 30.5 cm high with a brightly illuminated square arena of 43.2 x 43.2 cm. For this test, 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 analyzed using the Activity Monitor software (Med Associates, Inc.), considering two previously defined zones: a central and an outer zone. Time spent in each zone was recorded and further analyzed.

2.4.6 Forced Swimming Test

Learned helplessness was evaluated in the forced swimming test (FST) as previously described (Mateus-Pinheiro et al., 2013a; Porsolt et al., 1977). Animals were placed in a transparent glass cylinder filled with water (25°C; 50 cm of depth) and submitted to a 5 min pretest session 24h before conducting the assays. Five min trials were recorded using a video camera and analyzed through EthoLog 2.2 (Ottoni, 2000) and EthoVision XT 10.0 (Noldus Information Technology, Netherlands) software. Immobility time was assessed and an increase in immobility time was considered as learned-helplessness. This test was performed at the 6th week of the uCMS protocol for all animals.

2.4.7 Novel Object Recognition test

In order to assess cognitive functions, namely recognition memory, novel object recognition test (NOR) was performed as previously described (Bevins and Besheer, 2006; Dere et al., 2007; Winters et al., 2008; Ennaceur, 2010). The test apparatus consists of a black acrylic box (50 x 50 x 150 cm) illuminated with a white lamp (100-140 lux). The test is phased in 4 days and was performed at week 4 (only by the prevention groups) and week 6 (all groups) of the uCMS protocol. Objects differing from each other in color, shape and texture were used in each time point.

Day 1 – Habituation – On the first day animals were allowed to habituate to the apparatus in a 10 min trial, consisting of an exploratory phase.

Day 2 – Sample phase – In a second day, the animals had to explore two identical objects placed at the back left and right corners of the apparatus for 10 min. Within an interval of approximately one hour, a second sample object exposure (3 min) was performed. This time, the left object was repositioned (spatial recognition) in a new corner of the apparatus (frontal left corner). Due to the spatial repositioning, this second trial gives an insight of hippocampus function and works as a memory "reinforcement" of the sample object.

Day 3 – Long-term memory assessment – The right sample object was replaced for a new object 24h after memory reinforcement, and animals were allowed to explore the two objects for 3 min. This trial consists of a choice phase were long-term memory was evaluated.

Day 4 – Short-term memory assessment – On the last day, short-term memory was assessed, with different objects from the previous days. Similarly to day 1, animals were presented with to identical objects in a first trial of 10min exploring. Then, with 1hour interval, the object on the right is switched for a new object and a second trial of 3 min is performed.

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 was crucial to define what we considered as the object exploration by the animal; for that, we assumed exploration of an object as touching it with the nose or directing the nose to the object at a distance of less than 2 cm.

For a schematic representation of the test see Figure 2.3.

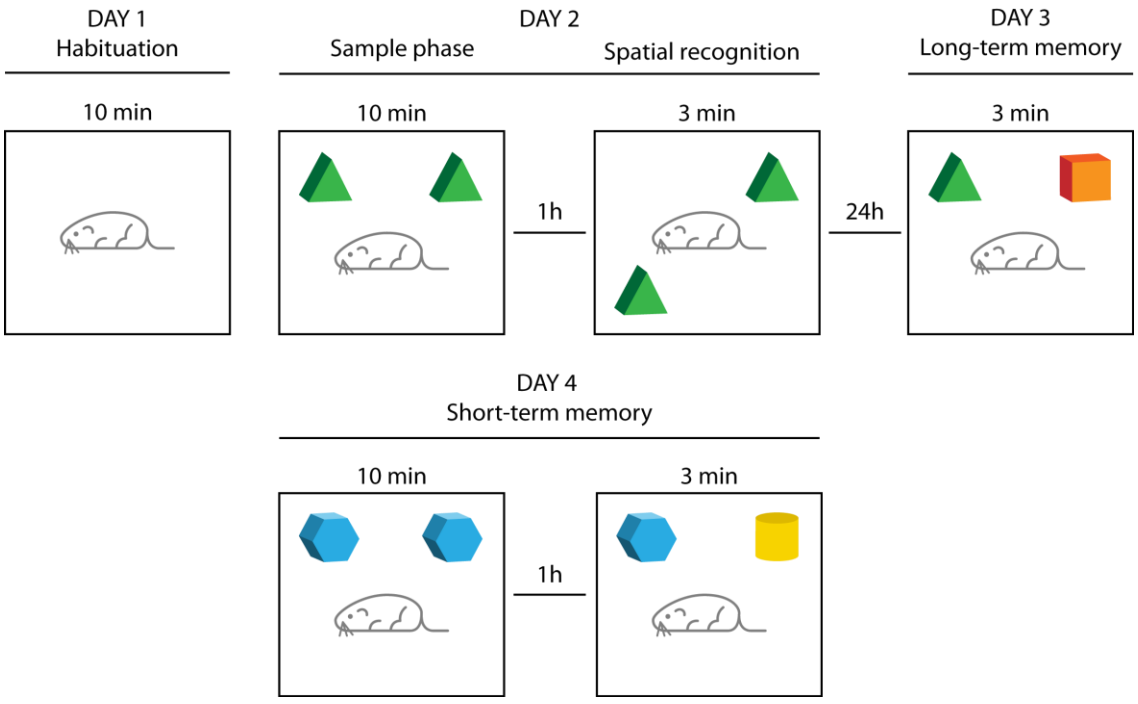

Figure 2.3 Schematic representation of the Novel Object Recognition (NOR) protocol. The test consists of 4 phases. DAY 1, Habituation phase, animals explore the apparatus for 10 min. DAY 2, Sample phase, animals explore two identical objects in a trial of 10 min then 1h later the left object position is changed and a second trial is performed (spatial recognition). DAY 3, Long-term memory choice phase, the left object is the same from day 2 and the right object switches for a new object and a single trial of 3 min is performed. DAY 4, Short-term memory choice phase, with new objects the animal preforms a first trial of 10 min exploring two identical objects then 1h later the right object switches for a new one and a second trial of 3 min is performed.

2.4.8 Morris Water Maze: Reference memory and Reversal tasks

The Morris water maze (MWM) test was performed to evaluate cognitive functions in the 6th week of the uCMS protocol as previously described (Bessa et al., 2009a). The test included a reference memory task (hippocampus-dependent) and a reversal learning task (behavior flexibility, dependent on the prefrontal cortex). In both tasks the animal had to explore a black pool (diameter 170 cm; depth: 50 cm) filled with water (22°C) above a hidden platform (31 cm), divided in 4 imaginary quadrants. The room was dimly lit and spatial clues were fixed to the walls surrounding the pool. The reference memory task consists of a three days protocol. Throughout the protocol the platform was kept in the same position and four consecutive trials of 2 min were conducted daily. In each trial the animal was placed facing the wall of the pool and finished the trial once it reached the platform. Moreover, in each trial the animal was placed in a different quadrant. Every time an animal was unable to find the platform within the trial time, the animal was guided to the platform and allowed to stay in it for 30 seconds.

At the fourth day, a reversal task was performed; for this task the platform was positioned at the opposite quadrant from previous days. This task was conducted in four trials of 2 min, and the percentage of time travelled in the new quadrant was measured. The performance of the animals was recorded using a video-tracking system.

2.5 Measurement of Plasma Corticosterone Levels

Corticosterone levels were measured in blood serum of all animals using a [^{125}I] radioimmunoassay (RIA) kit (MP Biomedicals, Costa Mesa, CA). Blood sampling (tail venipuncture) was performed at 8:00-9:00 pm (night), at week 6 of the uCMS protocol, one day prior to behavior tests. Blood serum was separated by centrifugation (13 000 rpm, 10 min) and kept at -20° C before analysis.

2.6 Tissue Processing

Animals were anesthetized with sodium pentobarbital (Eutasil, 60 mg/Kg, i.p.; Ceva Saúde Animal, Portugal). For molecular analysis (Western blot and Real Time RT-PCR) and neuronal

morphology analysis (Golgi-Cox staining) animals (n=4-6 per group) were transcardially perfused with 0.9% saline; brains were then extracted from the skull and divided into two hemispheres: the right hemisphere was macrodissected for molecular analysis and the left hemisphere was used for 3D morphological analysis. For astrocytic morphology analysis (immunohistochemistry procedures) animals (n=3-4 per group) were transcardially perfused with 0.9% saline and 4% paraformaldehyde. Brains were collected and embedded in Neg-50 Frozen Section Medium (ThermoScientific, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and further frozen at -20°C. Twenty micrometers coronal sections of prefrontal cortex (PFC) and extending over the entire length of the hippocampus were cut in a cryostate. Slides will be maintained at -20°C for future analyzes. For the purpose of this thesis, the immunohistochemical analyzes were not performed due to time constrains.

2.7 MOLECULAR ASSAYS

2.7.1 Western Blot

For protein quantification the Western blot (WB) technique was applied. Total lysates of macrodissected hippocampal dentate gyrus (DG; right hemisphere) and PFC (right hemisphere) were mechanically homogenized in HEPES-buffered sucrose solution (0.32 M sucrose; 4 mM HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) pH 7.4) with protease inhibitors 25x (1 tablet per 2 mL of H_2O; Complete EDTA free, Roche, Basel, Switzerland), 10% sodium dodecyl sulfate (SDS) and Nonidet P-40 (NP-40). The homogenate was centrifuged (3000 rpm, 20 min at 4°C), and supernatant was stored at -80°C until use. A Quick Start™ Bradford (BioRad Laboratories, München, Germany) protein assay was done to determine the total amount of protein in each homogenate (3 measurements per homogenate) and different concentrations (0.5, 1, 3, 5, 8, 10, 16 μg/mL) of bovine serum albumin (BSA, 0.1mg/mL in distilled water; Sigma Chemicals, St Louis, MO, USA) were used to create a standard curve for each run. Samples were measured at 595 nm in a spectrometer microplate reader (model 680, BioRad Laboratories, München, Germany), and volumes corresponding to 50 μg of total protein were then calculated. For the SDS-Page step, laemmli buffer (LB) was added to each sample and the volume was adjusted with HEPES-buffered sucrose solution. The samples run in a 10% acrylamide gel. Transference of SDS-gel to a nitrocellulose membrane was performed with a

Trans-Blot® Turbo™ Transfer System (BioRad Laboratories, München, Germany) according to manufactures' instructions. After transferring of the total protein, the membrane was incubated in TBS (Tris-buffered saline) with 5% of milk (Molico; Nestlé Portugal S.A., Portugal) for 1 h blockage. After blocking unspecific bindings, the membrane was incubated with primary antibody (**Table 1**) overnight at 4°C with agitation. In the next day, 3 washes of 10 min with 1% milk/TBS-Triton-X100 (T, ThermoScientific, Thermo Fisher Scientific, Inc., Waltham, MA, USA) solution were performed before incubation with secondary antibody (**Table 2**) for 2 hours at room temperature (RT). Bands were revealed using the chemiluminescent Clarity™ Western ECL substrate (BioRad Laboratories, München, Germany) and visualized by Bio-Rad Chemidoc and Quantity One software.

Samples homogenates were loaded onto the same gel accordingly to possible comparisons between two groups (2 groups per gel and 4 animals per group). For each gel, actin (see **Table 1** and **Table 2** for antibodies information) was used as housekeeping and an internal control was added in order to minimize procedural variations in the revelation. Results are presented as fold-change of protein levels between the respective experimental groups after normalization to actin and internal control levels.

Table 1. Dilutions and information about the primary antibodies (Ab) used for western-blot analysis.

Primary Ab	Specie	Working dilution	Company
GLT-1	Guinea Pig	(1:1500)	Chemicon®, Millipore
β-Actin	Mouse	(1:5000)	Ambion®, Life Technologies

Table 2. Dilutions and information about the primary antibodies (Ab) used for western-blot analysis.

Secundary Ab / Antigenicity	Working dilution	Company
Guinea Pig HRP	(1:10000)	Chemicon®, Millipore
Mouse HRP	(1:8000)	BioRad

2.7.2 RNA isolation, cDNA synthesis and real time PCR analysis

Total RNA was isolated from macrodissected tissue of the remaining right hippocampus (Cornus Ammonis (CA) regions, DG previously used for WB analysis) and PFC samples using the Direct-zol RNA miniPrep kit (Zymo Research, Irvine, CA, USA), according to manufacturer's

instructions. Briefly, tissue was mechanically homogenized using a syringe and 20 G needle in 600 µl of Qiazol Reagent (Qiagen, Valencia, CA, USA).

Total RNA (500 ng) was reverse-transcribed using qScript™ cDNA SuperMix (Quanta Biosciences™, Gaithersburg, Md, USA). Primers for Glutamate transporter-1 isoform-a (GLT-1a) and isoform-b (GLT-1b) for real time RT-PCR were designed using Primer-BLAST software (NCBI, **Table 3**). Reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, LLC, CA, USA) using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus, ROX (Solis Biodyne, Tartu, Estonia). Target gene expression levels were normalized against the housekeeping gene Beta-2-Microglobulin (B2M) and the relative expression was calculated using the ΔΔCt method. Results are presented as fold-change of mRNA levels between the respective experimental groups after normalization to B2M levels.

Table 3. Primers' sequences for real time RT-PCR and the corresponding amplicon size.

Name in use	Gene symbol	Gene name	Sequences		Product size (bp)
GLT-1a	*Slc1a2 isoform a*	Solute carrier family 1 (glial high affinity glutamate transporter), member 2, isoform a	**Sense**	CTCTGGCGGCCAATGGAAAG	119
			Antisense	CCATAAGATACGCTGGGGAGTTT	
GLT-1b	*Slc1a2 isoform b*	Solute carrier family 1 (glial high affinity glutamate transporter), member 2, isoform b	**Sense**	CACCATCCCCTGCATCCATTCT	166
			Antisense	GCGGATGTGGGAATCTGGTGAAA	
B2M	*B2M*	Beta-2 microglobulin	**Sense**	GTGCTTGCCATTCAGAAAACTCC	136
			Antisense	AGGTGGGTGGAACTGAGACA	

2.8 MORPHOLOGICAL ANALYSIS: GOLGI-COX STAINING

After perfusion with 0.9% saline, brains were removed and kept in the dark for 15 days in Golgi-Cox solution (Bessa et al., 2009b). Subsequently, the brains were transferred to a 30% sucrose solution and kept in the refrigerator, in the dark, for more 2-5 days. The tissue was sectioned (200 µm) in a vibratome (MicroHM-650V) and collected in 6% sucrose. Sections were blotted onto gelatin-coated microscope slides and subsequently alkalinized in 18.7% ammonia.

Slices were developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated and xylene cleared before coverslipping (Bessa et al., 2009b).

Three-dimensional (3D) dendritic morphology of neurons was assessed in the dorsal and ventral hippocampal DG following already described criteria (Patrício et al., 2014; Pinto et al., 2014). Two to three animals per group (only the Prevention groups were analyzed) and 8 neurons per animal were analyzed. Dendritic branches were reconstructed at 1000x (oil) magnification, for each selected neuron. For that, a motorized microscope (Axioplan 2; Carl Zeiss, LLC, United States) and Neurolucida software (MBF Bioscience, Williston, VT) were used. Three-dimensional analysis of those neurons was performed using NeuroExplorer software (MBF Bioscience, Williston, VT) and the measurements from individual neurons from each animal were averaged. Total dendritic length and branching was compared among experimental groups. For each neuron, dendritic spine density (number of spines/dendritic length) of proximal and distal branches was analyzed. Selected branches were either parallel or at acute angles to the coronal surface of the section.

2.9 DATA ANALYSIS

Statistical analysis was carried out through GraphPad software (GraphPad Software, Inc., San Diego, CA, USA). A repeated measures ANOVA was used to analyze cognitive-learning task performance (MWM) and One-Way ANOVA was use to analyze the remaining behavioral results. F-values and P-values are indicated along the text. Significant differences between groups were analyzed by Tuckey post-hoc test and the correspondent P-values are indicated in the figures. T-test was used to evaluate differences between two groups whenever appropriate. Statistical significance was accepted for $P \leq 0.05$. Data is presented generally as group mean ± standard error of the mean (SEM).

III. RESULTS

3.1 VALIDATION OF THE UCMS MODEL OF DEPRESSION

uCMS exposure is known to disrupt the hypothalamic-pituitary-adrenal (HPA) axis (Ottenweller et al., 1994) and therefore the measurement of corticosterone (produced by the adrenals) levels in the blood provides a correlate of the stress-level in the tested animals (Patrício et al., 2014; Sousa et al., 1998; Ventura-Silva et al., 2013). Control rats display a corticosterone peak at diurnal zenith (night time) (D'Agostino et al., 1982), which is affected in animals exposed to chronic stress.

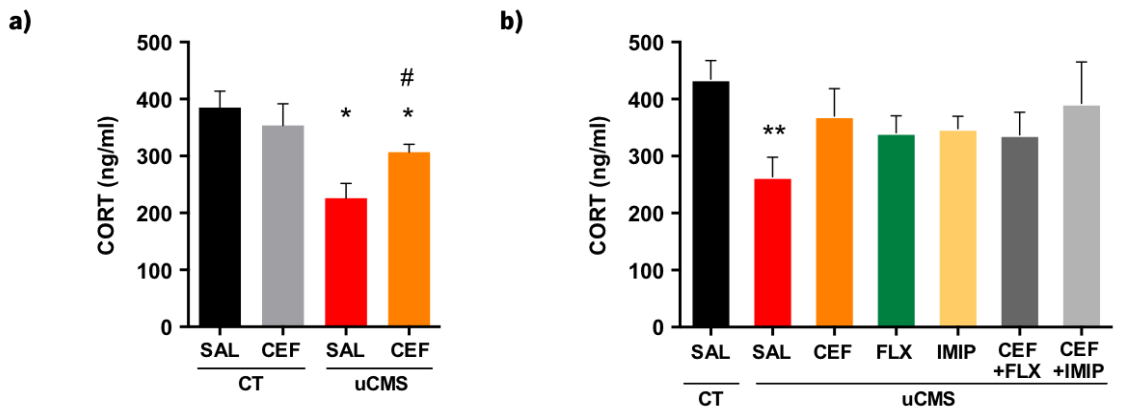

Figure 3.1 Corticosterone levels measured at night, for validation of the uCMS model. a) Corticosterone levels of prevention groups. **b)** Corticosterone levels of treatment groups. CORT, corticosterone; CT, control; uCMS, chronically stressed animals; SAL, saline; CEF, ceftriaxone; FLX, fluoxetine; IMIP, imipramine. Data is represented as mean ± SEM. *, different from CT+SAL; #, different from uCMS+SAL. *, #, P<0.05; **, P<0.01. n=7-10 animals per group.

Corticosterone levels were measured at the end of the uCMS protocol (6th week) both in the prevention and treatment groups. In the prevention groups, the exposure to stress disrupted the circadian rhythm of corticosterone secretion, decreasing the zenith levels when comparing to the control group (t_6=2.93, P= 0.026 Figure 3.1 a). Ceftriaxone (CEF) administration prevented the effects of stress on corticosterone levels (uCMS+CEF; t_{11}=2.56, P= 0.026 to CT animals; t_7= 2.74, P=0.029 to uCMS animals), whereas CEF administration to unstressed animals did not alter the corticosterone levels (CT+CEF; Figure 3.1 a) when compared to controls.

In the treatment groups, again, uCMS+SAL group presents lower levels of corticosterone when compared to control animals (t_7=3.60, P=0.009) and CEF seems to revert partially this effect of stress (uCMS+CEF; P>0.05 to uCMS animals; P>0.05 to CT animals; Figure 3.1 b). Stressed animals treated with the antidepressants (ADs) fluoxetine and imipramine alone or in combination with CEF (CEF+FLX; CEF+IMIP) also reverse partially, although not statistically significant, the corticosterone levels to those of control animals (Figure 3.1 b).

3.2 ASSESSMENT OF BEHAVIOR DIMENSIONS AFFECTED BY UCMS

uCMS exposure typically produces deficits in three behavioral dimensions that are commonly affected in depression – anxiety, mood and cognition (Bessa et al., 2009a). In order to address the potential of CEF either to prevent or treat these deficits, a multidimensional behavioral analysis was performed.

3.2.1 Study of the role of ceftriaxone in the prevention of uCMS-induced behavior alterations

Three behavioral dimensions putatively affected by stress (mood, anxiety, cognition) were assessed in the prevention groups. In order to assess longitudinal effects, behavior tests were performed at two different time-points: at the 4th and 6th weeks of the uCMS protocol (Figure 2.2). Sweet drive test (SDT), sucrose preference test (SPT) and forced swim test (FST) were used to assess mood alterations. Novelty suppressed feeding (NSF), elevated plus maze (EPM) and open field (OF) tests were used to assess anxiety-related behaviors. Finally, to assess cognitive functions, the novel object recognition (NOR) and Morris water maze (MWM) tests were performed.

In order to assess anhedonic behavior, SDT and SPT tests were performed (Figure 3.2 a and b). In the SDT, uCMS animals presented significantly lower preference levels comparing to control group (t_8=4.47, P= 0.002) in the 4th week of the uCMS protocol. CEF does not alter the performance of control animals, but prevents partially the installation of the anhedonic behavior in the uCMS-exposed animals (Figure 3.2 a). Again, at the 6th week of the uCMS protocol, the

uCMS animals present an anhedonic behavior when compared to control group (t_8=2.46, P=0.039; Figure 3.2 a). Moreover, CEF maintains the partial prevention of the anhedonic behavior caused by stress exposure (Figure 3.2 a).

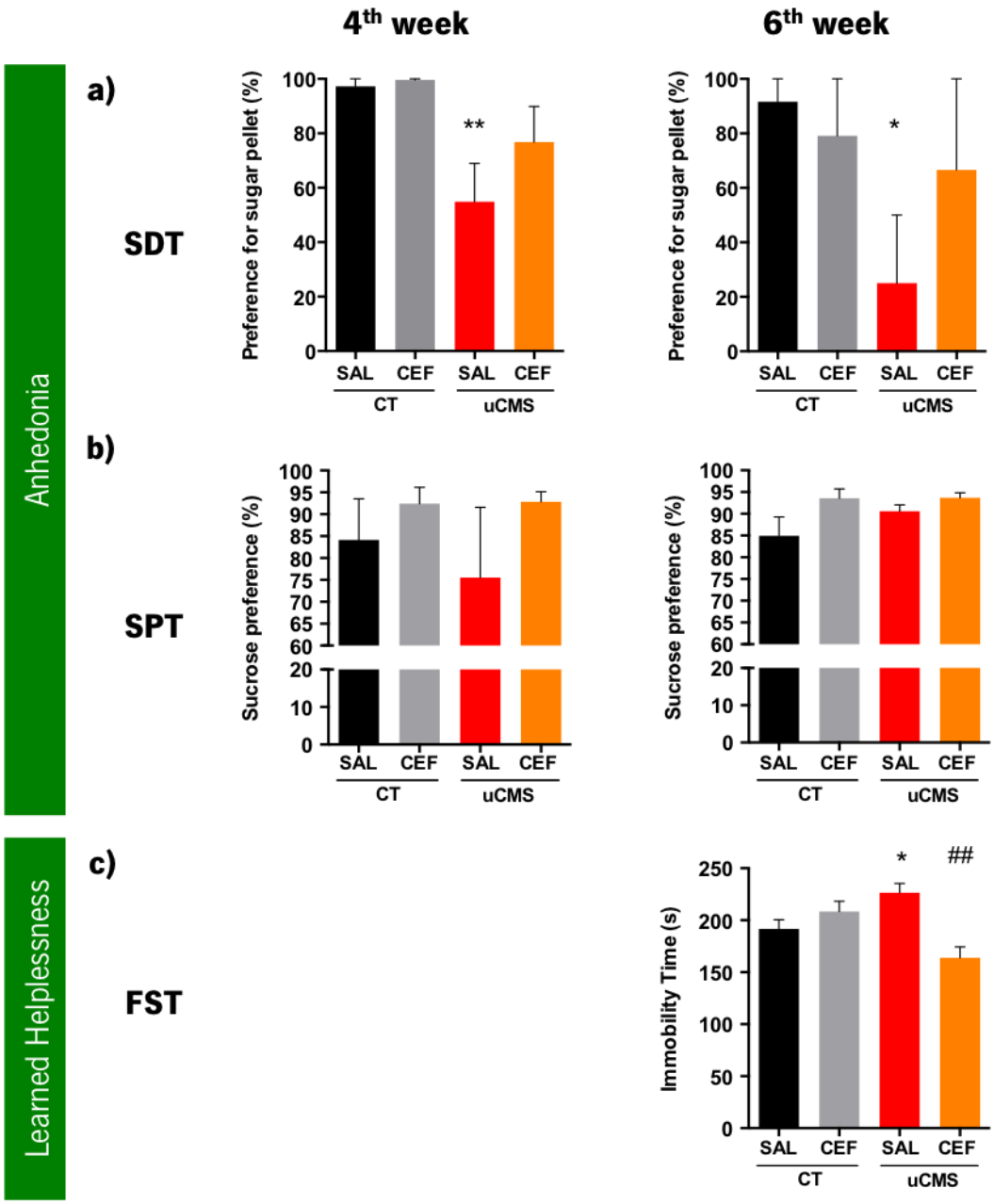

Figure 3.2 Assessment of depressive-like behavior in the prevention groups at the 4th and 6th weeks of the uCMS protocol. a), **b)** Anhedonia was assessed through the sweet drive test (SDT) and sucrose preference test (SPT) at the 4th and 6th weeks. **c)** At the 6th week the forced swim test (FST) was used to assess learned helplessness. CT, control; uCMS, stressed animals; SAL, saline (vehicle); CEF, ceftriaxone. Data is represented as mean ± SEM. *, different from CT+SAL; #, different from uCMS+SAL. *, P<0.05; **, ##, P<0.01. n=5-10 animals per group.

In the SPT, uCMS exposure did not induce lower preferences for sucrose solution, when comparing to control animals at the 4th neither at the 6th week of the uCMS protocol ($P > 0.05$). Treatment with CEF did not produce significant differences; therefore the results obtained in this test were inconclusive (Figure 3.2 b).

The second component of mood, learned helplessness, was assessed at the 6th week of the uCMS protocol by the FST. In this test, as expected, stressed animals spent significantly more time immobile when comparing to control animals (uCMS+SAL vs CT+SAL, $t_7=2.72$, $P=0.030$; Figure 3.2 c). CEF prevents installation of learned helplessness produced by uCMS exposure, as CEF-treated animals spent significantly less time immobile comparing to uCMS+SAL animals ($t_{12}=3.50$, $P=0.004$; Figure 3.2 c).

The prevention groups were also tested for anxious-like behavior through the NSF test, at the 4th week of the uCMS protocol. In this test, no differences between control and stressed animals were observed (Figure 3.3 a). Moreover, administration of CEF had no effect both on controls and uCMS (Figure 3.3 a). No differences were observed in the food consumption of these animals (Figure 3.3 a, right panel).

At the end of the uCMS protocol (6th week), anxious-like behavior was also assessed through the EPM and OF tests. In the EPM, the control group administered with CEF spent less time in the open arms, suggesting a more anxious phenotype when compared to untreated control rats (CT+CEF vs CT+SAL, $t_{10}=2.45$, $P=0.034$; Figure 3.3 b). Similarly, uCMS+CEF animals spent significantly less time in the open arms compared to uCMS+SAL animals ($t_{11}=3.36$, $P=0.006$; Figure 3.3 b). uCMS alone did not influence the behavior in this task (Figure 3.3 b). For confirmation of the CEF effect a second test for anxious-like behavior was performed. In the OF test, all 4 groups spent similar time in the center (Figure 3.3 c), which did not confirm the effects of CEF in the EPM.

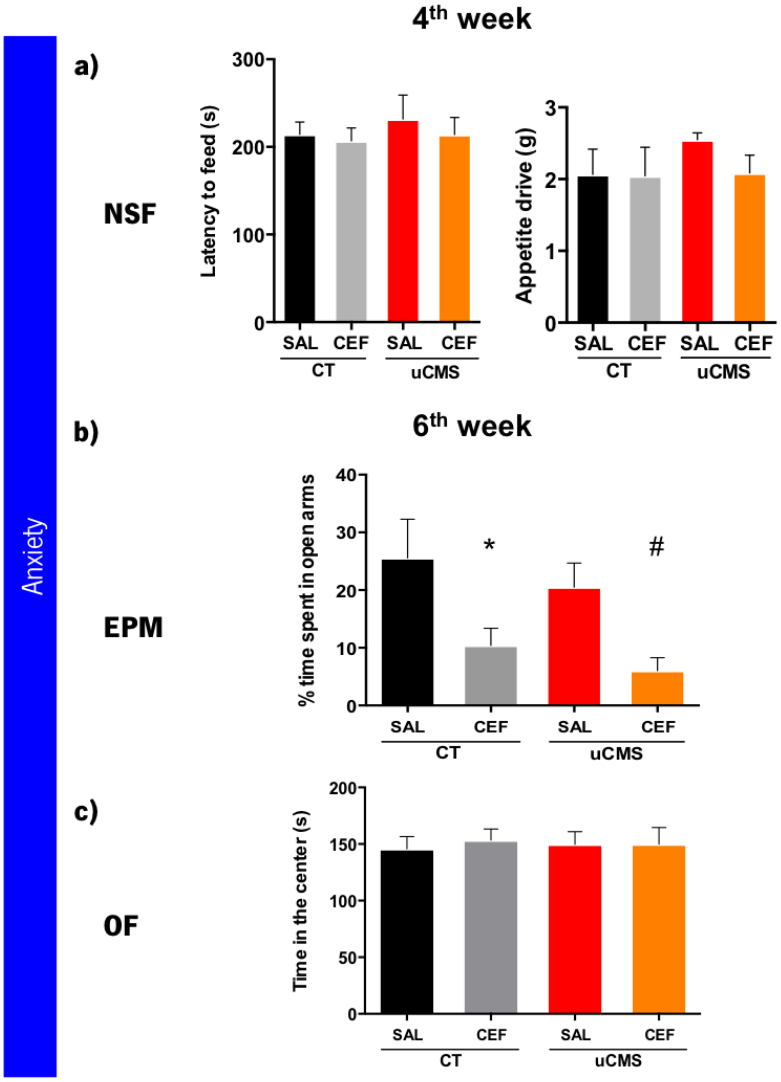

Figure 3.3 Assessment of anxious-like behavior of the prevention groups at the 4th and 6th weeks of the uCMS protocol. a) At the 4th week anxious-like behavior was assessed through novelty suppressed feeding (NSF). At the 6th week, **b)** elevated plus maze (EPM) and **c)** open field (OF) tests were used to assess anxious-like behavior. CT, control; uCMS, stressed animals; SAL, saline (vehicle); CEF, ceftriaxone. Data is represented as mean ± SEM. *, different from CT+SAL; #, different from uCMS+SAL. *, #, P<0.05. n=7-10 animals per group.

Cognitive evaluation was performed at the 4th and 6th of the uCMS protocol through the NOR test, to assess short-term and long-term memory of the animals from prevention groups. At the 4th week, no differences were observed between groups, neither in short-term memory nor in long-term memory analysis (Figure 3.4 a). At the 6th week, differences were observed in the short-term recognition memory task (Figure 3.4 a). In particular, stressed animals explored the new object less time than control animals (uCMS+SAL vs CT+SAL; t_{11}=3.01, P=0.012; Figure 3.4 a). However, this cognitive impairment was not prevented by CEF administration. No differences between all experimental groups were observed in the long-term memory task at the 6th week.

Reference memory and behavior flexibility was also assessed at the 6th week of the uCMS protocol through the MWM test. In the reference memory task no differences were observed in escape latencies (time to find the platform; Figure 3.4 b). Regarding behavior flexibility, no differences were observed between groups in time spent in the new quadrant (Figure 3.4 b).

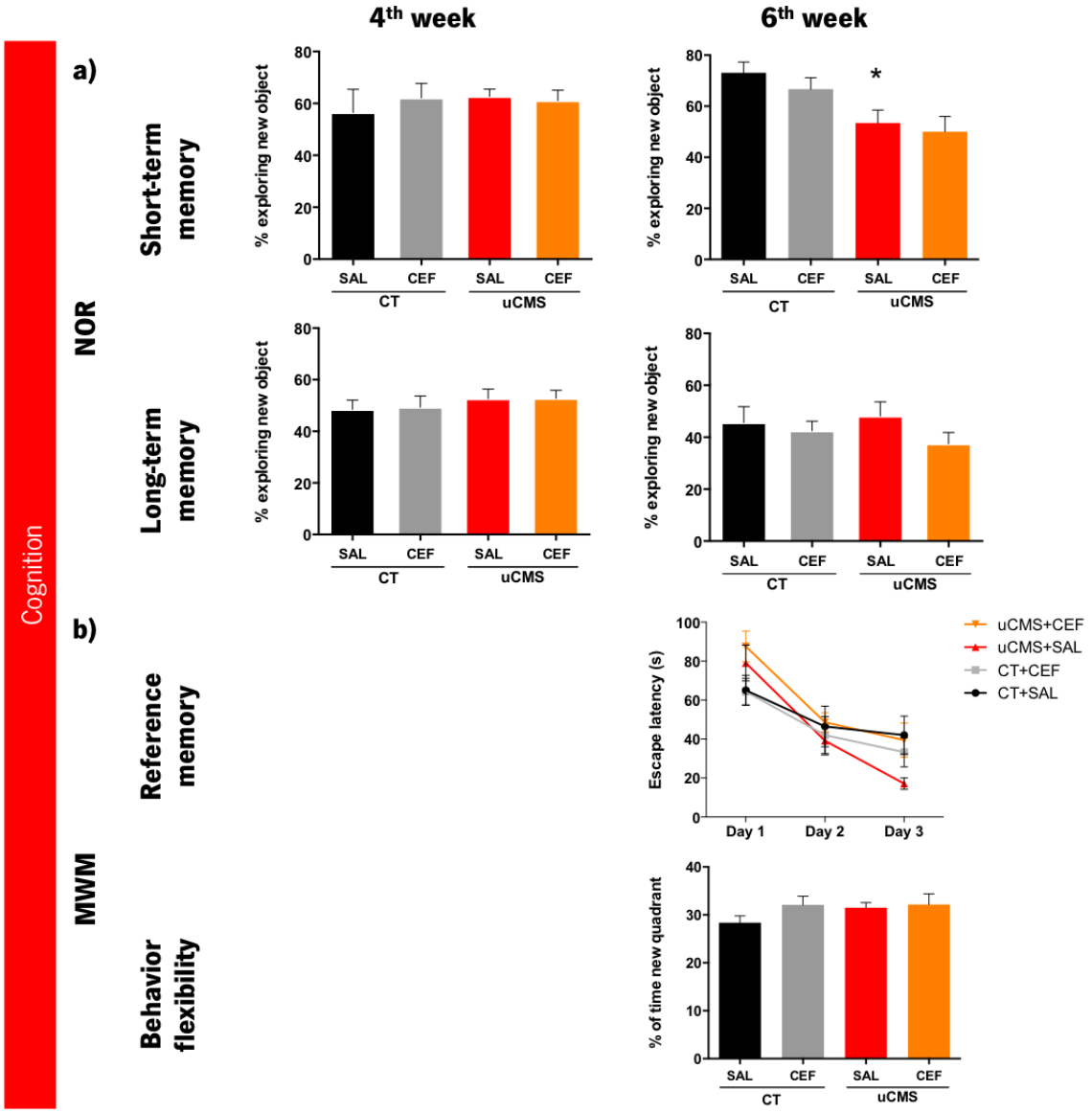

Figure 3.4 Cognitive assessment of prevention groups at the 4th and 6th weeks of the uCMS protocol. a) Short-term memory and Long-term memory were assessed through the novel object recognition (NOR) test at the 4th and 6th weeks of the uCMS protocol. **b)** At the 6th week of the uCMS protocol, reference memory and behavior flexibility of prevention animals was evaluated through the Morris water maze (MWM). CT, control; uCMS, stressed animals; SAL, saline (vehicle); CEF, ceftriaxone. Data is represented as mean ± SEM. *, different from CT+SAL. *, P<0.05. n=7-10 animals per group.

3.2.2 Study of the impact of ceftriaxone in the reversion of uCMS-induced behavior alterations

To assess the potential of CEF to treat the impairments in the three behavior dimensions affected by stress, a similar behavior analysis was performed. Animals from treatment groups were tested at the end of the uCMS protocol (6th week) for mood, anxiety and cognition. SDT and SPT were performed to assess anhedonia and FST was performed to assess learned helplessness. NSF and EPM tests were performed to assess anxious-like behavior. Cognition was assessed through the NOR and MWM behavioral tests.

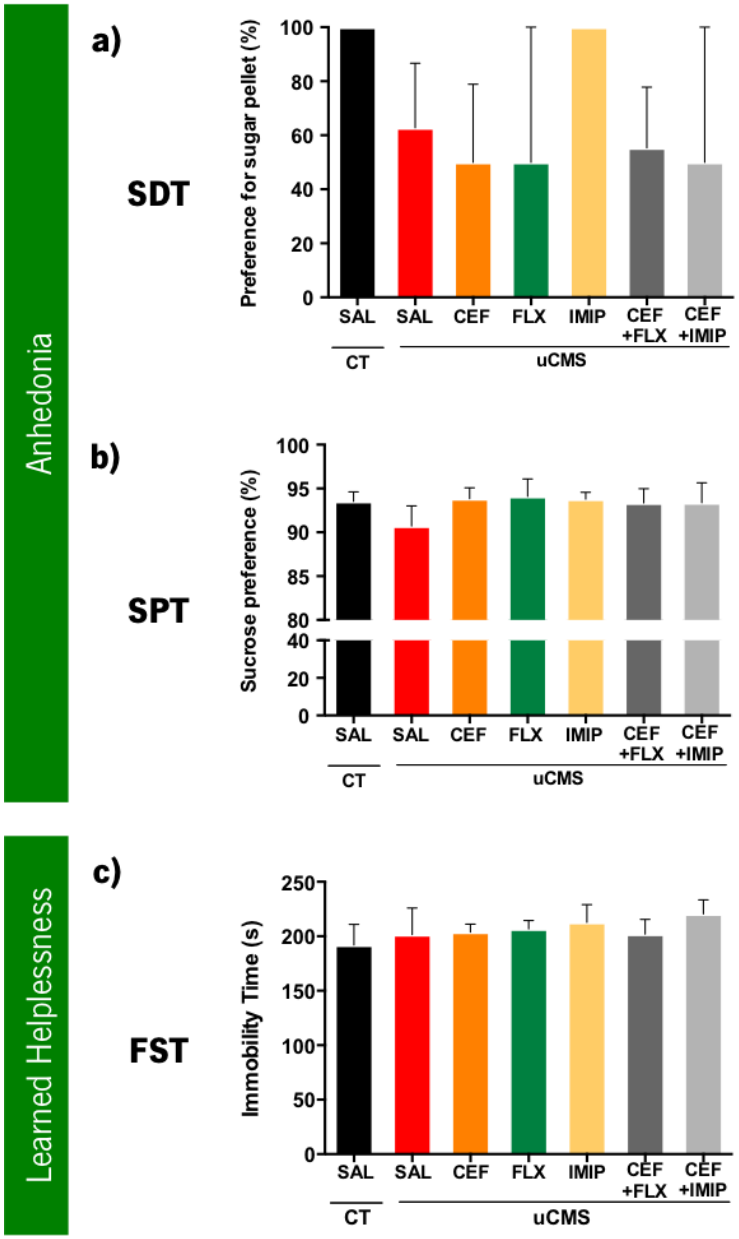

Figure 3.5 Assessment of depressive-like behavior of the treatment groups at the 6th week of the uCMS protocol. a), **b)** Anhedonia was assessed through and sucrose preference test (SPT). **c)** forced swim test (FST) was performed to assess learned helplessness. CT, control; uCMS, stressed animals; SAL, saline; CEF, ceftriaxone. Data is represented as mean ± SEM. n=5-10 animals per group.

Regarding anhedonia, in the SDT and SPT no differences were observed between groups (Figure 3.5 a and b). However, a trend for lower sucrose or sweet preference in the uCMS+SAL

groups comparing to control groups was observed in both tests, although not statistically significant (Figure 3.5 a and b).

In depressive-like behavior, measured by the FST, no differences were observed in time spent immobile between uCMS animals and controls (Figure 3.5 c). Stressed animals treated with CEF or ADs had similar performance when compared to untreated stressed animals (uCMS+SAL). Since uCMS animals did not present depressive phenotype these results are inconclusive.

At the end of the uCMS protocol, treatment groups were also tested for anxious-like behavior, through the NSF and EPM tests. In the NSF test, untreated uCMS animals (uCMS+SAL) had higher latency to feed when compared to the control group (t_{12}=2.41, P=0.033; Figure 3.6 a). This effect was not reverted by CEF (uCMS+CEF), suggesting no positive effect of this treatment. Interestingly, animals treated with fluoxetine (uCMS+FLX) displayed less anxious behavior when compared to uCMS+SAL ($F_{(5,\ 40)}$=4.06, P=0.004, P_{ajd}=0.041; Figure 3.6 a). This effect was not observed in animals treated with imipramine (uCMS+IMIP). Animals treated with the combination of CEF plus fluoxetine (uCMS+CEF+FLX) or plus imipramine (uCMS+CEF+IMIP) had lower latencies to feed when compared to CEF treatment alone (uCMS+CEF+FLX vs uCMS+SAL $F_{(5,\ 40)}$=4.06, P=0.004, P_{ajd}=0.025; uCMS+CEF+IMIP vs uCMS+SAL, $F_{(5,\ 40)}$=4.06, P=0.004, P_{ajd}=0.048; Figure 3.6 a). Appetite drive was measured to exclude possible bias to the test (Figure 3.6 a). Appetite drive for treatment animals seems to be in accordance to the results in latency to feed. In fact, animals treated with CEF in combination with imipramine, showed higher appetite drive and lower latency to feed (Figure 3.6 a).

In the EPM test, untreated stressed animals (uCMS+SAL) spent significantly less time in the open arms when compared to controls, denoting an anxious phenotype (t_{10}=2.86, P=0.017; Figure 3.6 b). This effect was not reversed by CEF (t_{10}=3.53, P=0.005; Figure 3.6 b). Fluoxetine and imipramine partially reverted the stress-induced anxious-like behavior, as animals from these groups spent more time in the open-arms (Figure 3.6 b) than uCMS animals. Moreover, this effect was similar also when these ADs where given in combination with CEF. Altogether this data points out that CEF administration does not seem to revert the anxiety-related behaviors triggered by uCMS exposure.

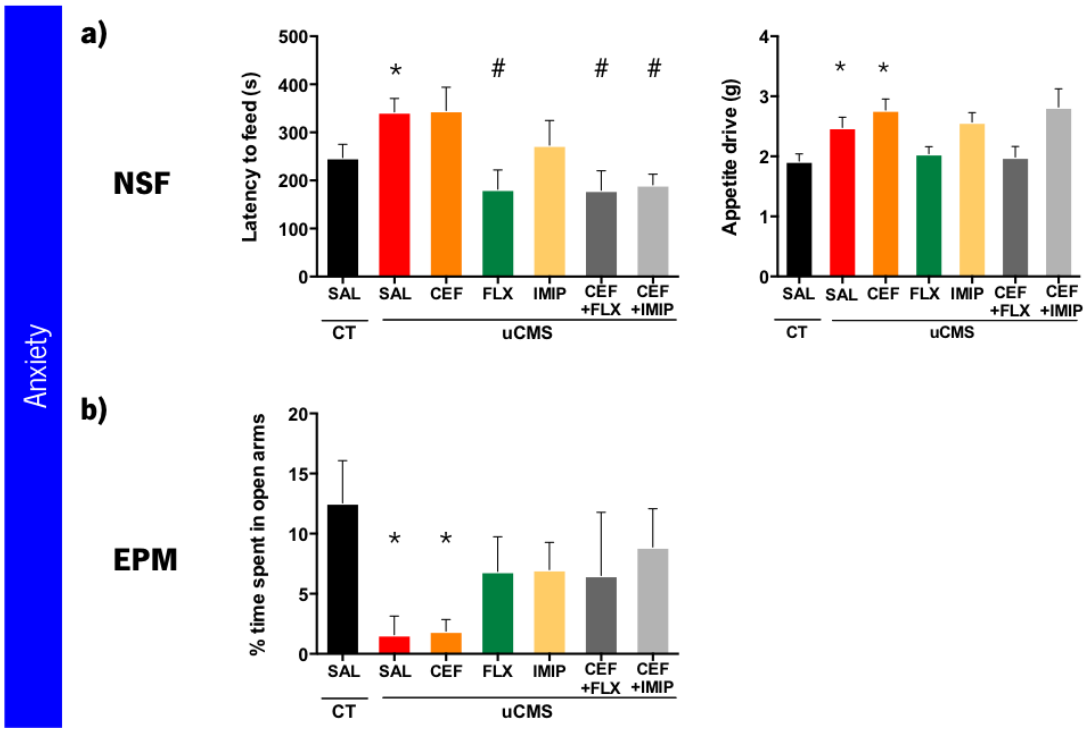

Figure 3.6 Anxious-like behavior assessment of the treatment groups at the 6th week of the uCMS protocol. a) Novelty suppressed feeding (NSF) and **b)** elevated plus maze (EPM) tests were used to assess anxiety in the animals. CT, control; uCMS, stressed animals; SAL, saline; CEF, ceftriaxone. Data is represented as mean ± SEM. *, different from CT+SAL; #, different from uCMS+SAL; *, #, P<0.05. n=7-10 animals per group.

Cognitive function of the treatment groups was also assessed at the end of the uCMS protocol. Animals were tested for spatial and object recognition through the novel object recognition (NOR) test. Differences between control group and untreated stressed animals were observed in short-term memory at the new object recognition (t_7=2.92, P=0.022; Figure 3.7 a). This effect was partially reverted by CEF or AD administration, yet only fluoxetine treatment succeeded to revert the effect significantly ($F_{(5,32)}$=2.94, P=0.027; Figure 3.7 a). Differences between control and stressed animals were also observed for an additional measure of short-term memory, based on a spatial rearrangement of the familiar objects (spatial recognition; t=2.26, P=0.050; Figure 3.7 a, right panel). Regarding long-term recognition memory, no significant effects were observed between groups.

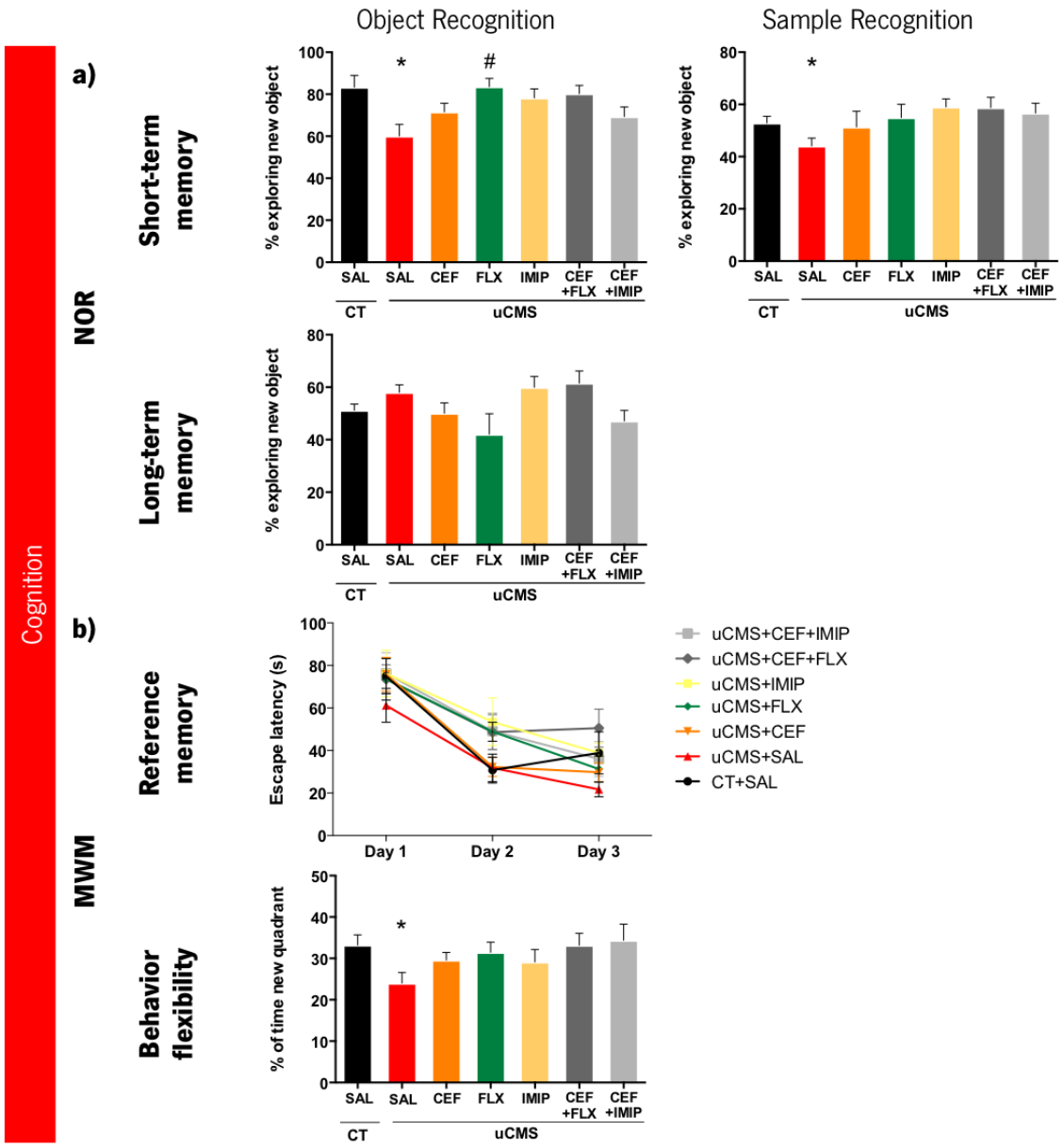

Figure 3.7 Cognitive evaluation of the treatment groups at the 6th week of the uCMS protocol. a) Short-term memory (measured through novel object and as spatial recognition) and Long-term memory were assessed through novel object recognition (NOR) test. **b)** Reference memory and behavior flexibility of treatment animals was evaluated through the Morris water maze (MWM) test. CT, control groups; uCMS, stressed animals; SAL, saline; CEF, ceftriaxone. Data is represented as mean ± SEM. *, different from CT+SAL. #, different from uCMS+SAL; *, #, P<0.05. n=7-10 animals per group.

Reference memory state was assessed in the MWM test. In this test, animals from all groups performed the task similarly, yielding no significant differences among groups (Figure 3.7 b). In the behavior flexibility task untreated uCMS animals spent less time exploring the new quadrant when compared to the control groups (t_8=2.49, P=0.038; Figure 3.7 b), suggesting deficits in

behavior flexibility. Ceftriaxone seems to partially revert this deficit, as these animals behaved similarly to controls. This effect was also true for the ADs alone, or in combination with CEF (Figure 3.7 b).

3.3 Cellular and molecular correlates of the ceftriaxone prevention of uCMS-related effects

The behavior analysis points out a role for CEF in the prevention of the depressive phenotype observed in the tested subjects after uCMS exposure. However, the potential use of CEF as treatment for depressive-like behavior remains inconclusive. Therefore, brain tissue obtained from the Prevention Groups was analyzed in order to assess cellular and molecular correlates that may help to understand the mechanisms underlying the prevention of uCMS-related effects.

3.3.1 Ceftriaxone effect in the expression of glutamate transporter GLT-1

CEF administration is known to increase the expression of glutamate transporter-1 (GLT-1) (Rothstein *et al.*, 2005). In order to study the alterations caused by stress in the levels of GLT-1 and the putative effect of CEF in the prevention of stress-induced effects, mRNA GLT-1 expression was quantified by real time (RT)-PCR analysis. Additionally, western blot (WB) analysis was performed for quantification of GLT-1 protein levels. Previous studies have related impairments in the function of prefrontal cortex (PFC) and hippocampus in animals exposed to the uCMS protocol to their depressive phenotype (Bessa *et al.*, 2009a; Mateus-Pinheiro, Pinto, *et al.*, 2013; Patrício *et al.*, 2014). Therefore levels of gene transcription and protein expression related to GLT-1 were performed in tissue macrodissected from those brain regions of animals of the prevention groups (CT+SAL, CT+CEF, uCMS+SAL, uCMS+CEF).

Gene expression levels was determined for the two isoforms of GLT-1 transporter: isoform-a (GLT-1a) and isoform-b (GLT-1b; Figure 3.8). In the PFC, neither uCMS exposure nor CEF administration had significant impact in the levels of GLT-1 mRNA.

In the dorsal hippocampus the administration of CEF significantly decreased the level of GLT-1a transcript in control animals (t_6=4.52, P= 0.004; Figure 3.8 b). However, in this brain region, neither uCMS exposure nor CEF administration had significant impact on gene transcript levels for GLT-1a and GLT-1b (Figure 3.8 b).

In the ventral hippocampus, CEF administration seems to interfere with GLT-1b but not GLT-1a gene expression (Figure 3.8 c). In this area of the brain, the transcript levels of GLT-1a are not altered between control and stressed animals or after CEF administration in both conditions. Differently, stress exposure decreases the levels of GLT-1b transcripts relatively to CT animals (t_6=2.81, P=0.031; Figure 3.8 c). Interestingly, administration of CEF triggers the increase of GLT-1b transcripts in the uCMS+CEF animals when comparing to uCMS animals (t_7=4.33, P=0.003; Figure 3.8 c). In summary, CEF administration seems to prevent the effect of stress on the GLT-1b transcript levels in the ventral hippocampus (Figure 3.8 c).

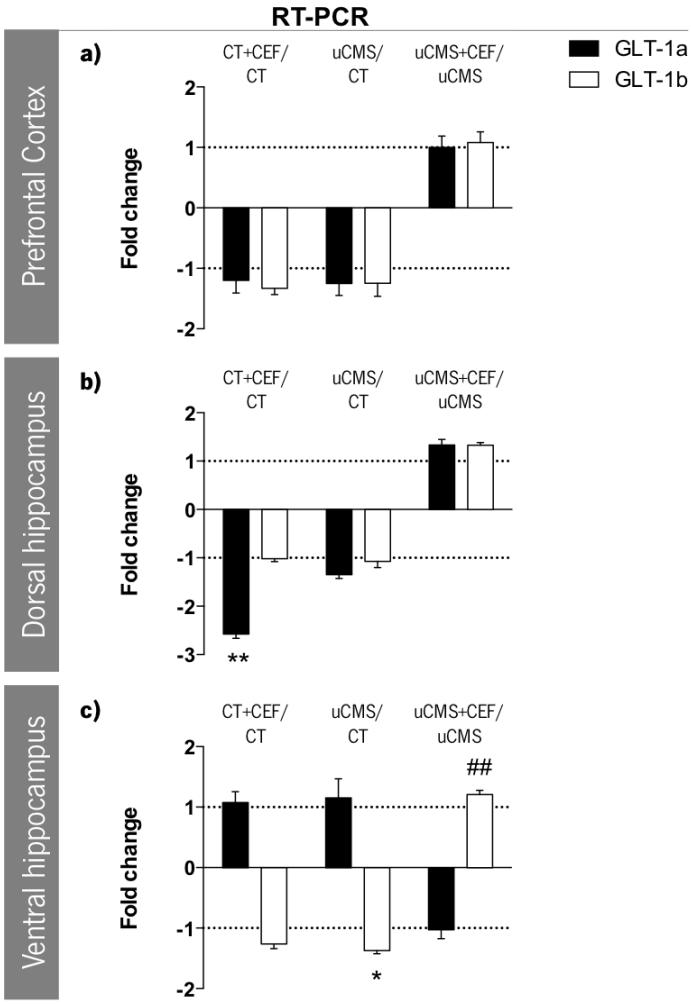

Figure 3.8 GLT-1 isoforms -a and -b gene expression in the prefrontal cortex and hippocampus. GLT-1 isoform-a (GLT-1a; black bars) and isoform-b (GLT-1b; white bars) expression as fold change values. **a)** Fold change values in the prefrontal cortex, dorsal hippocampus **(b)**, and ventral hippocampus **(c)**. CT, control; uCMS, stressed animals; CT+CEF, control group administrated with ceftriaxone; uCMS+CEF, stressed animals administrated with ceftriaxone. Data is represented as mean ± SEM. *, different from CT. #, different from uCMS. *, P<0.05; **, ##, P<0.01. n=4-6 animals per group.

3.3.2 Ceftriaxone effect in the protein levels of glutamate transporter GLT-1

For GLT-1 protein quantification, WB analysis was performed for brain tissue containing the PFC, dorsal and ventral dentate gyrus (DG) of the hippocampus (Figure 3.9). Similarly to RT-PCR, fold change analysis of the relative values for GLT-1 protein quantification was performed. In this analysis both isoforms were quantified together, as it was not possible to discriminate the bands formed (61 and 62 kDa).

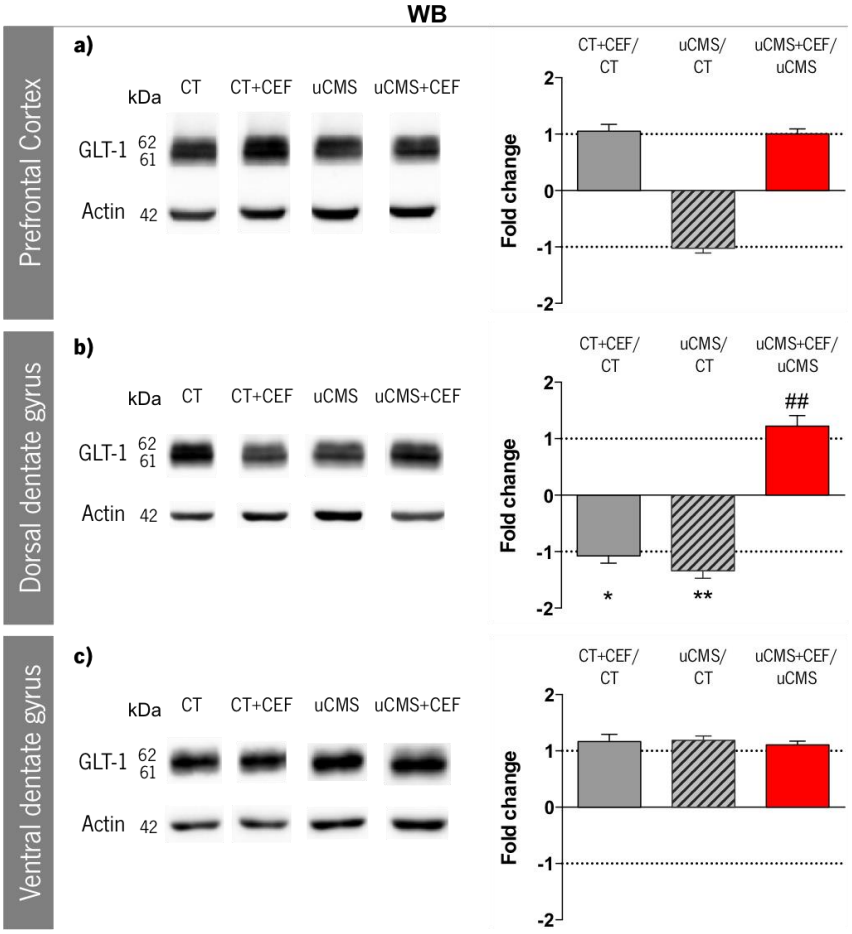

Figure 3.9 GLT-1 protein quantification by western blot of prefrontal cortex and dentate gyrus. Fold change values of GLT-1 quantification in the prefrontal cortex **(a)**, dorsal dentate gyrus **(b)**, and ventral dentate gyrus **(c)**. Left panel, representative blots for each condition; right panel, relative fold change analysis for each group and condition; CT, control group; uCMS, stressed animals; CT+CEF, control group administrated with ceftriaxone, uCMS+CEF, stressed animals administrated with ceftriaxone. Data is represented as mean ± SEM. *, different from CT; #, different from uCMS. *, P<0.05; **, ##, P<0.01. n=4-6 animals per group.

Similarly to the distribution of transcript levels, in the PFC no significant differences were found in the amount of GLT-1 protein among groups (Figure 3.9 a).

In the dorsal DG, GLT-1 protein levels in the CT+CEF group were significantly decreased when compared to the control group (t_8=2.92, P=0.019; Figure 3.9 b). Exposure to uCMS seems to decrease the levels of GLT-1 protein in a statistically significant manner (t_8=4.12, P=0.003; Figure 3.9 b). Interestingly, GLT-1 levels remained high by administration of CEF when comparing to uCMS animals (t_8=3.68, P=0.006; Figure 3.9 b).

In the ventral DG, there was no effect of stress exposure or CEF administration, both in control and stressed animals, in GLT-1 protein levels. For all comparisons, fold change differences were very close to 1 and no statistically significant results were observed, suggesting that the level of regulation is mild (Figure 3.9 c).

3.3.3 Assessment of ceftriaxone impact on neuronal 3D morphology

uCMS-triggered depressive-like behavior has been shown to be correlated with alterations in the neuronal structure and morphology in the hippocampal DG. These alterations are translated in dendritic atrophy, decreased number of ramifications, and alterations in spine distribution (Bessa et al., 2009b; Mateus-Pinheiro et al., 2013a; Patrício et al., 2014). In order to assess cellular alterations promoted by CEF in the Prevention Groups, neuronal 3D morphological alterations were assessed through the septo-temporal DG (dorsal and ventral DG). Total dendritic length, branching and spine densities were assessed in this analysis.

The data obtained by 3D reconstruction of dorsal DG neurons is presented in Figure 3.10. CEF administration to control animals induced an increase in total dendritic length (t_{25}=3.07, P=0.005) of dorsal DG neurons. As expected, stressed animals (uCMS+SAL) presented decreased neuronal dendritic length when compared to control animals (t_{23}=2.40, P=0.025). Interestingly, CEF administration prevented the installation of the dendritic atrophy caused by chronic stress-exposure (t_{28}=2.04, P=0.051). Regarding neuronal branching, no differences were observed between control and uCMS animals. CEF alone induced an increase in neuronal branching in the CT+CEF group (t_{33}=2.32, P=0.026), but failed to alter the number of branches in stressed animals.

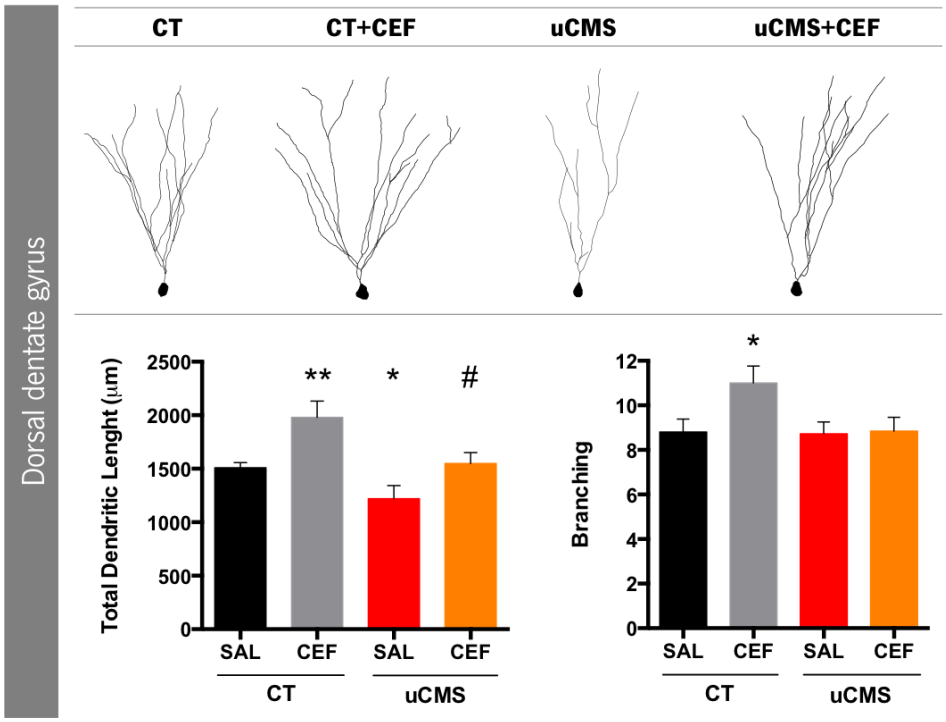

Figure 3.10 Morphological analysis of neurons from the dorsal dentate gyrus. Total dendritic length and branching of neurons in the dorsal dentate gyrus. CT, control groups; uCMS, stressed animals; SAL, saline; CEF, ceftriaxone. Data is represented as mean ± SEM. *,# different from CT group. *,# P<0.05; **, P<0.01. n=15-22 neurons per group.

Regarding the density of spines in the dorsal DG, uCMS exposure leads to a significant decrease in the number of spines, when comparing to the control group (CT+SAL; t_{19}=2.23, P=0.038; Figure 3.11 b). The administration of CEF prevented the decrease of spine density significantly (uCMS+CEF; t_{26}=2.52, P=0.018).

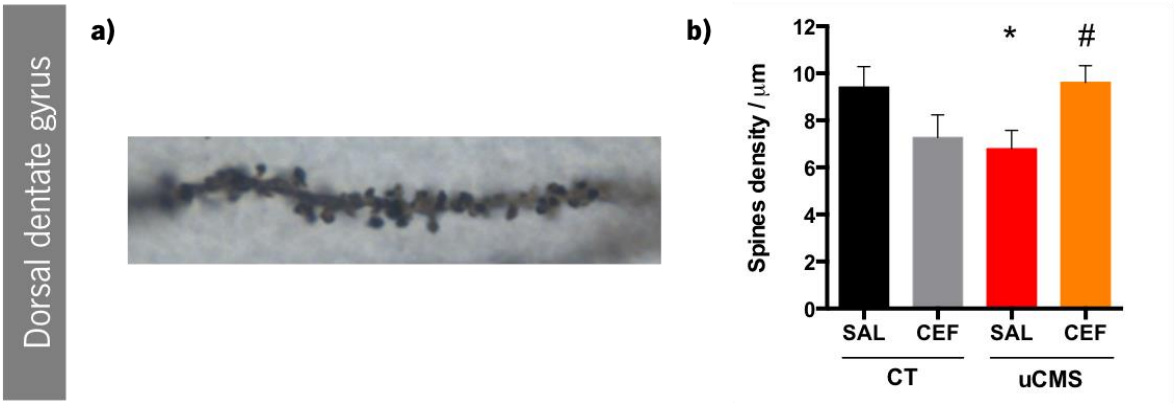

Figure 3.11 Analysis of spine densities in the dorsal dentate gyrus. a) Representative image of spines in the dorsal dentate gyrus. **b)** Spine density of the dendrites belonging to neurons at the dorsal dentate gyrus. CT, controls; uCMS, stressed; SAL, saline; CEF, ceftriaxone. Data represented as mean ± SEM. *, different from CT group; #, different from uCMS group. *, #, P<0.05. n=15-22 neurons per group.

In the ventral DG (Figure 3.12), no significant effects of stress exposure were found. Although, a typical trend for increased neuronal dendritic length was observed in the uCMS+SAL group when compared to control group (CT+SAL). Administration of CEF to control animals increased by itself the neuronal dendritic length (CT+CEF; t_{20}=2.38, P=0.027), yet similar administration to uCMS animals (uCMS+CEF) kept dendritic lengths close to those of controls (CT+SAL).

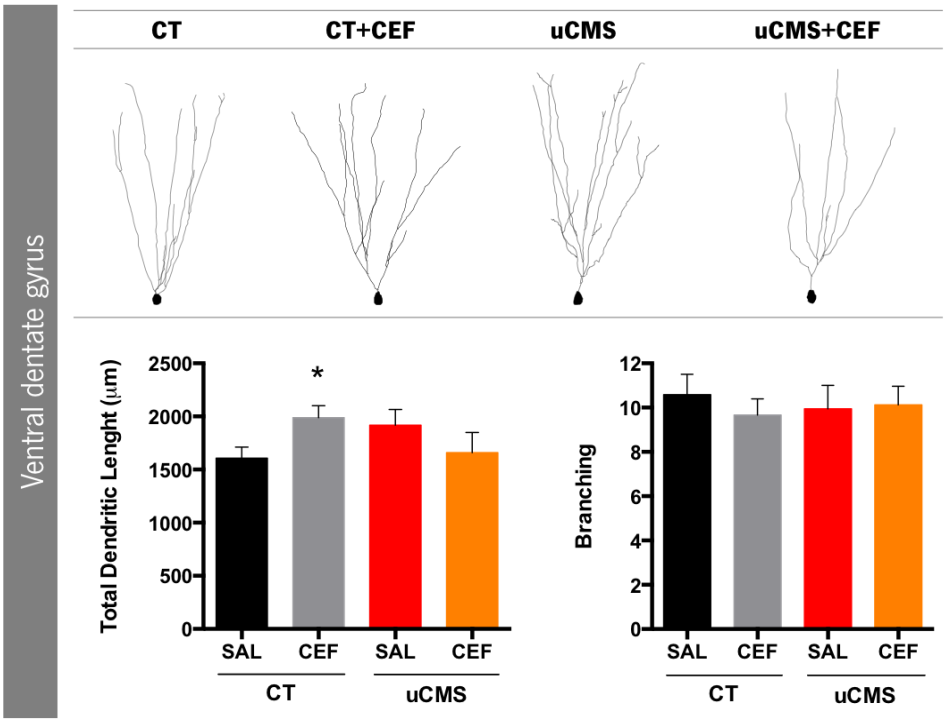

Figure 3.12 Morphological analysis of neurons from the ventral dentate gyrus. Total dendritic length and branching of neurons in in the ventral dentate gyrus. CT, control groups; uCMS, stressed animals; SAL, saline; CEF, ceftriaxone. Data is represented as mean ± SEM. *, different from CT group. *, P<0.05. n=12-17 neurons per group.

Regarding the analysis of total spine density in dendrites of the ventral DG neurons, no significant differences were observed between groups, neither after uCMS exposure, nor by CEF treatment (Figure 3.13 b).

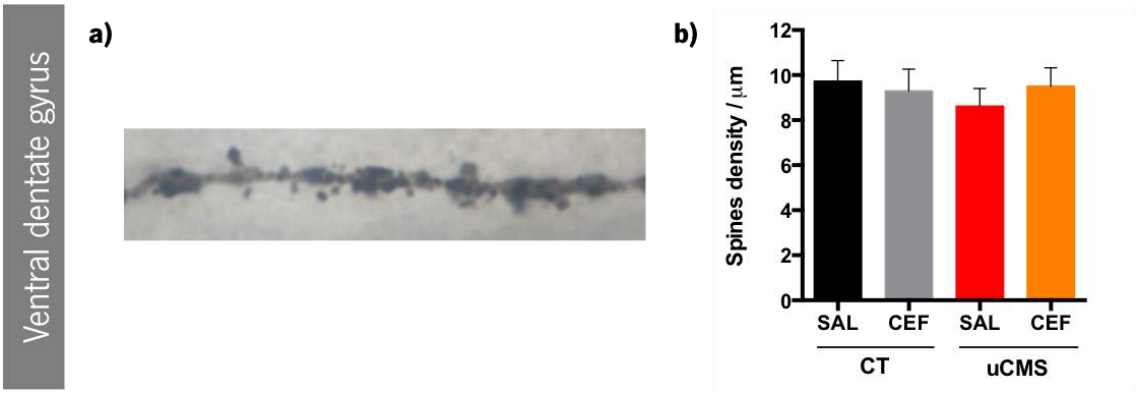

Figure 3.13 Analysis of spine densities in the ventral dentate gyrus. a) Representative picture of spines in the ventral dentate gyrus. **b)** Total spine density of the dendrites belonging to neurons at the ventral dentate gyrus. CT, control groups; uCMS, stressed animals; SAL, saline; CEF, ceftriaxone. Data is represented as mean ± SEM. n=12-17 neurons per group.

IV. DISCUSSION AND CONCLUSIONS

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The main goal of this work was to assess the potential of ceftriaxone (CEF) to prevent the installation or revert the deleterious effects of chronic stress exposure in a rat model of depression (uCMS).

In this study we show that the administration of CEF prevented the installation of depressive-like behaviors in this model, yet it failed to revert the deleterious effects induced by uCMS exposure. Indeed, CEF failed to treat the installed anxious-like behavior; although it seems to revert partially the cognitive deficits caused by uCMS exposure.

As previously described, the circadian distribution of corticosterone in control rats is characterized by a night peak, typical of the beginning of the active phase of the day (D'Agostino et al., 1982). This circadian regulation of corticosterone secretion was found to be altered in animals exposed to stressful events, suggesting a disruption of the hypothalamic-pituitary-adrenal (HPA) axis (Ottenweller et al., 1994). In fact, it is known that stress is accompanied by HPA axis hyperactivity, resulting in deregulated glucocorticoid secretion-pattern into the blood (Schoenfeld and Gould, 2012). In our results, a decrease of the corticosterone night peak in the untreated stressed animals (uCMS+SAL) was observed, which was prevented by CEF administration in the Prevention Groups of animals, and partially reversed by CEF treatment in the Treatment Groups, similarly to groups administered with antidepressants (ADs; fluoxetine and imipramine). These results confirmed the induction of the uCMS protocol in both animal sets and disclosed a putative role of CEF in the regulation of the HPA-axis balance.

In the Prevention Groups, the behavioral analysis was performed one week after the last administration of CEF to evaluate its putative effect in the prevention of the installation of the deleterious effects caused by stress exposure. Also, it was important to evaluate if this effect would remain throughout the chronic exposure to stressors for 3 additional weeks. For that purpose, behavioral analyses at the end of the 6th week of the uCMS protocol were performed. A positive effect of CEF in preventing the installation of anhedonic effects of stress was observed in the Sweet Drive Test; yet, this observation was not confirmed in the Sucrose Preference test. Inconclusive results in the SPT could be related to less discriminative potential to assess

anhedonic behavior compared to SDT, as previously described (Mateus-Pinheiro et al., 2014). Also, in the mood domain, CEF was effective in preventing the learned helplessness symptom observed in the stressed animals by the Forced Swim Test. These results suggest that CEF has preventive potential of mood impairments to the exposure of chronic stress. Regarding the analyses of anxiety and cognitive function, these were inconclusive, as we could not observe a stress effect in these two dimensions as previously described (Bessa et al., 2009a; Mateus-Pinheiro et al., 2013a; Patrício et al., 2014). In fact, uCMS exposure rendered effective alterations in corticosterone regulation, suggesting that the uCMS protocol worked in these animals. Therefore, at this point we still cannot rule out an effect of CEF also on these dimensions.

The second important aim of this study was to understand the potential of CEF treatment to revert the uCMS-induced deficits. For that, behavior tests were performed using stressed animals treated either with CEF, fluoxetine, imipramine (ADs) or with a combination of CEF and ADs. The efficacy of different classes of ADs to revert the deleterious effects of stress in the several behavioral dimensions commonly affected by stress was already verified and described (Bessa et al., 2009b; Duman, 2014; Mateus-Pinheiro et al., 2013a; Patrício et al., 2014). Regarding the Treatment Groups, CEF did not revert anxious-like behavior both in the EPM and NSF tests. However, fluoxetine was able to revert the effects of stress and it is hypothesized that the beneficial effects of the combination of CEF with either fluoxetine or imipramine may be an effect of the ADs alone. In addition, in the cognitive assessment, CEF could partially revert the impairments caused by stress on short-term memory, both in novelty recognition and spatial rearrangement, evaluated in the NOR test. In the MWM, CEF could partially revert the negative effects on behavior flexibility seen in uCMS animals. In both tests, CEF exerted an effect similar to ADs. The function of the prefrontal cortex (PFC) is linked to the computation of these cognitive tasks and is a brain region severely affected by stress (Cerqueira et al., 2007; Czéh et al., 2008; Jay et al., 2004; Shansky and Morrison, 2009). Therefore, brain tissue of these animals should be further studied to seek for cellular/molecular correlates that may underlie the rescue of PFC function by CEF-triggered mechanisms. In a number of behavioral tests, the uCMS groups did not present the marked phenotypic deficits described in the literature (Bessa et al., 2009b)(eg. Anxiety-like behavior in the Prevention Groups) when compared to the control groups. Although in many of the cases a clear tendency was visible, this lack of differences may be justified by the

control groups. Since CEF is administered by intraperitoneal injections, controls suffered also sham injections that may function as a repeated aggression/stress. In further studies, a novel non-injected control group should be added to exclude this possibility.

In order to understand the molecular and cellular mechanisms underlying the prevention of uCMS-induced mood impairments by CEF, brain tissues from animals in the Prevention Groups were analyzed. This analysis was performed in brain regions typically affected by uCMS such as the PFC, hippocampus in general and dentate gyrus (DG) in particular (Bessa et al., 2009b; Mateus-Pinheiro et al., 2013a). The goal of this analysis was to investigate a putative effect of CEF on dendritic remodeling, a major cellular effect of uCMS exposure namely by correlating it to expression levels of GLT-1 transporters or mRNA levels.

Rothstein *et al.* (2005) had previously reported that CEF as an increasing effect on GLT-1 astrocytic transporter expression. Therefore, in order to further study the CEF effect in the prevention of the alterations caused by stress, we performed RT-PCR analysis to assess mRNA GLT-1 expression levels and WB analysis to quantify GLT-1 protein in the brain regions analyzed. Detailed analysis of brain tissue collected from the tested animals revealed that the prevention of the depressive-like behavior was accompanied with an increase in the GLT-1 transcript and protein expression of GLT-1 transporter in hippocampal areas with important roles in these behaviors. Regarding the PFC, no significant differences were observed in GLT-1a and b transcript levels of uCMS animals. This lack of effect was also reflected in the protein levels. In the dorsal hippocampus, it was observed that gene expression of GLT-1a isoform is downregulated in control animals administrated with CEF (CT+CEF), which matches the decreased levels of GLT-1 protein in the dorsal DG of these animals. Despite of the lack of differences observed for GLT-1 transcript levels after uCMS or CEF administration, in the dorsal hippocampus, in the dorsal DG, CEF appears to prevent the effects of stress by maintaining the GLT-1 protein levels high. Interestingly, the mRNA of GLT-1b is downregulated in the ventral hippocampus of uCMS animals; CEF has prevented this effect through an upregulation of GLT-1b transcript. In the ventral hippocampus, CEF seems to interfere significantly only with the GLT-1b isoform. These results suggest a balance effect between both isoforms. Indeed, GLT-1 protein levels in the ventral DG do not change when comparing each group to respective controls – CT+CEF vs CT, uCMS vs CT and uCMS+CEF vs uCMS. This constant stage of GLT-1 protein level in the DG is probably reflecting the balance observed in the gene transcript in the remaining

hippocampus. Therefore, it may be hypothesized that depending on the physiological state of the animal and on the sub-region of the hippocampus analyzed, CEF appears to modulate the GLT-1 transporter isoforms transcript affected by the given condition. In further analysis, microdissection of hippocampal subregions should be performed to access intra-hippocampal molecular differences.

Since dendritic atrophy in the DG may underlie the depressive-like behavior caused by uCMS-exposure (Bessa et al., 2009b; Mateus-Pinheiro et al., 2013a; Patrício et al., 2014) the morphology of DG neurons was assessed. In the dorsal DG of the hippocampus a significant effect of CEF to prevent dendritic atrophy and spine densities was observed in uCMS animals. In fact, this effect of CEF was similar to the effect of commonly used ADs on neuron dendritic remodeling in comparison to animals exposed to chronic stress (Bessa et al., 2009b). However, the effect of CEF seems to be specific to the dorsal DG, since in the ventral DG this preventive effect was not clear. It was previously evidenced that along the DG septo-temporal axis there is a heterogeneous structure and function (Kheirbek et al., 2013; Tanti and Belzung, 2013), being the dorsal DG responsible mainly for learning and memory processes, whereas ventral DG is related to anxiety and emotional regulation (Tanti and Belzung, 2013). Regarding the behavioral results for the prevention groups, it would be also expected an impact on the ventral DG neuronal 3D morphology. Although ADs promote region specific effects on adult hippocampal neurogenesis along the DG septo-temporal axis (Felice et al., 2012; O'Leary et al., 2012), the implication of morphological changes in the DG granule neurons is yet to be fulfilled. Interestingly, it has been recently recognized that morphological and physiological changes along the septo-temporal axis of the hippocampus are contrastingly affected by stress exposure (Pinto et al., 2014). In summary, the decreased GLT-1 protein expression in the dorsal DG of uCMS animals seems to match the observed total spines density decrease and dendritic atrophy. Importantly, CEF prevented these effects, which seem to correlate with the prevention of the anhedonic behavior induced by uCMS, thus being the major discovery of this study. Because the main carriers of GLT-1 are astrocytes, future work should tackle alteration in these cells, in the different conditions studied. In particular, astrocyte numbers and morphology should be compared with GLT-1 protein and transcript levels and correlated with CEF preventive effect. A direct correlation to astrocytic markers (such as GFAP) and GLT-1 levels should be expected.

Since CEF is used in the clinics as an antibiotic, it may be possible to ascribe some of the behavior observations to the alterations of the microbiome in the studied animals. Emerging evidence on the importance of stress-related microbiotic alterations in the CNS has been taken into consideration, suggesting bidirectional interplay between the gut and the brain (Foster and McVey Neufeld, 2013; Moloney et al., 2014). This interplay encompasses a communication between neuronal networks and hormonal signaling, including humoural signaling molecules, promoting an alteration of gastrointestinal and brain function (Mayer, 2011; Moloney et al., 2014; Rhee et al., 2009). In fact, treatment with antibiotics can cause a fluctuation in the composition of an individual flora, as it has been reported to significantly reduce the microbial number and diversity in healthy adult mice (Bech-Nielsen et al., 2012). Although, antibiotic administration has been suggested to promote a beneficial effect on behavioral dimensions such as anxiety and exploratory behavior (Bercik et al., 2011), in our study CEF administration in control animals exerted an opposite effect causing higher anxious-like behavior in the EPM behavioral paradigm. In this case, the effect was not confirmed in additional anxiety tests, therefore this observation was rather inconclusive. It has already been described that the epithelial barrier of the gastrointestinal tract is compromised as a result of stress. As a consequence, the gastrointestinary tract permeability increases and translocation of gram-negative bacteria across the mucosal occurs, leading to a further immune response characterized by the production of inflammatory mediators. These mediators could be correlated to an increase of IgM and IgA against LPS of enterobacteria on serum concentrations in MDD patients (Maes et al., 2008; Moloney et al., 2014). Moreover, a link between emotional stressors and negative alterations in the gut microflora has been revealed for both humans and animal studies (Bailey et al., 2011; Collins and Bercik, 2013; O'Mahony et al., 2009). In fact, in this study we could observe positive effects of CEF administration on anhedonic behavior of stressed animals. In infection animal models known to have altered microbiota profile, increased anxious-like behavior is associated to a reduction in the BDNF mRNA or protein levels, which can be restored by treatment with probiotics (Bercik et al., 2010, 2011). This work can be transposed to AD treatment effects on restoring the levels of BDNF expression that is decreased after stress exposure (Duman and Monteggia, 2006; Martinowich and Lu, 2008). Furthermore, the use of CEF antibiotic to prevent or treat the installation of chronic stress suggests an effect not only on the behavioral mood domain but also on molecular levels of its target, the GLT-1 transporter.

Altogether, these findings help to support the emerging need to unveil the physiological causes of depression and therefore, to develop new strategies for novel therapeutics based on molecular targets involved in affected mechanisms, such as the GLT-1 transporter. Here, we have shown that CEF prevented the installation of chronic stress-related depressive behavior, by sustaining the levels of GLT-1 transporters in the dorsal DG. Under chronic stress-exposure, increase of excitatory neurotransmitter release seems to be counteracted by the action of GLT-1, which in turn seems to prevent the typical dendritic atrophy in the dorsal DG and consequent depressive-like behavior, typical of the model of depression used in this study. This work may pave the way to the development of new more directed drugs to target GLT-1 expression without possible secondary deleterious effects of the antibiotic on the microbiota-brain-gut axis and other systems.

V. FUTURE PRESPECTIVES

5. FUTURE PRESPECTIVES

The present work has explored the mechanisms by which ceftriaxone (CEF) antibiotic impacts on depression-related behavior, GLT-1 transcript and protein expression levels and also on dendritic remodeling. At this stage, a number of additional questions arise which should be addressed in future work:

- The manifestation of the effects of stress remains inconclusive for behavioral paradigms such as open field (OF) or elevated plus maze (EPM), since the performance of controls is similar to that of stressed animals. Control animals may present a minor level of stress induced by the repetitive sham injections. Therefore, this possibility should be excluded by establishing an additional group of non-injected controls, in a future experimental set.

- The interesting effects of CEF in the reversion of the cognitive deficits triggered by uCMS-exposure should be further addressed, namely by seeking cellular and molecular correlates in brain areas responsible for these tasks (e.g. prefrontal cortex).

- In order to complement the analyses of 3D morphological neuronal alterations, further astrocytic morphological changes should be accessed. In particular, at astrocyte numbers and morphology should be correlated with GLT-1 protein and transcript levels and compared to CEF preventive effects. A direct correlation to astrocytic markers (such as GFAP) and GLT-1 levels is expected. This evaluation could be performed by means of immunohistochemistry techniques such as 3,3'-Diaminobenzidine (DAB) staining or immunofluorescence double-lab§eling of these cells with glial fibrillary acidic protein (GFAP) and GLT-1 transporter.

- Alternative methods for targeting overexpression of GLT-1 in astrocytes, by using a adeno-associated virus type 8 (AAV8)-Gfa2 vector (Li et al., 2014) and characterization of CEF effects using a transgenic BAC-GLT-1-e-GFP reporter mice (Lepore et al., 2011) to access GLT-1 should be implemented. Indeed, during this project collaboration was established with the Thomas Jefferson University (Lepore lab) in order to exchange expertise in these models in the near future.

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VI. REFERENCES

6. REFERENCES

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