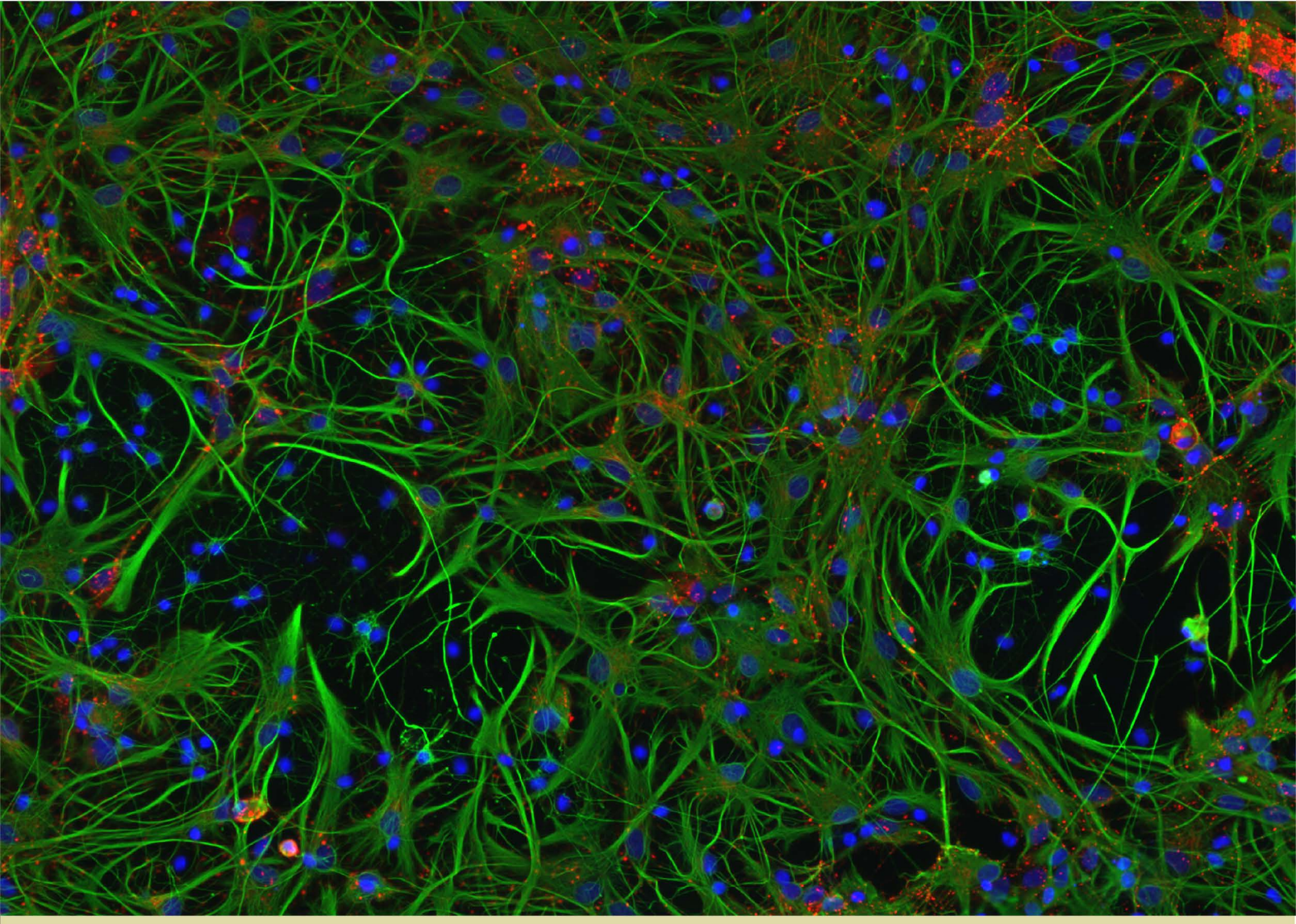

Patrícia Inês Pires dos Santos

The role of astrocytic A_{2A} receptors in depressive-like conditions

Dissertação de Mestrado em Biologia Celular e Molecular

Agosto 2017

· U C ·

Universidade de Coimbra

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The role of astrocytic A_{2A} receptors in depressive-like conditions

O papel dos recetores A_{2A} astrocíticos em condições depressivas

Patrícia Inês Pires dos Santos

Dissertação apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada pela orientação científica da Doutora Paula Canas (Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e do Professor Doutor Ângelo Tomé (Departamento de Ciências da Vida, Universidade de Coimbra).

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Cover image: dexamethasone-treated primary astrocytes from rat brain cultured *in vitro* for 15 days (green: Glial fibrillary acidic protein (GFAP); red: connexin 43; blue: nuclei)

The experimental work described in this dissertation was performed at the Center for Neuroscience and Cell Biology (CNC), in Coimbra, in the "Neuromodulation group" ("Purines at CNC").

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“When you realize there is something you don't understand, then you're generally on the right path to understanding all kinds of things.”

Jostein Gaarder

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ABBREVIATIONS

A_1R	Adenosine A_1 receptor
$A_{2A}R$	Adenosine A_{2A} receptor
$A_{2B}R$	Adenosine A_{2B} receptor
A_3R	Adenosine A_3 receptor
ACTH	Adrenocorticotropic hormone
AD	Alzheimer's disease
AMP	Adenosine monophosphate
AR	Adenosine receptor
ANOVA	Analysis of variance
ANS	Autonomic nervous system
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BBB	Blood-Brain barrier
BCA	2-(4-carboxyquinolin-2-yl)quinoline-4-carboxylic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
Ca^{2+}	Free calcium ion
$[Ca^{2+}]_i$	Intracellular calcium concentration
CAPS	3-(cyclohexylamino)propane-1-sulfonic acid
CBF	Cerebral blood flow
CBX	Carbenoxolone
CD73	Ecto-5'-nucleotidase
CRF	Corticotropin-releasing factor
CLAP	Cocktail of proteases inhibitors
CNS	Central nervous system
CTR	Control
Cx	Connexin
Cx26	Connexin 26
Cx40	Connexin 40
Cx43	Connexin 43
DBS	Deep brain stimulation
DEX	Dexamethasone
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Enhanced chemiluminescence (ECL)
DSM-5	Diagnostic Statistical Manual 5th edition
DTT	1,4-bis(sulfanyl)butane-2,3-diol
EAAT1	Excitatory amino-acid transporter 1
EAAT2	Excitatory amino-acid transporter 2
EAAT3	Excitatory amino-acid transporter 3
EAAT4	Excitatory amino-acid transporter 4
EDTA	2,2',2",2'"-(Ethane-1,2-diyldinitrilo) tetraacetic acid
ECL	Enhanced chemiluminescence
ECT	Enhanced chemiluminescence (ECL)
FBS	Fetal Bovine Serum
GABA	Gama-amino butyric acid

GC	Glucocorticoids
GFAP	Glial fibrillary acidic protein
GJ	Gap junction
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter
Glu	Glutamate
GR	Glucocorticoid receptors
GS	Glutamine synthetase
HCs	Hemichannels
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HPA	Hypothalamic-pituitary-adrenal
HD	Huntington's disease
INMLCF	Instituto Nacional de Medicina Legal e Ciências Forenses, I. P.
MDD	Major depressive disorder
MR	Mineralocorticoid receptors
NMDA-R	N-methyl-D-aspartate receptor
NO	Nitric oxide
NS	Not significant
PBS	Phosphate buffer saline
PD	Parkinson's disease
P_{2X}R	Purinergic 2X receptors
P_{2Y}R	Purinergic 2Y receptors
PFC	Prefrontal cortex
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulfonyl fluoride
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
SCH58261	7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4 triazolol[1,5c]pyrimidine
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SUI	Suicide
TBS	Trizma buffered saline
TBS-T	TBS with tween 20
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
TMS	Transcranial magnetic stimulation
VGLUT	Vesicular glutamate transporters
VRAC	Volume-regulated anion channels

ABSTACT

Major Depressive Disorder (MDD), the most common of all psychiatric disorders, is defined by episodes of low mood or anhedonia combined with several other symptoms that decrease interest in daily activities. This pathology seems to be associated with alterations in brain regions that control emotional and stress responses. There is a direct relation between cumulative stress and the incidence of depression. The etiology of depression is not fully understood and anti-depressive treatments are not 100% effective, which reinforces the importance of research work to discover new targets for better treatments. So far, there are several hypotheses to explain what can cause the disease. This work will focus mainly, on dysfunction of hypothalamic-pituitary-adrenal (HPA) axis and glutamatergic hypothesis as potential explanations for MDD.

The HPA axis impairments are associated to negative feedback dysfunction, leading to an increased level of glucocorticoids. These hormones are well established to affect synaptic plasticity and to contribute for synaptic atrophy in several brain regions involved in emotional processing and memory, namely amygdala, ventral striatum, hippocampus and prefrontal cortex. On the other hand, glutamatergic hypothesis, suggests that depression results in part from an imbalance of neuronal circuits and dysregulation of glutamate neurotransmission. Glutamate is the major excitatory neurotransmitter in the brain, and plays an essential role in synaptic plasticity that is impaired in depression. Moreover, an overstimulation of glutamate receptors can result in excitotoxicity, and neuronal death. The processing of information in synapses is defined by neurons and also by astrocytes and microglia, which interact with synapses in an activity-dependent manner. In MDD there are alterations of the inter-relations between glial cells and neurons, leading to astrocytic and microglia dysfunction and consequently an aberrant gliotransmitters release among other reported alterations, which results in inadequate encoding of information.

Adenosine is a neuromodulator that can control synaptic transmission and plasticity directly and through their effects on astrocytes. Moreover, the consumption of caffeine it is inversely correlated with the incidence of depression and suicide. The only molecular targets of caffeine at moderate doses are A_1 and A_{2A} receptors (A_1R, $A_{2A}R$), which are the main adenosine receptors in the brain. In stressed conditions $A_{2A}R$ are up-regulated and their over-expression induces anhedonia and depressive like-behavior and, conversely, $A_{2A}R$ blockade prevents mood and memory deficits induced by chronic unpredictable stress, through a control of synaptic dysfunction and astrogliosis.

The main goal of this work was to study the role of astrocytic A_{2A} receptors in depressive-like conditions: i) incubate astrocytic cultures with a stress mediator and evaluate the release of

gliotransmitters, namely glutamate and ATP; ii) validate the gliosomal preparation in human postmortem tissue; and later on, iii) try to evaluate alterations in glial proteins and adenosine receptors in suicide completers.

To comprehend the effect of stressors in gliotransmission we exposed astrocyte primary cell culture to DEX and we observed an increase in extracellular glutamate and ATP levels in DEX-exposed cells when compared to non-treated cells. We also observed that this increase in extracellular ATP levels was maintained until 24h of DEX-incubation. Based on this increase of extracellular gliotransmitters levels, we then started to look into to the possible release mechanisms.

Moreover, we observed that DEX induce alterations in the astrocytes morphology and an increase in connexins (Cx43) as also as glutamine synthetase (GS) levels.

In addition we observed a tendency for DEX to induce an increase of $A_{2A}R$ levels, which corroborated the prevention effect observed, by the blockade of $A_{2A}R$, in preventing DEX induced alterations (change in the astrocytic morphology, increase of Cx43 immunoreactivity and ATP release), with an exception for glutamate release. In addition, for the first time, it was described a physical interaction of $A_{2A}R$ with Cx43.

To attain the second goal, it was performed an optimization and validation of the gliosomal preparation. Moreover, we observed an increased excitatory amino acid transporter 1 (EAAT1) density in suicide completers, whereas levels of glial fibrillary acidic protein (GFAP), Cx43 and GS remained unaltered in suicide completers when compared with age-matched controls.

At last, it was also observed that synaptic and glial markers change with age. We described an up-regulation of GFAP density; a down-regulation of synaptophysin and GS levels; and no alterations in syntaxin, Cx43 and EAAT1 densities with aging.

In sum, this study contributed to improve knowledge about the role of astrocytic $A_{2A}R$ and Cx43 and to reinforce them as possible targets to alleviate the consequences of depression. Moreover, this study also provided information about protein alterations present in brains of suicide completers, which might contribute to the development of novel biomarkers to improve diagnosis and treatment of depression and mood disorders.

Keywords: Major Depressive Disorder; suicide; astrocytes; glucocorticoids; adenosine A_{2A} receptor; glutamate; ATP

RESUMO

A depressão *major* (MDD, do inglês *Major Depressive Disorder*), é um dos distúrbios do foro psiquiátrico mais comum. A depressão *major*, é caracterizada por episódios de humor negativo ou anedonia combinados com vários outros sintomas que diminuem o interesse nas atividades do quotidiano. Esta patologia parece estar associada a alterações em regiões cerebrais que controlam respostas emocionais e relacionadas com o estresse. Existe uma relação direta entre o estresse acumulado e a incidência de depressão. A etiologia da depressão não é totalmente compreendida e os tratamentos antidepressivos não são 100% efetivos, o que reforça a importância do trabalho de pesquisa para descobrir novos alvos para melhores tratamentos. Até ao momento, existem várias hipóteses para explicar o que pode causar a doença. No entanto, este trabalho incidirá principalmente na disfunção do eixo hipotálamo-hipófise-adrenal (HPA, do inglês *hypothalamic-pituitary-adrenal*) e hipóteses glutamatérgicas como potenciais explicações para o aparecimento da MDD.

Alterações no eixo HPA estão associadas a disfunções do *feedback* negativo, levando a um aumento dos níveis de glucocorticóides. Está bem estabelecido que essas hormonas afetam a plasticidade sináptica e contribuem para a atrofia sináptica em várias regiões cerebrais envolvidas no processamento emocional e na memória, nomeadamente amígdala, estriado ventral, hipocampo e córtex pré-frontal. Por outro lado, a hipótese glutamatérgica, sugere que a depressão resulta em parte de um desequilíbrio de circuitos neuronais e desregulação da neurotransmissão de glutamato. O glutamato é o principal neurotransmissor excitatório no cérebro, e desempenha um papel essencial na plasticidade sináptica que se encontra diminuída na depressão. Além disso, uma estimulação anormal dos recetores de glutamato pode resultar em excitotoxicidade e morte neuronal. O processamento de informação em sinapses é definido por neurónios e ainda por astrócitos e microglia, que interagem com as sinapses dependentemente da atividade. Na MDD há alterações nas inter-relações entre células gliais e neurónios, levando a uma disfunção astrocítica e microglial e consequentemente a uma libertação aberrante de gliotransmissores entre outras alterações relatadas, o que resulta num processamento inadequado de informação.

A adenosina é um neuromodulador que pode controlar a transmissão sináptica e a plasticidade diretamente e através dos seus efeitos sobre os astrócitos. Além disso, o consumo de cafeína é inversamente correlacionado com a incidência de depressão e suicídio. Os únicos alvos moleculares da cafeína, quando consumida em doses moderadas, são os recetores A_1 e A_{2A} para a adenosina (A_1R, $A_{2A}R$), que são os principais recetores de adenosina no cérebro. Em condições estressantes, existe um aumento dos níveis dos $A_{2A}R$. A sua sobreexpressão induz anedonia e comportamentos depressivos e, inversamente, o bloqueio dos $A_{2A}R$ evita défices de memória e

distúrbios de humor induzidos pelo estresse crónico imprevisível, através do controlo da disfunção sináptica e da astroglióse.

O objetivo principal deste trabalho foi estudar o papel dos recetores astrócitos A_{2A} em condições de depressão: i) incubar culturas astrocíticas com um estresssor e avaliar a libertação de gliotransmissores, nomeadamente glutamato e ATP; ii) validar a preparação gliossomal em tecido *postmortem* humano; e mais tarde, iii) tentar avaliar as alterações nas proteínas gliais e nos recetores de adenosina em suicidas.

Para compreender o efeito dos estressores na gliotransmissão, expusemos a cultura de células primárias de astrócitos a DEX e observámos um aumento nos níveis extracelulares de glutamato e ATP em células expostas a DEX quando comparadas às células não tratadas. Observámos também que esse aumento nos níveis de ATP extracelular foi mantido até 24 horas de incubação com DEX. Com base nesse aumento dos níveis de gliotransmissores extracelulares, começámos a analisar possíveis mecanismos de libertação.

Além disso, observámos que a DEX induz alterações na morfologia dos astrócitos e um aumento nos níveis das conexinas 43 (Cx43), assim como nos níveis de glutamina sintetase (GS).

Além disso, observamos uma tendência para a DEX induzir um aumento nos níveis dos $A_{2A}R$, que corroborou o efeito de prevenção observado pelo bloqueio dos $A_{2A}R$, na prevenção de alterações induzidas pela DEX (alteração na morfologia astrocítica, aumento da imunoreatividade das Cx43 e libertação de ATP), com uma exceção para a libertação de glutamato. Adicionalmente, pela primeira vez, descreveu-se uma interação física dos $A_{2A}R$ com as Cx43.

Para alcançar o segundo objetivo, foi realizada uma otimização e validação da preparação dos gliossomas. Posteriormente, nos suicidas observámos um aumento da densidade de transportadores de aminoácidos excitatórios 1 (EAAT1, do inglês *excitatory amino acid transporter* 1), enquanto os níveis de proteína ácida fibrilar glial (GFAP, do inglês *glial fibrillary acidic protein*), Cx43 e GS permaneceram inalterados aquando comparados com controlos da mesma idade.

Por último, também se observou que os marcadores sinápticos e gliais mudam com a idade. Descrevemos um aumento da densidade de GFAP; uma dimuição dos níveis de sinaptofisina e GS; e ausência de alterações nas densidades de sintaxina, Cx43 e EAAT1 com o envelhecimento.

Em suma, este estudo contribuiu para melhorar o conhecimento sobre o papel dos $A_{2A}R$ astrócitos e das Cx43 e reforçá-los como possíveis alvos para aliviar as consequências da depressão. Além disso, este estudo também forneceu informações sobre alterações proteicas presentes em cérebros de suicidas, o que poderá contribuir para o desenvolvimento de novos biomarcadores para melhorar o diagnóstico e tratamento de depressão e distúrbios de humor.

Palavras-chave: Transtorno Depressivo *Major*; suicídio; astrócitos; glucocorticóides; recetores A_{2A} para a adenosina; glutamato; ATP

CHAPTER 1

INTRODUCTION

1.1. MAJOR DEPRESSIVE DISORDER AND DISEASE HYPOTHESIS

1.1.1. Major Depressive Disorder definition

Major Depressive Disorder (MDD) (also known as simply depression), the most common of all psychiatric disorders (Mayberg et al., 2005), is clinically defined by the continuous presence of episodes of low mood or anhedonia combined with several other symptoms that decrease interest in daily activities for more than 2 weeks. According to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V) criteria, a patient should have at least five of the following persistent symptoms to be diagnosed with depression: (i) **Depressed mood** – most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad, empty or hopeless) or observation made by others (e.g., appears tearful); (ii) **Anhedonia** – decreased interest or pleasure in almost all activities most of the day; (iii) **Changes in appetite/weight** – significant weight loss (5%) or decrease or increase in appetite; (iv) **Changes in sleep** – insomnia or hypersomnia; (v) **Changes in movement** – psychomotor agitation or retardation; (vi) **Fatigue or loss of energy**; (vii) **Guilt/worthlessness** – feelings of worthlessness or excessive or inappropriate guilt; (viii) **Concentration** – diminished ability to think or concentrate, or indecisiveness; (ix) **Suicidality** – recurrent thoughts of death (not just fear of dying) or suicide, or has suicide plan.

Since the definition of MDD has a temporal component, this disorder is often described in terms of "episodes", a period of time where symptoms are present but potentially not permanent. Indeed, the goal of treatment for depressed patients is to relieve these symptoms and end an episode. The duration of episodes can be in order of weeks to years, and patients can experience multiple episodes over their lifetime. However, disease phenotypes of MDD include not only episodic and persistent mood alterations, but also diverse cognitive, autonomic, motoric and endocrine abnormalities, and effects on multiple organ system (Manji et al., 2001).

The neurobiology and neuronal circuitry underlying this pathology is not fully understood, nevertheless, some hypotheses that try to explain the etiology of depression have been emerged, such as, genetic vulnerability, deficiency of monoamines, alterations in neurotrophic factors (e.g. BDNF), alterations in the activity of hypothalamic-pituitary-adrenal (HPA) axis (discussed in section 1.1.2), dysfunction of specific brain regions, dysregulation of glutamatergic (discussed in section 1.1.3) and GABAergic system (Hasler, 2010). Additionally, depression occurs idiopathically, so various risk factors as appear as reflect of limited understanding of its etiology, such as, stressful life events, cancers (such as pancreatic adenocarcinoma and breast tumours), endocrine abnormalities (hypothyroidism and hypercortisolism) and side effects of drugs, among many others (Krishnan and Nestler, 2008). Moreover, in the majority of people, depression results from the interplay between a genetic predisposition and some environmental factors, such as

stress, and for this reason depression has been defined as a stress-related disorder (Krishnan and Nestler, 2008).

In spite of all the knowledge about depression, we are still far from being able to complete the puzzle. Part of the current understanding about this pathology has come from animal models of the disease. However due to the unique and complex features of human depression, such as depressed mood, low self-esteem and suicidality, the generation of valid and insightful depression models that truly mimics patient symptoms has been difficult (Krishnan and Nestler, 2011; Yan et al., 2010). So far, some stress-induced animal model has been created, such as, the learned helplessness (Chourbaji et al., 2005), the chronic mild stress (Willner, 2005), the social defeat stress (Golden et al., 2011) and the early life stress (Ladd et al., 2000). Besides these models, other approaches are used, such as, pharmacological, lesion and mutant models (Yan et al., 2010). As previously mentioned, the major difficulty in research of depression pathogenesis is the fact that animal models only represent some of the MDD features, but not the pathogenesis of the disease (Yan et al., 2010). Therefore, an alternative to try to understand the neurobiological basis of depression is the study of the brain tissue from suicide completers, since the vast majority of people that commit suicide are depressive patients or had a depressive episode (Coryell and Young, 2005; Rihmer, 2007).

Several brain regions, major components of the limbic-reward circuitry, such as prefrontal cortex (PFC) and hippocampus, appear to be involved in depression pathogenesis (reviewed in (Bagot et al., 2014; Russo and Nestler, 2013). This is supported by neuroimaging studies in depression patients or evaluation of postmortem brain samples from suicide completers that provided evidence of abnormalities in those brain regions. These studies demonstrated alterations in the cerebral blood flow (CBF), glucose metabolism (Mayberg, 2003). In addition, volumetric alterations were also observed in hippocampus (Bremner et al., 2000; Caetano et al., 2004; Frodl et al., 2006; Sheline et al., 2003), basal ganglia (Bonelli et al., 2006; Hickie et al., 2007; Pillay et al., 1998), caudate nucleus, putamen and globus pallidus (Lorenzetti et al., 2009) and Brodmann area 25 (BA25) (Gotlib et al., 2005; Pizzagalli et al., 2004). Dysfunction in these specific brain areas appears to be related with neuronal atrophy and synaptic and glial loss, which result in disruption of circuits (Kang et al., 2012; MacQueen and Frodl, 2011). Furthermore, in depression it was also reported alterations to the serotonergic, dopaminergic, noradrenergic (Moylan et al., 2013), cholinergic, GABAergic and glutamatergic neurotransmission systems (Manji et al., 2001).

Several treatments have been developed to try to relieve the symptoms associated to depression. The majority of approved classes of antidepressants act on controlling monoamine levels (Krishnan and Nestler, 2008). Antidepressants and certain forms of psychotherapy and

electroconvulsive therapy (ECT) (Nemeroff and Owens, 2002) are some of the several available therapeutics, albeit, these treatments are not 100% effective, showing complete remission in less than half of patients and partial or intolerant responses to treatment in other cases (Nemeroff and Owens, 2002). Based on neuroanatomical research, new treatment techniques are emerging, such as deep brain stimulation (DBS) (Holtzheimer and Mayberg, 2010; Malone et al., 2009; Mayberg et al., 2005) and transcranial magnetic stimulation (TMS) (Carpenter et al., 2012; Janicak et al., 2013), but the long-term benefits and side effects are still unknown. For these reasons, it is important to understand the etiology of depression and to discover new biomarkers and potential targets to develop new anti-depressant therapies.

1.1.2. The HPA axis hypothesis

One of the hypothesis to explain depression is the HPA axis hypothesis. Actually, half of the depressive patients display an hyperactivity of this axis, and the number increases to 80% in severely depressed patients (Holsboer, 2000; Pariante, 2003; Pariante and Miller, 2001; Young et al., 1991). In fact, patients with depression have hypercortisolemia, an increased baseline concentration of the glucocorticoid (GC) cortisol in the blood and presumably in the brain (Wong et al., 2000). In addition, it was described alterations in glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) in depression (reviewed in (Anacker et al., 2011)) and anxiety (reviewed in (Lucassen et al., 2014)).

Stress is a state of threatened homeostasis evoked by stressors (intrinsic or extrinsic adverse forces) and the stress response is a countermeasure to restore physiological and behavior homeostasis. This response is primary mediated by the brain in two major steps. Firstly, as a response to a stressor, there is an activation of the autonomic nervous system (ANS), in particular the sympathetic nervous system (SNS). Sympathetic activity climaxes by catecholamine secretion (e.g. norepinephrine), which in turn leads to heartbeat acceleration, dilation of the pupils and raised heart pressure (Barrett et al., 2016; Welberg, 2013). The second phase consists in the hypothalamic-pituitary-adrenal (HPA) axis response (Figure 1). However, under chronic stress conditions, there is an excessive and prolonged HPA axis stimulation that leads to persistently elevated glucocorticoids and increase the difficulty to maintain homeostasis (Duman and Aghajanian, 2012; de Kloet et al., 2005; Lucassen et al., 2014).

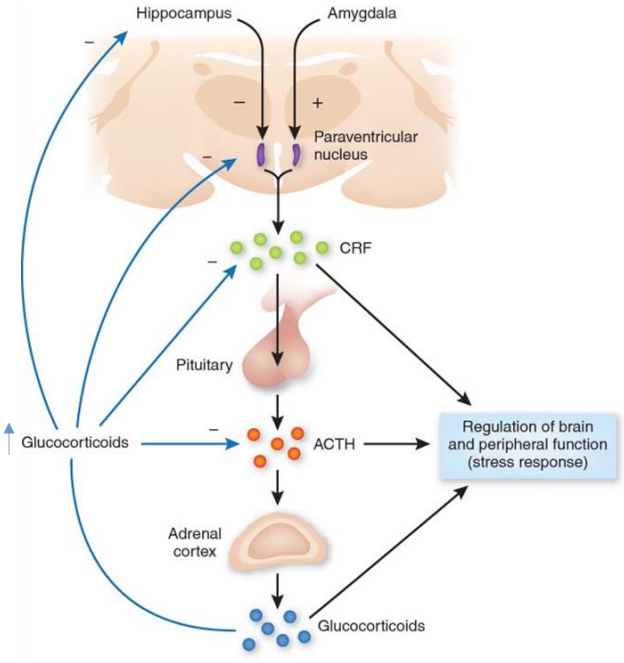

Figure 1 | Hypothalamic-pituitary-adrenal (HPA) axis regulation. Corticotropin-releasing factor (CRF), the principal central biologic effector of the stress response, is secreted from the hypothalamus into the anterior pituitary by the capillaries of the hypophyseal portal vessels. In the pituitary, CRF stimulates adrenocorticotropic hormone (ACTH) synthesis and secretion. ACTH is transported via systemic circulation to the adrenal gland, where it enhances glucocorticoid (e.g. cortisol) synthesis and secretion. Interestingly, glucocorticoid, is involved in a negative feedback loop that inhibits CRF and ACTH secretion, plus stimulating catabolic processes in an attempt to restore homeostasis. Adapted from: Hyman, 2009.

In the brain, GCs will bind to both glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), preferentially binding to MR due to its higher affinity (Sapolsky, 1996). However, upon a stressful event, elevated levels of GCs will promote their binding to GR, (Myers et al., 2014) (Figure 2).

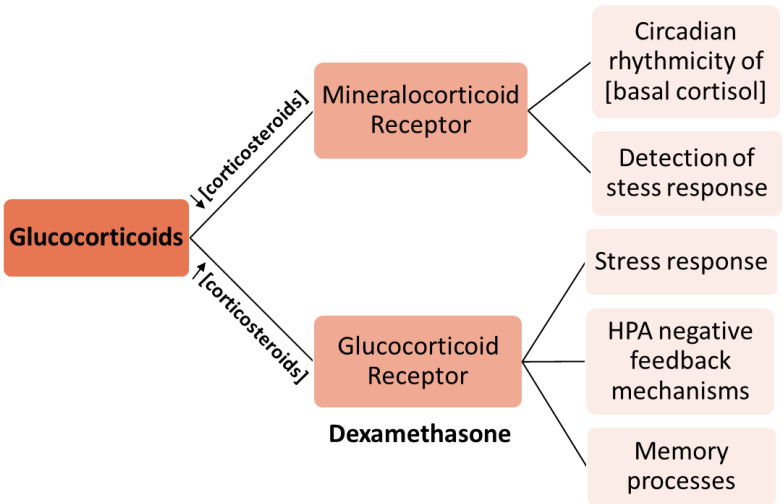

Figure 2 | General associations mediated by glucocorticoids. Glucocorticoids can act through two receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). These receptors have differential affinities for different glucocorticoids. The difference in affinity for glucocorticoids has led to the general associations of MR action

with lower concentrations of corticosteroids (e.g. circadian rhythmicity of basal cortisol concentrations, initiation/detection of stress response) and GR action with higher concentrations of corticosteroids (e.g. stress response, HPA negative feedback mechanisms, recovery/memory processes). Dexamethasone is the primary GR-selective agonist and this is the motive that dexamethasone is important to understand stress response.

So far, there are several hypotheses that involve glucocorticoid receptors have been formulated to explain how this phenomenon could occur (Oitzl et al., 2010). In the glucocorticoid cascade hypothesis, chronic stress induces an excessive release of glucocorticoids and a downregulation of glucocorticoid receptors in the brain, which result in cellular damage to the brain (Conrad, 2008). In the balance hypothesis, an imbalance in the glucocorticoid receptors in specific brain regions is thought to alter signaling in neural circuits, resulting in alterations in behavior and HPA axis signaling (reviewed in (Lucassen et al., 2014)). These hormones are also well established to affect synaptic plasticity and to contribute for synaptic atrophy in several brain regions involved in emotional processing and memory, namely amygdala, ventral striatum, hippocampus and PFC (reviewed in Russo and Nestler, 2013).

1.1.3. The glutamatergic hypothesis

Another hypothesis to explain how depression occurs in the brain is the well-developed glutamate hypothesis, which states that depression results in part from an imbalance of neuronal circuits and dysregulation of glutamate neurotransmission. This hypothesis started with the observation in the early 90's, that N-methyl-D-aspartate receptor (NMDA-R) antagonists possess antidepressant-like action (Trullas and Skolnick, 1990).

Overall, the brain can be compared to a glutamatergic excitatory engine that is tightly regulated by gamma-aminobutyric acid (GABA) and other neurotransmitters. Therefore, any alteration to the ratio glutamate/GABA may contribute to the development of a pathological situation. In fact, it has been reported that, an augmented extracellular glutamate concentration has a key role in neurodegenerative diseases, since it can cause excitoxicity, and later on, neuronal degeneration (Hardingham and Bading, 2010). So, a rapid removal of this neurotransmitter in excess is necessary, to assure a proper functioning of the brain, such as a normal synaptic plasticity. Glutamate reuptake is performed mostly by astrocytes, by specific sodium-dependent high-affinity glutamate transporters, such as glutamate aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1), which correspond to human EAAT1 and EAAT2, respectively (Anderson and Swanson, 2000; Bak et al., 2006; Chaudhry et al., 1995; Palacin et al., 1998). After the reuptake, the astrocytes convert glutamate into glutamine, via the glutamine synthetase pathway,

and then release it into the extracellular space. Glutamine is taken up by neurons, which use it to generate again glutamate and GABA, the main excitatory and inhibitory neurotransmitters respectively (Bak et al., 2006). In addition GABA can also be taken up and metabolized by astrocytes (Jursky et al., 1994).

In depression, glutamatergic neurotransmission is known to be increased in limbic-thalamo-cortical circuits (Drevets et al., 2008) whereas GABAergic transmission is downregulated (Sanacora et al., 1999). These alterations may be due to changes observed in genes related to glutamatergic and GABAergic transmission (Sequeira et al., 2009). Alterations of both excitatory and inhibitory circuits in the PFC of rodents has been found to play also a pivotal role in depressive-like behaviors (Manji et al., 2001). Clinical studies have also reported alterations in glutamate metabolites in the brains of depressed individuals (Auer et al., 2000; Hasler et al., 2007). Furthermore, the glutamate hypothesis of depression is further supported by recent studies that revealed antidepressant drugs target directly or indirectly glutamatergic signaling (Banasr et al., 2010; Berman et al., 2000).

1.2. ASTROCYTES IN THE CNS

1.2.1. Astrocytes definition

The nervous system is composed of neurons and non-neural cells that have distinct roles in supporting the brain physiology. Non-neural cells are called glial cells, or neuroglia, and they support and protect neurons and perform other functions. In the past, neurons have received the majority of attention in neuroscience research, with little regard for other cell types, because of their well-accepted role in transmitting information in the brain. However, nowadays, the scientific paradigm changed and researchers are also focusing their attention in glia cells, since it was discovered that these type of cells are responsible for multiple complex and essential functions in the healthy CNS and can also have a role in pathological conditions. Glial cells are classified in two main groups: microglia and macroglia. Macroglia are generally subdivided into four specialized cell types: ependymal cells, Schwann cells, oligodendroglia, and astroglia.

Astrocytes are estimated to be the most abundant glial cell in the brain and can be categorized into subpopulations based on their morphology and location in the brain (Miller and Raff, 1984). Astrocytes have been classically categorized into two main subtypes based on differences in cellular morphology and anatomical locations, fibrous astrocytes and protoplasmic astrocytes (Cajal and Ramon, 1911). Fibrous astrocytes are found throughout all white matter and exhibit a morphology of many long and less complex fiber-like processes as compared with protoplasmic astrocytes (Cajal and Ramon, 1911; Wang and Bordey, 2008) (Figure 3A). Protoplasmic astrocytes are typically found in gray matter and demonstrate non-overlapping arrangements where each astrocyte generate many finely branching processes in globoid distribution and appears to occupy a unique domain, an anatomy referred to as "tiling" (Oberheim et al., 2012) (Figure 3B). According to microscopic analyses, the processes of protoplasmic astrocytes envelop synapses and the processes of fibrous astrocytes contact nodes of Ranvier, and both types of astrocytes form gap junctions between distal processes of neighboring astrocytes (Sofroniew and Vinters, 2010).

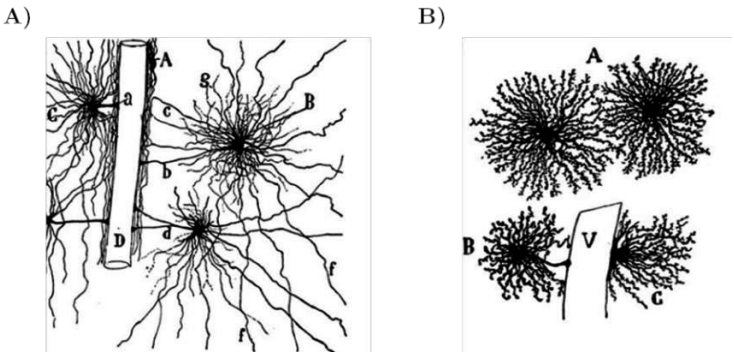

Figure 3 | Classical drawings of fibrous astrocytes (A) and protoplasmic (B) from adult human cortex. From: Cajal and Ramon, 1911.

1.2.2. The role of astrocytes in the CNS

Astrocytes, as dynamic components of brain function, are known to play many different functions:

(i) responsible for metabolic support and nutrition (Eulenburg and Gomeza, 2010; Simard and Nedergaard, 2004);

(ii) regulate brain-blood barrier (BBB) (Mishra et al., 2016; Pascual et al., 2005);

(iii) control oxidative stress in the brain (Parpura et al., 2012);

(iv) play a role in immune function (Sofroniew, 2015);

(v) regulate information processing and synaptic connectivity in the brain (Allen et al., 2012; Christopherson et al., 2005; Chung et al., 2013; Henneberger et al., 2010; Min and Nevian, 2012; Panatier et al., 2011; Singh et al., 2016; Yang et al., 2003);

(vii) control sleep and waking states (Parpura et al., 2012).

In the 90's, existence of bidirectional communication between neurons and astrocytes was identified, and the concept of 'tripartite synapse' was proposed (Araque et al., 1999). It represents a new concept in synaptic physiology wherein, in addition to the information flow between the pre- and postsynaptic neurons, astrocytes exchange information with the synaptic elements by responding to synaptic activity with the overall goal of modulating synaptic transmission (Araque et al., 1999). More recently, a new synaptic player was added, the microglia cells changing the concept of 'tripartite synapse' to a 'quad-partite synapse' concept. Several studies revealed that microglia phylopodia dynamically interact with astrocytes and neurons to regulate and support brain homeostasis (Hanisch and Kettenmann, 2007; Raivich, 2005; Wake et al., 2013). In fact, it was reported that microglia can interact with synapses in an activity-dependent manner (Biber et al., 2007; Kettenmann et al., 2013; Wake et al., 2013). Microglia can also affect neurotransmission through the release of a variety of signals ranging from chemokines (Schafer et al., 2012), cytokines (Rebola et al., 2011), BDNF (Parkhurst et al., 2013), NO (Zhan et al., 2014), glutamate and D-serine (Scianni et al., 2013) or purines (George et al., 2015; Pascual et al., 2012). These functions imply that astrocytes and microglia are active participants in brain activity, rather than passive elements in maintaining the extracellular space and can had a new level of transmission of information in neuronal networks (Araque et al., 1999; Halassa et al., 2007).

Several studies suggest that astrocytes in a response to neurotransmission release, increase their intracellular calcium levels ($[Ca^{2+}]_i$), and control neuronal excitability through gliotransmitters release (Haydon and Carmignoto, 2006). More in detail, transporters, ion channels and receptors expressed on astrocytes surface detect synaptic activity and consequently activate second messenger pathways, most of them involving Ca^{2+} (Perea and Araque, 2005). This induces the

release of gliotransmitters that can act on neurons and also on neighboring glia. There are various gliotransmitters that mediate astrocyte-neuron signaling, for instance, cytokines, D-serine, glutamate, GABA, ATP and adenosine, among others (Haydon and Carmignoto, 2006; Kimelberg et al., 1990; Ota et al., 2013). According to several studies, gliotransmitters can be released by several different mechanisms. These mechanisms include:

(i) through Ca^{2+}-dependent exocytosis (Parpura et al., 1994);

(ii) through functional HCs, constituted mainly by connexin 43 (Cx43) assembles (Chever et al., 2014; Orellana and Stehberg, 2014; Torres et al., 2012);

(iii) release through reverse uptake of plasma membrane glutamate transporters (Szatkowski et al., 1990);

(iv) through volume-regulated anion channels (VRAC), which open cell swelling (Benfenati et al., 2009; Malarkey and Parpura, 2008; Mongin and Orlov, 2001);

(v) release through ionotropic purinergic receptors (Duan et al., 2003; Malarkey and Parpura, 2008).

In this thesis, we will focus our attention in two particular gliotransmitters: glutamate (described in more detail in 1.2.2.2) and ATP (described in more detail in 1.2.2.3).

1.2.2.1. Astrocytic 'syncytium'

Astrocytes display a form of excitability that is based on variations in the cytosolic calcium concentration (Cornell-Bell et al., 1990) that is possible thanks to the organized astrocytes network called the astrocytic 'syncytium' through different connexins hemichannels (Decrock et al., 2015; Theis and Giaume, 2012; Wallraff et al., 2004) (Figure 4).

Connexins (Cxs) are transmembrane proteins involved in cell-cell communication through Gap junctions (GJs). Astrocytes express the highest levels of Cxs proteins of all cells in brain (Giaume et al., 2013). Despite the 11 Cxs detected in the rodent brain (Nagy et al., 2004), Cx43 and Cx30, and possibly Cx26, are the major subunits express in astrocytes (Giaume and McCarthy, 1996; Lynn et al., 2011; Perez Velazquez et al., 1996; Wasseff and Scherer, 2011).

This inter-astrocytic transport of calcium waves can be responsible for the release of several gliotransmitters such as glutamate, adenosine 5'-triphosphate (ATP), D-serine, NO, neurotrophins, prostaglandins or cytokines (Haydon and Carmignoto, 2006; Volterra and Meldolesi, 2005) that can influence neuronal function (Fellin and Carmignoto, 2004; Haydon,

2001; Newman, 2003a). The GJs and hemichannels (HCs) functions are strictly regulated by nerve cells and other brain cell populations, for instance endothelial cells, via release of several intra- and extracellular chemicals. These functions are also vulnerable to microenvironmental alterations that happens under physiological and pathological conditions (Orellana et al., 2016).

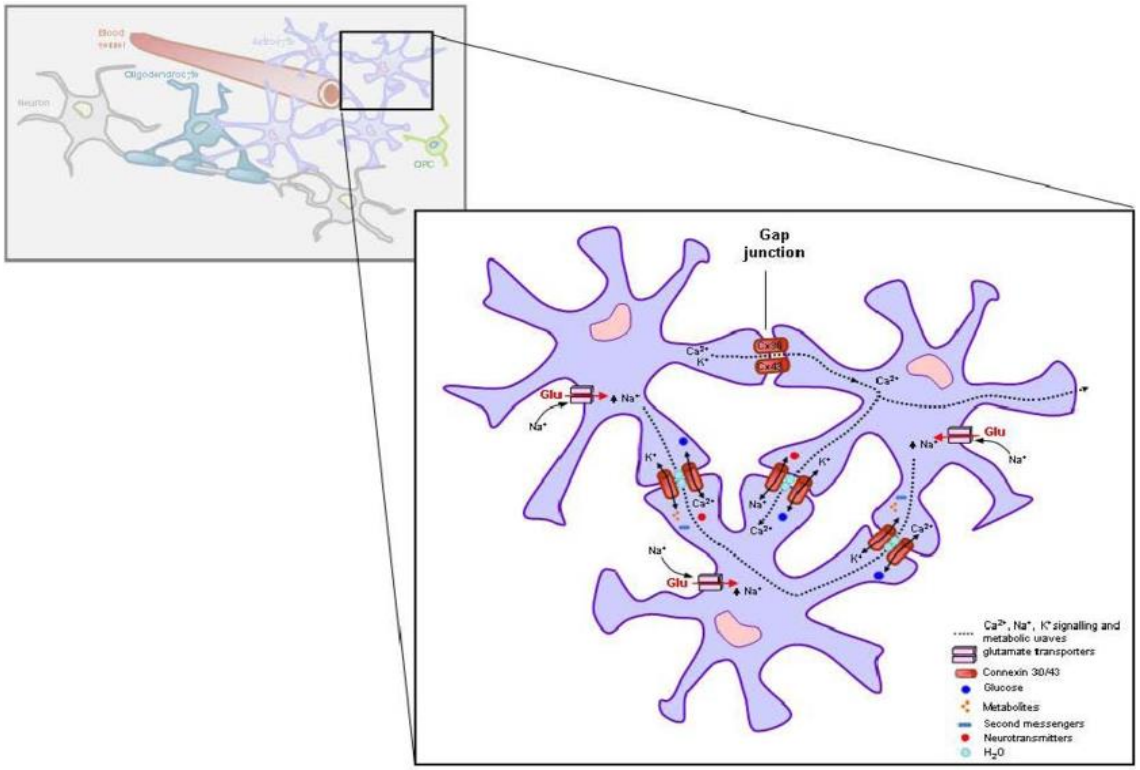

Figure 4 | Astrocytic syncytium. Astrocyte syncytium formed by astrocytes interconnected via gap junctions formed by connexin (Cx) pores contribute for the exchange of small molecules (water, glucose, metabolites, second messengers and neurotransmitters) and ions (Ca^{2+}, K^+ and Na^+). Both Na^+ and Ca^{2+}, diffuse through gap junctions generating signaling and metabolic waves. From: Lanciotti et al. 2013.

1.2.2.2. Glutamate release

Glutamate is an amino acid derivative of glucose that is abundant in biological systems, especially in the CNS and it is involved in nearly all CNS functions from primary sensory perception to cognition (Persson and Rönnbäck, 2012). In opposite to other neurotransmitters, glutamate is an integral element of protein, energy and ammonia metabolism of all cells. Thus, its intracellular concentration is high (close to 10 mM) compared to extracellular concentration (less than 2μM) (reviewed in (Persson and Rönnbäck, 2012). In addition, glutamate is the neurotransmitter, in 70% of the excitatory CNS synapses (Purves et al., 2001). Both neuronal and glial cell membranes have glutamate receptors and glutamate transporters. Although this excitatory amino acid is an essential element in regulating the physiological balance within the CNS, excessive activation of glutamate receptors contributes to excitotoxicity.

Astrocytes account for clearing glutamate (>90%) and other neurotransmitters from the synaptic cleft contributing to neurotransmission (Bak et al., 2006; Chaudhry et al., 1995; Palacin et al., 1998). The two main glutamate transporters expressed in astrocytes are EAAT1 (i.e. GLAST) and EAAT2 (i.e. GLT-1). While EAAT1 is robustly expressed in cerebellum and olfactory system, the EAAT2 is more ubiquitously expressed throughout the brain, exhibiting different neuroanatomical expression patterns (Lehre et al., 1995). After release from the presynaptic membrane to synaptic clef, this excitatory amino acid is taken up from the synapse through rapid and efficient reuptake systems present, mainly, in astrocytes (Anderson and Swanson, 2000; Bak et al., 2006; Chaudhry et al., 1995; Palacin et al., 1998; Schousboe, 2003). In astrocytes, glutamate is converted to glutamine via glutamine synthetase (GS), an enzyme uniquely expressed in astrocytes in the brain. Glutamine is shuttled back to neurons and deaminated to glutamate by neuron-specific phosphate-activated glutaminase without any problems of toxicity (Attwell, 2000; Bak et al., 2006) (Figure 5).

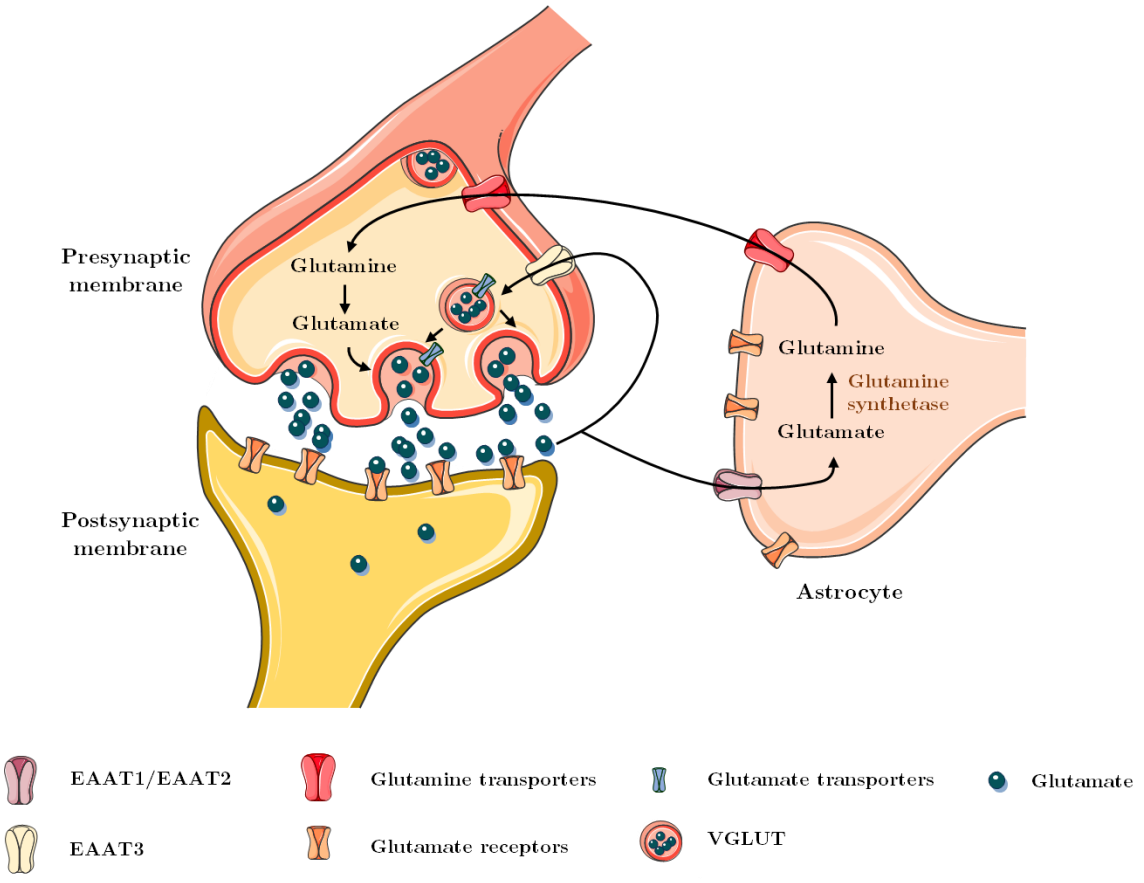

Figure 5 | The tripartite glutamate synapse. Neuronal glutamate (Glu) is synthesized *de novo* from glucose (not shown) and from glutamine (Gln) provided by astrocytes. Glu is then packaged into synaptic vesicles by vesicular glutamate transporters (vGluTs) that will fuse with the presynaptic membrane. After release into the extracellular space, glutamate binds to glutamate receptors on the postsynaptic and presynaptic membranes of neurons and astrocytes, initiating various biological responses. Glu that did not bind to receptors is cleared from the synapse through excitatory amino acid transporters (EAATs) located on neighbouring astrocytes (EAAT1 and EAAT2) and, to a lesser extent, on neurons (EAAT3 and EAAT4). Within the astrocyte, Glu is converted to Gln by glutamine synthetase and the Gln is subsequently released by glutamine transporters and taken up by neurons to complete the glutamate–glutamine cycle.

Given the critical role of glutamatergic neurotransmission in the CNS, it is not surprising that dysfunction of glutamate transporters and glutamate-induced excitatory toxicity have been linked to a wide range of neurological diseases, such as, Alzheimer's disease (AD) (Masliah et al., 1996), stroke and ischemia (Lee Martin et al., 1997), trauma (Watase et al., 1998), epilepsy (Tanaka et al., 1997), Parkinson's disease (PD) (Hazell et al., 1997), Huntington's disease (HD) (Liévens et al., 2001), demyelinating diseases (Korn et al., 2005), neuropsychiatric disorders such as depression and bipolar disorder (Hasler, 2010; McCullumsmith and Meador-Woodruff, 2002; Simpson et al., 1992).

1.2.2.3. ATP release

Prior studies demonstrate that astrocytes can release ATP *in vitro* (Guthrie et al., 1999; Maienschein et al., 1999; Newman, 2003b; Zhang et al., 2003) and *in vivo* (Cao et al., 2013a; Gordon et al., 2005; Pascual et al., 2005), mediating the coupling between astrocytes and between astrocytes and neurons. In culture and *in vivo*, elevation of the Ca^{2+} signal within one astrocyte leads to a Ca^{2+} wave that propagates through the astroglial network. Cell culture studies showed that ATP is able to mediate Ca^{2+} waves, a form of astrocyte communication (Guthrie et al., 1999). In fact, astrocytes utilized ATP, that is an important extracellular signaling molecule, to communicate with another astrocyte as well as to neurons (Haydon and Carmignoto, 2006; Ota et al., 2013).

Astrocytic purines can adjust synaptic efficacy modulating to accord various plasticity events in different regions of brain. This balanced modulation requires that astrocytes change basal synaptic transmission. Inhibition of excitatory transmission has been shown in different paradigms where ATP released by astrocytes is degraded into adenosine, which then acts on presynaptic adenosine A_1 receptors (A_1R) to inhibit excitatory transmission (Pascual et al., 2005). Interestingly, astrocyte-derived adenosine can also act on neighboring synapses increasing neuronal signal by inhibiting adjacent synapses (Halassa et al., 2009a). Recent findings demonstrate that this mechanism is involved in sleep regulation (Halassa et al., 2009b), cognitive impairment (Stone et al., 2009) and depressive-like behaviors (Cao et al., 2013a). Moreover, astrocytes have also been shown to increase basal synaptic transmission through activation of facilitatory presynaptic A_{2A} receptors ($A_{2A}R$) (Panatier et al., 2011). For these reasons, astrocytes can differentially influence synaptic plasticity using a balanced A_1R-$A_{2A}R$ modulation.

1.2.3. Astrocytes in depression condition

In terms of classification as a disease condition, MDD was originally viewed as a neuron-based disorder. However, antidepressants target proteins expressed by multiple brain cell types, and specific mechanisms and cell types involved in antidepressant action remain largely unknown. Researchers suspected that depression would lead to cell death and decreased cell density in brain regions associated with depression. In fact, it was reported a decrease in cell density in areas such as the prefrontal cortex (PFC) (Rajkowska et al., 1999) and hippocampus (Stockmeier et al., 2004), consistent with both neurons and glial cells (reviewed in (Rial et al., 2015)). They also found alterations in the inter-relations between glial cells and neurons, leading to astrocytic and microglia dysfunction and consequently an aberrant plasticity, which results in inadequate encoding of information (Rial et al., 2015). Other studies revealed that a glial ablation (through the administration of a gliotoxin) into the PFC is sufficient for the animal to present a depressive-like behaviors (Banasr and Duman 2008). Moreover, in postmortem human tissue, it was observed a decreased expression of the astrocyte marker GFAP and S100β in the PFC (Miguel-Hidalgo et al., 2000), anterior cingulate cortex (Gittins and Harrison, 2011), hippocampus (Cobb et al., 2016; Gos et al., 2013), and amygdala (Altshuler et al., 2010). These findings suggest that astrocyte alteration can indeed be causative or at least a contributing factor to the neuropathophysiology of depression biology and imply further studies to understand this relationship.

Overall, astrocytes may also modulate stress signaling responding to glucocorticoids (GCs). The information about glucocorticoid regulation in astrocytes is scarce, however, it suggests that stress hormones do have specific effects on this cell type. Astrocytes express both glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Bohn et al., 1991) and thus are sensitive to glucocorticoid regulation. The hypertrophy of GFAP has also been associated with glucocorticoid action (O'Callaghan et al., 1989). Glutamate uptake can decrease in cultured astrocytes by action of glucocorticoids (Virgin et al., 1991). It is also known that GCs regulate messenger RNAs specifically in astrocytes relative to other cell types (Carter et al., 2013a, 2013b).

Maintaining in mind that astrocytes express the highest levels of Cxs proteins of all cells in brain (Giaume et al., 2013) and are responsible to maintain astrocytic 'syncytium' that regulate some astrocytic functions, it is not surprising, that it was observed alterations in Cxs in depressive patients. Studies with postmortem samples from patients with MDD and suicide completers have shown that Cx43 and Cx30 are significantly downregulated in brain regions involved in mood regulation, such as PFC , (Miguel-Hidalgo et al., 2014), hippocampus (Medina et al., 2016), locus coeruleus, mediodorsal thalamus and caudate nucleus (Nagy et al., 2015). Other studies shown that epigenetic mechanisms such as DNA methylation mediate alterations in astrocytic Cxs gene expression in suicide completers with depression (Nagy et al., 2015, 2016). In 2016, Nagy and

colleagues also observed an increase in the repressive chromatin mark H3K9me3 for both Cx30 and Cx43 in suicide completers when compared with control. Furthermore, studies in animal models of depression shown alterations in both Cx43 mRNA and protein levels in PFC (Sun et al., 2012). Furthermore, it was reported that antidepressive drugs could influence the expression of Cx 43 in astrocytes (Jeanson et al., 2015; Quesseveur et al., 2015). These findings suggest that astrocytic Cxs may be a potential therapeutic target for the treatment of depression, however more research is required to confirm this theory.

1.3. ADENOSINE

The purine nucleoside adenosine is virtually present in every cell type and exerts important regulatory functions in the CNS, as it can act as a homeostatic transcellular messenger and a neuromodulator (Cunha, 2001; Fredholm et al., 2005). Adenosine can act on at least four subtypes of guanine nucleotide binding protein (G protein) coupled receptors (Fredholm et al., 2005; Takahashi et al., 2008). Adenosine can control synaptic transmission and plasticity directly, and through their effects on astrocytes (Matos et al., 2012a)(Fredholm et al., 2005; Matos et al., 2012a). At the synaptic level, it mediates the information flow and neuronal excitability by controlling neurotransmitter release and consequently the action of some receptor systems (Cunha, 2001; Fredholm et al., 2005; Gomes et al., 2011), independently of imbalances in energy metabolism (Cunha, 2001).

Adenosine can be generated through several mechanisms throughout the entire organism. ATP is an important precursor of extracellular adenosine, contributing to its rapid genesis, and being the main source in some occurrences (e.g. in necrotic or apoptotic cells) (Fredholm et al., 2011). The ATP release from astrocytes is converted extracellularly into adenosine by ectonucleotidases such as ecto-5'-nucleotidase (CD73) (Augusto et al., 2013; Kreutzberg et al., 1978). Then, astrocytes recapture adenosine via bi-directional nucleoside transporters (Peng et al., 2005) and rapidly convert to AMP (and then back to ATP) via adenosine kinase (ADK), which is also predominantly expressed in astrocytes in the adult brain (Boison et al., 2010) (Figure 6).

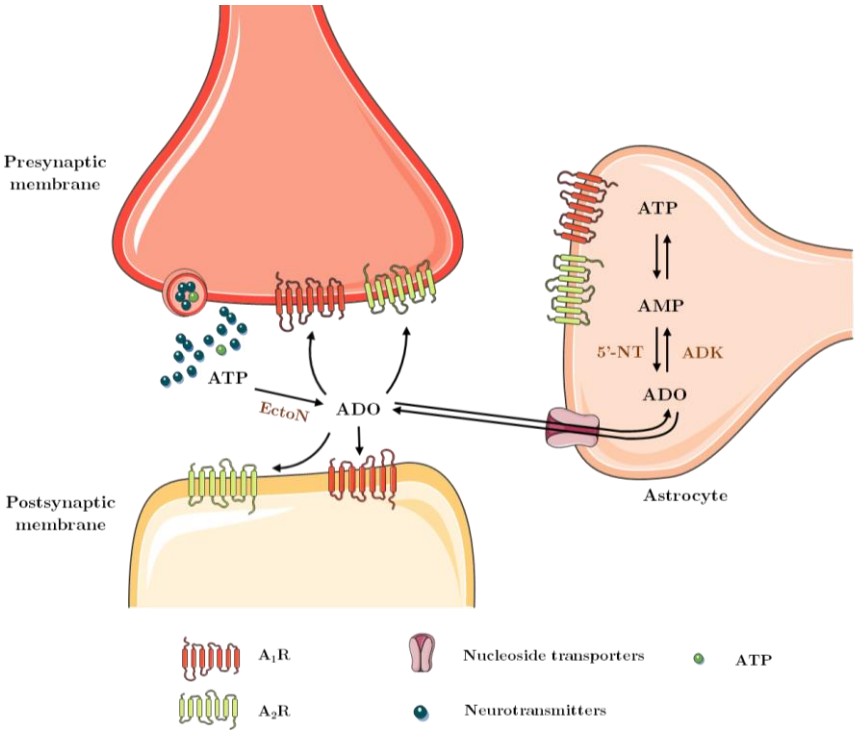

Figure 6 | The tripartite adenosine synapse. The extracellular levels of adenosine are largely regulated by astrocyte-based adenosine cycle. A major source of synaptic adenosine is vesicular release of ATP (green dot) followed by its

extracellular degradation to adenosine (ADO) via ectonucleotidases. The adenosine levels are equilibrated due to nucleoside transporters (NT). Intracellular metabolism of ADO depends on the activity of adenosine kinase (ADK), which, together with 5'-nucleotidase (5'-NT), forms a substrate cycle between AMP and adenosine. Thus, intracellular metabolism of adenosine via ADK drives the influx of adenosine into the cell.

1.3.1. Adenosine receptors

There are four adenosine metabotropic receptors (AR), these have been denominated adenosine A_1, A_{2A}, A_{2B} and A_3 receptors (A_1R, $A_{2A}R$, $A_{2B}R$, A_3R) (Fredholm et al., 2001). Adenosine is a full agonist of all these receptors. Inosine can also act as a partial agonist of A_1 and A_3 receptors (Fredholm et al., 2011; Jin et al., 1997). These receptors are formally divided in two categories: A_1R and A_3R are coupled to inhibitory G-proteins and $A_{2A}R$ and $A_{2B}R$ are coupled to stimulatory G-proteins (Lopes et al., 1999; Takahashi et al., 2008).

1.3.1.1. Adenosine $A_{2A}R$ in the brain

Previous studies reported that A_{2A} receptors are highly abundant in the basal ganglia (Schiffmann et al., 1991; Svenningsson et al., 1999), however, nowadays they are recognized to display a widespread distribution in the brain (Fredholm et al., 2005; Lopes et al., 2004) and mostly located in synapses (Rebola et al., 2005a). More specifically, $A_{2A}R$ can be located in dendritic spines and post-synaptic densities (Rebola et al., 2005a) of asymmetric contacts between cortico-thalamic glutamatergic projections (Cunha, 2005) and striatopallidal medium spiny GABAergic neurons (Schiffmann et al., 1991; Svenningsson et al., 1999). A minority of these $A_{2A}R$ are also located in the presynaptic terminals of limbic and neocortical regions of the brain, being also present in glial cells (microglia and astrocytes) (Küst et al., 1999; Li et al., 2001; Moreau and Huber, 1999; Nishizaki et al., 2002).

These receptors can exert different functions: can control the release of neurotransmitters, either glutamate (e.g. (Lopes et al., 2002)), GABA (e.g. (Cunha and Ribeiro, 2000)), acetylcholine (e.g. (Jin and Fredholm, 1997; Rebola et al., 2002)) or serotonin (e.g. (Okada et al., 2001)) and the function of ionotropic glutamate receptors (Di Angelantonio et al., 2015; Azdad et al., 2009; Dias et al., 2012; Guntz et al., 2008; Higley and Sabatini, 2010; Rebola et al., 2008; Sarantis et al., 2015; Scianni et al., 2013; Wirkner et al., 2004). In glial cells, $A_{2A}R$ can control Na^+/K^+-ATPase (Matos et al., 2013), glutamate uptake (Matos et al., 2012a, 2012b), and the production of pro-inflammatory cytokines (Dai et al., 2010; Madeira et al., 2015; Merighi et al., 2015; Rebola et al., 2011). Overall, $A_{2A}R$ can mediate motor activity, working memory, psychiatric behaviors and sleep-awake cycle, inflammation and angiogenesis (Cunha, 2016).

Moreover, for $A_{2A}R$ to play their function can activate several signaling pathways: cAMP-dependent kinase/PKA, and phosphorylate cAMP responsive element-binding protein (CREB) (Josselyn and Nguyen, 2005); MAPKs (De Cesare et al., 1999; Cheng et al., 2002; Du et al., 2000) and can recruit β-arrestin via a GRK-2 dependent mechanism (Khoa et al., 2006). In addition, $A_{2A}R$ can also interact or form heterodimers with others receptors such as A_1 (Ciruela et al., 2006; Ribeiro, 1999), dopamine D2 receptors (Fredholm, 1995), group I metabotropic glutamate 5 (Tebano et al., 2005), NMDA (Ribeiro, 1999) and cannabinoid CB_1 (Carriba et al., 2007).

1.3.2. Adenosine, stress and MDD

The adenosinergic system is able to modulate mood states (Gomes et al., 2011), however its role in depression is very complex. Some therapeutic strategies, such as ECT and sleep deprivation, use short and long term adaptations that are directly associated with the adenosinergic system to control mood disorders (Cunha et al., 2008b). Additionally, some antidepressants can also target adenosine receptors (Gomes et al., 2011). Moreover, in the past, several retrospective studies were published with caffeine, that is a non-selective antagonist of A_1 and A_{2A} receptors (Chen et al., 2013; Fredholm, 2007; Fredholm et al., 2007; El Yacoubi et al., 2003), and they are the only known molecular target of caffeine, at nontoxic doses (Cunha et al., 2008b; Fredholm et al., 2005). Those studies reported that caffeine intake in humans can: improve psychomotor performance and cognition (Cunha et al., 2008b; Jégou et al., 2003; Lara, 2010); and reduce depression incidence by intake of moderate coffee, in a dose-dependent manner (Lucas et al., 2011, 2014; Smith, 2009). More than a decade of research suggest that suicide risk is lower among persons with higher consumption of coffee (Kawachi et al., 1996). At low to moderate doses, caffeine has psychostimulant effects, contrary to higher doses, wherein the reverse effect can be observed (Lucas et al., 2011), triggering behavioral modifications, both anxiety disorders as well as depressive-like conditions.

In animal models of depression, nonselective activation of $A_{2A}R$ by adenosine and its analogues appears to induce depressive-like behaviors in animal models of depression (Hunter et al., 2003) which can be reversed by administration of antidepressants (Kulkarni and Mehta, 1985). On the other hand, it was also shown that administration of adenosine can impair the antidepressant-like effects in animal models of depression, and those effects reinstated after treatment with an antagonist selective of $A_{2A}R$ (Kaster et al., 2004). Blockade or genetic depletion of $A_{2A}R$ reduced immobilization period or suppressed behavioral despair in the forced swim test and tail suspension test, respectively (El Yacoubi et al., 2001, 2003). Moreover, studies have shown that $A_{2A}R$ are up-regulated (Kaster et al., 2015) and their over-expression induces anhedonia and depressive

like-behavior (Coelho et al., 2014) and, conversely, A_{2A} blockage confers a robust neuroprotection in animal models of stress by preventing mood and memory deficits (Batalha et al., 2013; Kaster et al., 2015; Yin et al., 2015).

These findings, suggest that $A_{2A}R$ should be considered as a novel therapeutic target to manage depression and other mood disorders (Cunha et al., 2006).

CHAPTER 2

OBJECTIVES

The main goal of this work was to study the role of astrocytic A_{2A} receptors in depressive-like conditions. For that purpose, in this work two experimental approaches were used: (1) an *in vitro* model of stress – incubation of astrocyte primary cell culture with dexamethasone; (2) hippocampal gliosomes of suicide completers when compared with age-matched controls.

With the first approach, we evaluated:

- alterations in the release of gliotransmitters (glutamate and ATP) induced by dexamethasone and involved mechanisms;
- changes in different astrocytic markers immunoreactivity upon incubation with dexamethasone;
- if blockade of $A_{2A}R$ can prevent alterations induced by dexamethasone.

With the second approach, we evaluated:

- validation of gliosomal preparation;
- alterations in glial markers density in suicide when compared to age-matched controls;
- alterations in synaptic and glial markers density according to age.

CHAPTER 3

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Reagents

Table 1| Reagents used in the methodologies.

Reagent	Supplier
2,2',2",2'"-(Ethane-1,2-diyldinitrilo) tetraacetic acid (EDTA)	Sigma Aldrich
2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES)	Sigma Aldrich
3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS)	Sigma Aldrich
4',6-diamidino-2-phenylindole (DAPI)	Invitrogen
30% Acrylamide/ Bisacrylamide solution	Bio-Rad
Ammonium persulfate (APS)	Sigma Aldrich
Adenosine 5'-triphosphate (ATP) Assay Mix	Sigma-Aldrich
Adenosine 5'-triphosphate (ATP) Standard	Sigma-Aldrich
BCA Kit	Thermo Scientific
Bicine	Sigma-Aldrich
Bio-Rad protein assay dye reagent	Bio-Rad
Bovine Serum Albumin (BSA)	Sigma Aldrich
Bromophenol blue	Sigma Aldrich
Calcium Chloride ($CaCl_2$)	Sigma Aldrich
CLAP (cocktail of proteases inhibitors)	Sigma Aldrich
Dako fluorescent mounting medium	DAKO
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
Dithiothreitol (DTT)	Sigma Aldrich
DNase	Sigma Aldrich
Dulbecco's Modified Eagle Medium (DMEM)	Sigma Aldrich
Enhanced chemiluminescence (ECL)	Thermo Scientific
Fetal Bovine Serum (FBS)	Alfagene
Glucose	Sigma Aldrich
Glutamate Assay Kit	ABCAM
Glycerol	Sigma Aldrich
Horse serum	Gibco by Life Technologies
Magnesium chloride ($MgCl_2$)	Fluka Analytical
Magnesium sulfate ($MgSO_4$)	Fluka Analytical
Methanol	Sigma Aldrich
Paraformaldehyde	Sigma Aldrich
Penicillin-Streptomycin (Pen-Strep)	Gibco by Life Technologies
Percoll	GE Healthcare
Penylmethanesulfonylfluoride (PMSF)	Sigma Aldrich
Poly-D-Lysine	Sigma Aldrich
Potassium chloride (KCl)	Sigma Aldrich
Potassium Dihydrogen Phosphate (KH_2PO_4)	Sigma Aldrich
Sodium Bicarbonate ($NaHCO_3$)	Sigma Aldrich
Sodium dodecyl sulfate (SDS)	Bio Rad
Sodium Chloride (NaCl)	Sigma Aldrich
Sucrose ($C_{12}H_{22}O_{11}$)	Sigma Aldrich
TEMED	Sigma Aldrich
Triton-X-100	Sigma Aldrich
Trizma base	Sigma Aldrich
Trypan blue	Sigma Aldrich
TryplE™ Express (1X)	Gibco by Life Technologies
Trypsin	Sigma Aldrich
Tween 20	Fluka Analytical

3.1.2. Drugs

Table 2 | Drugs used throughout cell cultures.

Drug	Supplier	Concentration	Function
3β-Hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate (Carbenoxolone disodium salt; $C_{34}H_{48}O_7Na_2$)	Sigma Aldrich	50 μM	Hemichannel/gap junction inhibitor
7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4 triazolol[1,5c]pyrimidine (SCH 58261; $C_{18}H_{15}N_7O$)	Tocris bioscience	50 nM	$A_{2A}R$ selective antagonist
(Brilliant Blue G; $C_{47}H_{48}N_3NaO_7S_2$)	Sigma Aldrich	100 nM	P_2X_7 purinergic receptor antagonist
(Dexamethasone; $C_{22}H_{29}FO_5$)	Tocris bioscience	100 nM	Synthetic Glucocorticoid
(*R*)-4-(2-Amino-1-hydroxyethyl)-1,2-benzenediol (Norepinephrine; $C_8H_{11}NO_3$)	Fluka chemie GmbH CH-9471 Buchs Biochemika 74498	30 μM	Noradrenergic receptor agonist

3.1.3. Antibodies

Table 3 | Primary and secondary antibodies used in immunoblotting.

Antibody	Supplier	Host	Type	Dilution
$A_{2A}R$	Santa cruz #SC-32261	Mouse	Monoclonal	1:250
GLAST (EAAT1)	Abcam #ab416	Rabbit	Polyclonal	1:1 000
Synaptophysin	Sigma #S5768	Mouse	Monoclonal	1:20 000
GFAP	Millipore #ab5804	Rabbit	Polyclonal	1:20 000
Connexin 43	Sigma-Aldrich #C6219	Rabbit	Polyclonal	1:8 000
Glutamine synthetase	Thermo Fisher Scientific #PA1-46165	Rabbit	Polyclonal	1:5 000
Syntaxin	Sigma-Aldrich #S0664	Mouse	Monoclonal	1:2 000
β-actin	Sigma-Aldrich #A5316	Mouse	Monoclonal	1:20 000
Anti-rabbit	Thermo Fisher Scientific #31462	Goat	Polyclonal	1:5 000
Anti-mouse	Thermo Fisher Scientific #31432	Goat	Polyclonal	1:5 000

Table 4 | Primary and secondary antibodies used in immunocytochemistry

Antibody	Supplier	Host	Type	Dilution
GFAP	Santa cruz #sc-6170	Goat	Polyclonal	1:200
Glutamine synthetase	Thermo Fisher Scientific #PA1-46165	Rabbit	Polyclonal	1:1 000 – 1:10 000
Connexin 43	Sigma-Aldrich #C6219	Rabbit	Polyclonal	1:600
Anti-goat 488	Invitrogen #A11055	Donkey	Polyclonal	1:250
Anti-mouse 488	Invitrogen #A-21202	Donkey	Polyclonal	1:250
Anti-rabbit 594	Invitrogen #A-21207	Donkey	Polyclonal	1:250

3.1.4. Experimental model systems

Depression affects up to 20% of the global population (Berton and Nestler, 2006) and will become the leading cause of disease, by 2030, according to the World Health Organization (Secretariat, 2011). The treatments for this recurring mood disorder that are currently available include psychotherapy, medications, and diverse brain stimulation therapies including electroconvulsive therapy (ECT), transcranial magnetic stimulation and vagus nerve stimulation (Mayberg et al., 2005). Unfortunately, only a third of patients achieve remission following first-line treatment with medication and a third of patients remain symptomatic following several medication and/or psychotherapy trials (Mayberg et al., 2005). One major reason for these shortfalls in treatment is the lack of a thorough understanding of the pathophysiology and pathogenesis of depression (Krishnan and Nestler, 2008). For that reason, it is really important to use a good experimental model system to understand this brain disorder and try to find a new potential therapeutic target. Nowadays, there are many experimental model, with different levels of complexity that contributed towards the understanding of depressions, such as: human postmortem analysis, animal disease models of depression, genetic models and primary cell cultures. However, by time limitation, in this thesis we focused only on two different approaches: the primary astrocyte cultures derived from rodents and the synaptosomes/gliosomes from human postmortem brain tissue.

The reasons to choose an astrocyte primary cell cultures, are the following: (i) being a simple model; (ii) control of physicochemical environment-pH, temperature, dissolved gases (O_2 and CO_2), osmolarity; (iii) regulation of physiological conditions-nutrient concentration, cell to cell interactions, hormonal control; (iv) the "absence" of other cell types so any effects observed with

these cultures are therefore astrocyte-specific; (v) replicability and speed of achieving results; (vi) study some functions in real time; (vi) test different anti-depressant therapies; among others. In spite of the fact that this system is extremely useful, there are some limitations. A major limitation encountered is that the cells are out of their normal environment and no culture system can fully mimic the complex events occurring *in vivo*. Moreover, the astrocyte primary cell cultures do not create a three-dimensional network that exist in *in vivo* cells, but rather a contact-inhibited monolayer, creating epithelioid-like cells devoid of synaptic contact and vascular elements. Overall, this simple model will allow studying directly the influence of glucocorticoids in some astrocytic functions, which would be difficult to perform in other models.

On other hand, we also choose human postmortem analysis to try to understand depression. Human postmortem brain samples provide pertinent and important opportunities to dissect particular regions of the brain and study molecular mechanism in an exact neuroanatomical location associated with depression. However, they also have several limitations, including longer postmortem interval, prior treatment with psycho active drugs and existence of others psychiatric disorders and/or alcoholism (Furczyk et al., 2013; Stockmeier and Rajkowska, 2004). Nevertheless, these limitations can be overcome by confirm the quality of samples conform certain standards, performing toxicological screening and examine the samples for any neuropathological abnormalities (Furczyk et al., 2013; Stockmeier and Rajkowska, 2004). With this in mind, it is reasonable to say that human postmortem brain samples are also a good tool in the research to enable a better understanding of the underlying pathology, as well for the development of new therapeutic approaches.

3.1.4.1. Animals

In our study, we used pups between 3- and 6-day postnatal Wistar rats that were obtained from littermates that exist at the CNC animal facilities. The animals were sacrificed by cervical dislocation and decapitation. Rats were handled according to European Union guidelines ((ORBEA 128_2015/04122015) and the certification of Direcção Geral de Alimentação e Veterinária (DGAV; 0421/000/000/2016 Ref 014420).

3.1.4.2. Human samples

Human brain samples (figure 7) were obtained at autopsies from the *Instituto Nacional de Medicina Legal e Ciências Forenses*, I.P. (INMLCF) in Coimbra, thanks to collaboration between pathologist Dr. Beatriz da Silva and our group, namely Rodrigo Cunha and Paula Canas. The

samples were collected according the procedures approved by the INMLCF and European Consortium of Nervous Tissues: BrainNet Europe II guidelines, to protect the identity of individual donors.

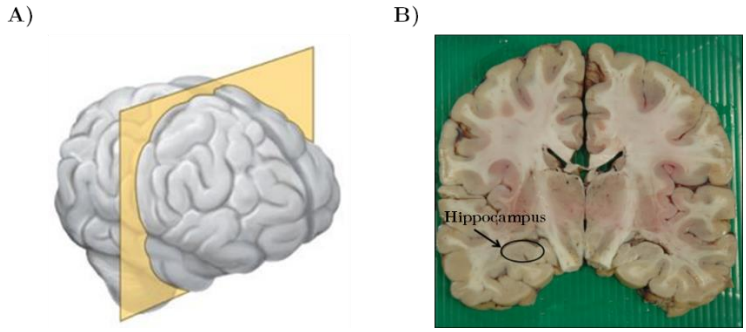

Figure 7 | Human brain coronal plane. Schematic image (A) and representative image (B) of coronal slice from human brain sample. Image kindly provided by Dr. Beatriz Silva from INMLCF.

The study was performed in male suicide completers and compared with age-matched controls, who died by natural causes or accidents. Individuals that consumed medically-prescribed psychoactive drugs at the time of death; with neuro-pathological features of neurological disorders or psychiatric conditions such as schizophrenia or phobia, eating disorders, addiction; with artificial respiration or in coma before death were excluded from the study.

The quality of the tissue samples used was taken into account and validated by measurement of ribonucleic acid (RNA) integrity number (RIN) and pH. The quality of tissue was evaluated previously by other members of the group (Paula Canas and Ana Xavier).

Table 5 | Demographic characteristics and cause of death of individual cases of suicide completers and age-matched controls.

Number	Sex	Age	Cause of death	Postmortem Interval (hours)	pH	Control or suicide
1	M	22	Car accident	31.3	6.59	Control
2	M	22	Car accident	32.7	6.64	Control
3	M	58	Hanging	20.5	6.52	Suicide
4	M	60	Work accident	22.5	6.53	Control
5	M	68	Hanging	23.6	6.77	Suicide
6	M	61	Drowning	63.5	6.72	Control
7	M	72	Car accident	26.7	6.70	Control
8	M	37	Cardiopathy	25.3	6.48	Control
9	M	71	Head concussion	64.7	5.98	Control
10	M	78	Hanging	72.8	6.45	Suicide
11	M	73	Car accident	16.5	5.83	Control
12	M	78	Drowning	61.6	6.54	Control
13	M	82	Hanging	38	6.48	Suicide
14	M	42	Embolism	16	6.44	Control
15	M	61	Hanging	21.7	6.46	Suicide
16	M	62	Work accident	66.7	6.57	Control
17	M	34	Burning Accident	87.7	6.26	Suicide
18	M	71	Hanging	67.2	6.57	Suicide

3.2. METHODS

3.2.1. Astrocyte Primary Cell Culture

Primary astrocyte cultures were prepared from cerebral cortices of 3–6-day postnatal Wistar rats according to previous described procedures (Harris et al., 1996; Matos et al., 2012a), but with some modifications. In brief, the mice were killed by cervical dislocation and decapitation and the brain was removed. The meninges were then removed and the cerebral cortices separated from olfactive bulb. The pre-frontal cortex was removed, chopped up and incubated with a digestive medium, TrypLE Express, and 0.5 mg/ml DNAse at 37 °C for 20–30 min. Then, the enzymatic digestion was stopped with Dulbecco's modified Eagle medium (DMEM) high glucose supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (10,000 U/mL), streptomycin (10 mg/mL), HEPES (25.18 mM), and sodium bicarbonate (9.99 mM) and the cell suspension centrifuged for 2 min at 115 g. Afterward, the obtained pellet was resuspended in astrocyte culture medium, DMEM, the number of cells in suspension was counted in a hemocytometer, using trypan blue dye. Then, the cells were plated onto poly-D-lysine-coated 75 cm^2 culture flasks, at a density at 1.14 x 10^5 cells/cm^2 (8.6 x 10^6 cells by flask), and maintained at 37 °C in a 5% CO_2/95% room-air humidified incubator. The cell culture medium was frequently replaced, every 2-3 days, until the mixed-glial cultures reached confluency, which was normally achieved after 10-15 days of culture *in vitro* (DIV). In order to separate microglial cells from the astrocytes monolayer, the mixed glial-cultures were shaken at 160-190 rotations per minute in an orbital shaker for 30 min-1h30min. Then, the medium with the up-layer detached microglial cells was discarded and fresh astrocytic culture medium was added to the astrocytes that remained in the flasks. The flasks were washed with PBS 1x containing EDTA (1 mM) and further detached by a mild trypsinization procedure using PBS 1x with 0.25% trypsin. Finally, the cells were reseeded with fresh astrocyte culture medium on poly-D-lysine-coated plates or coverslips (for immunocytochemistry), at a density of 0.5 x 10^6 cells/mL or 0.1 x 10^6 cells/mL, respectively, and maintained in culture for 2–3 days before the experiments beginning. Our cultures had a high percentage of astrocytes, which was confirmed previously by other members of group by immunostaining against astrocyte specific protein glial fibrillary acidic protein (GFAP) and microglial specific antibody CD11b (Matos et al., 2012a).

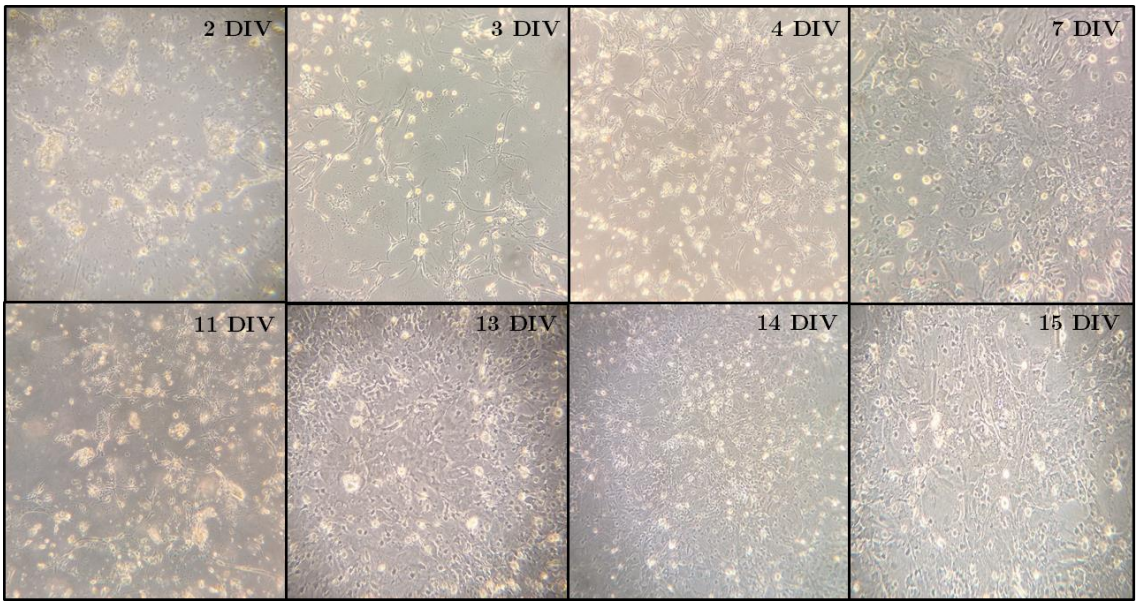

Figure 8 | Mixed-glial primary cell culture evolution during 15 days of culture *in vitro* (DIV). The images were taken in an optical microscope with a magnification of 200x.

3.2.2. Cell treatments

Cultured astrocytes were treated with Brilliant Blue G, carbenoxolone, dexamethasone and SCH58261 for different periods ranging from 5 min (acute effect), 1h (acute effect) to 24h (chronic effect). For 5 min and 1h incubations, the cultured cells were incubated with Na^+ medium (containing: 132 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1.2 Na_2HPO_4, 1.4 mM $MgCl_2$, 6 mM Glucose and 10 mM HEPES, pH 7.4), on the other hand, 24h incubations were performed with DMEM. The dexamethasone was reconstituted in dimethyl sulfoxide (DMSO) and added to astrocytic cultures to achieve a final concentration of 100 nM. The Brilliant Blue G was prepared in sterile type I water also as well carbenoxolone, at a concentration of 100 nM and 50 μM, respectively. The selective $A_{2A}R$ antagonist, SCH 58261 was reconstituted in DMSO to achieve a final concentration of 50 nM (supra-maximal concentration). Brilliant blue G, carbenoxolone and SCH58261 were added to the astrocyte culture medium or Na^+ medium 30 min before incubation with dexamethasone and maintained present throughout the incubation time period.

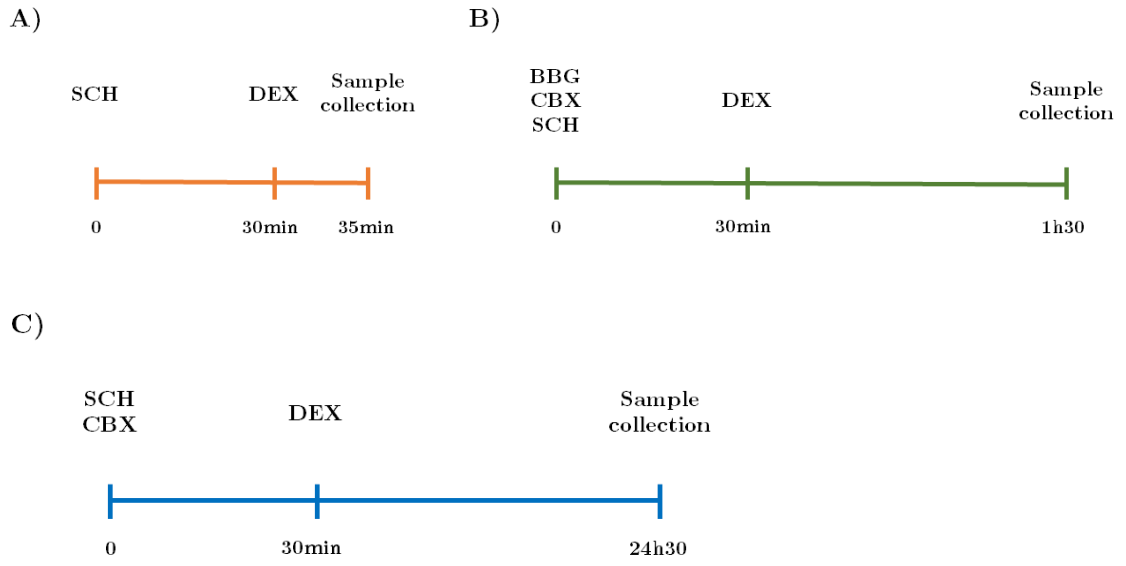

Figure 9 | Schematic overview of the experiments with pharmacological treatments. Cultured astrocytes were treated or not (control) with Brilliant Blue G (BBG), carbenoxolone (CBX), dexamethasone (DEX) and SCH58261 (SCH) for different periods of time, 5 min (A), 1h (B) or 24h (C).

3.2.3. Immunocytochemistry

Cultured astrocytes were placed onto 16mm poly-D-lysine-coated coverslips, fixed with 4% paraformaldehyde for 15 min and washed three times with phosphate-buffered saline (PBS) medium (140 mm NaCl, 3 mm KCl, 20 mm NaH_2PO_4, 1.5 mm KH_2PO_4, pH 7.4). The astrocytes were permeabilized in PBS with 0.2% Triton X-100 for 10 min at room temperature (RT) and then washed three times with PBS. Then, the cells were blocked with PBS with 3% bovine serum albumin (BSA) and 5% horse serum for 1h at room temperature. These preparations were washed twice with PBS with 3% BSA and incubated with the primary antibodies, namely anti-GFAP (1:200), anti-connexin43 (1:600) and anti-glutamine synthetase (1:1000) for 2 h at RT. After three washes with PBS with 3% BSA, the astrocytes were incubated in the dark for 1h at RT with AlexaFluor-488 (green) labelled donkey anti-goat IgG antibody, AlexaFluor-594 (red) labelled donkey anti-mouse or donkey anti-rabbit IG antibodies (1:250 for all). Incubation with DAPI dye (a marker of cell nuclei) (1:5 000) was carried out during 3 min. After washing with PBS and mounting onto slides with DAKO, the preparations were visualized by fluorescence microscopy (Zeiss, model Imager Z2, with Axiovision software 4.8) under a 200x magnification.

3.2.4. ATP Quantification

The extracellular levels of ATP were assessed by the high sensitivity luciferin-luciferase bioluminescence assay, as previously described (Cunha et al., 1996). Briefly, after an incubation period of 5 min, 1h or 24h with different drugs, cell supernatants were collected and kept at -80 °C. Cell supernatants (80 μL) were added to 40 μL of ATP assay mix in white 96-well plates kept inside a VICTOR^3 multilabel plate reader (Perkin Elmer^{TM}, with Wallac 1420 Manager software) to start luminescence recording (5 sec of acquisition). ATP levels were quantified by extrapolation of a standard curve and released ATP was normalized by the total protein content by the Bio-Rad protein method.

3.2.5. Glutamate Quantification

The extracellular levels of glutamate were assessed by a colorimetric assay, following the instructions provided by the manufacturer. Briefly, after an incubation period of 5 min, 1h or 24h with different drugs, cell supernatants were collected and kept at -80 °C. Cell supernatants (40 μL) were added to 10 μL of assay buffer and 100 μL of reaction mix (glutamate assay buffer, glutamate developer and glutamate enzyme mix) in a 96-well plate. Glutamate levels were quantified by extrapolation of a standard curve prepared by several dilutions of glutamate standard, using: 6, 4, 2, 1, 0.5 and 0 nmol/well of glutamate. The multi-well dish was incubated at 37° C, during 30 min, and, after that, the absorbance was read at 450 nm in a microplate reader spectrophotometer (Spectramax plus 384). Glutamate levels were quantified by extrapolation of a standard curve and released glutamate was normalized by the total protein content by the Bio-Rad protein method.

3.2.6. *In situ* Proximity Ligand Assay (PLA)

The Proximity Ligand Assay (PLA) was performed as previously described (Augusto et al., 2013; Söderberg et al., 2006) in cultured astrocytes. The samples were permeabilized in PBS with 0.2% Triton X-100 for 10 min at room temperature (RT) and then washed three times with PBS. Then, the cells were blocked with PBS with 3% bovine serum albumin (BSA) and 5% horse serum for 1h at RT. Subsequently, the cells were washed twice with PBS with 3% BSA and incubated with anti-A_2A (1:500) and anti-connexin43 (1:600) for 2h at RT. After washing 3 times in PBS with 0.2% Triton X-100, the cells were incubated for 2 h at 37 °C with the PLA secondary probes anti-rabbit PLUS and anti-mouse MINUS (1:5; Olink Bioscience) under gentle agitation. The PLA

probes are added together and diluted 1:5 in buffer Tris (0.1 M Tris, pH.7.4, and 0.9% w/v NaCl). Afterward, the cells were washed twice with Duolink II Wash Buffer A (Olink Bioscience) and incubated with the ligation-ligase solution (Olink Bioscience) for 30 min at 37 °C. After twice rinses during 2 min with Duolink II Wash Buffer A, the cells were incubated with DNA polymerase (1:40; Olink Bioscience) in the amplification solution (Olink Bioscience) for 100 min at 37 ºC. After wash twice the samples for 10 min with buffer B (SSC), a new wash was performed with 0.01% buffer B. After mounting onto slides with Duolink Mounting Medium (Olink Bioscience), the preparations were visualized by fluorescence microscopy (Zeiss, model Imager Z2, with Axiovision software 4.8) under a 400x magnification.

3.2.7. Protein quantification by BCA method

The amount of protein in each cellular suspension was determined by the bicinchoninic acid (BCA) protein assay. In this method, BSA is used as a protein standard. Therefore, a standard curve was drawn, by preparation of several dilutions of BSA in type I water, using: 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 μg/μL of BSA. In a 96 well dish, the standard curve was prepared in triplicate, by adding type I water and BCA reagent to each well containing different BSA concentrations. Synaptosomes and gliosomes samples were lysed with radioimmunoprecipitation assay buffer (RIPA), and were diluted accordingly. The diluted samples were added to several wells, in triplicates, as well as type I water and BCA reagent. The multi-well dish was incubated at 37° C, during 30 min, and, after that, the absorbance was read at 570 nm in a microplate reader spectrophotometer (Spectramax plus 384). The standard curve was then used to calculate the protein concentration in μg/mL of the different samples, taking into account the different dilutions.

3.2.8. Protein quantification by Bio-Rad method

Protein determination was performed using the Bio-Rad assay method. In a 96 well dish, a standard curve was construct, by preparation of several dilutions of BSA 0.1%, using: 5, 4, 2, 1, 0.5 and 0 μg/μL of BSA. The protein standard curve was prepared in triplicate, using BSA, type I water, lysis buffer (150 mM Tris) and Bio-Rad reagent. An aliquot of 2 μL of the sample was added to 78 μL of type I water in one 96-well plate followed by addition of 120 μL of 1:3 diluted Bio-Rad dye reagent to each well. The absorbance of each sample was read at 600 nm, after 15

min incubation at room temperature, in a microplate reader spectrophotometer (Spectramax plus 384). The absorbance of BSA standard samples was used to construct the standard curve and this was then used to calculate the protein concentration in μg/μL.

3.2.9. Synaptosomes and Gliosomes

Synaptosomes and gliosomes are functional nerve and glial subcellular re-sealed particles, respectively, which were separated from their axons and postsynaptic connections (Dunkley et al., 2008). These re-sealed terminals contain the complete presynaptic terminal, including mitochondria and synaptic vesicles, with the postsynaptic membrane and the postsynaptic density (PSD). Synaptosomes and gliosomes are commonly used to study synaptic function because they contain functional receptors, ion channels, proteins and enzymes, and moreover the intact molecular machinery for the release, uptake and storage of neurotransmitters, which closely resembles their function in nerve terminals *in vivo* (Bai and Witzmann, 2007; Dunkley et al., 1986, 2008).

3.2.9.1. Synaptosomes and Gliosomes Preparation

Synaptosomes and gliosomes were obtained from the hippocampus of brains from male suicide completers and age-matched controls (age range: 20-80 years) through a discontinuous Percoll gradient, as previously described (Canas et al., 2009a; Rebola et al., 2005a; Stigliani et al., 2006) but with some modifications (Dunkley et al., 2008). The tissue was homogenized in a medium containing 0.25 M sucrose and 10 mM HEPES (pH 7.4), using a glass-Teflon tissue grinder at 800 r.p.m for 10-12 strokes. The homogenate was centrifuged for 5 min at 1000 *g* at 4 °C (Avanti J-26 XPI centrifuge, rotor JA-25.50) to remove nuclei and debris, and the supernatant was gently stratified on a discontinuous Percoll gradient (2, 6, 10, and 23% v/v of Percoll in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4) (Figure 10). The mixture was centrifuged at 31000 *g* for 5 min at 4 °C (Avanti J-26 XPI centrifuge, rotor JA-25.50) and during the last deceleration of 2500 *g* to zero turned off braking speed (Dunkley et al., 2008). The layers between 2 and 6% of Percoll (gliosomal fraction) and between 10 and 23% of Percoll (synaptosomal fraction) were collected, washed in 10 mL of isotonic physiological solution (115 mM NaCl, 3 mM KCl, 1.2 mM KH_2PO_4, 25 mM HEPES, 10 mM glucose, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4), and further centrifuged at 30000 *g* for 20 min at 4 °C (Avanti J-26 XPI centrifuge, rotor JA-25.50) to remove myelin components and postsynaptic material from the gliosomal and

synaptosomal fractions, respectively. After that, the pellets were collected and washed in 1.5 mL of isotonic physiological solution, and further centrifuged at 16900 *g* for 20 min at 4 °C (Eppendorf 5418R centrifuge, rotor FA-45-18-11). To perform Western blot experiments, the gliosomal and synaptosomal fraction was resuspended in radioimmunoprecipitation assay buffer (RIPA) (50 mM Tris, 150 mM NaCl, 1.0% IGEPAL (NP-40), 0.5% sodium deoxycholate, 1mM EDTA and 0.1% SDS, pH 8.0) supplemented with protease inhibitor cocktail (1% CLAP), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM dithiothreitol (DTT) and the protein content was measured with BCA assay.

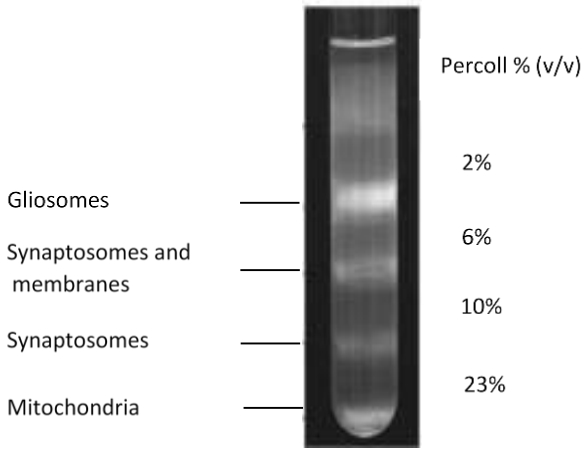

Figure 10 | Representation of the discontinuous Percoll gradient. Representative image of the discontinuous Percoll gradient after centrifugation at 31,000g during 5 min (adapted from Dunkley et al., 2008), where we can see different cellular components along the gradient. The gradient was built as follows (from bottom to top): 2.5 mL of a 23% (v/v) Percoll solution, 3 mL of a 10% (v/v) Percoll solution, 4 mL of a 6% (v/v) Percoll solution and 2.5 mL of a 2% (v/v) Percoll solution.

3.2.10. Western blotting for astrocytes lysates

After astrocytes treatment, they were lysed with 150 mM Tris buffer (pH 7.4) supplemented with protease inhibitor cocktail (CLAP), PMSF and DTT. The lysates were assayed for protein content with the Bio-Rad protein method. Afterward, the samples were denatured by addition of sample buffer 6x (500 mM Tris, 600 mM DTT, 10.3% SDS, 30% glycerol and 0.012% Bromophenol Blue, pH 6.8) and heat for 20 min at 70 °C and loaded into the Western blot gels for separation. Equal amounts of protein were then separated by electrophoresis on 10% SDS–polyacrylamide resolving gels (SDS-PAGE) (Table 6). After electrophoresis, the proteins on the polyacrylamide resolving gels were transferred to a nitrocellulose membrane, during 2 h, in a CAPS buffer (10 mM CAPS, pH 11, 10% methanol). Then, these membranes were blocked in TBS (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) with 0.1% Tween (TBS-T) and 5% nonfat dry milk, for 1h at room temperature (RT). Incubation with the primary antibodies, namely anti-GFAP, anti-Connexin-43

and anti-Glutamine synthetase (Table 3), all diluted in TBS-T with 5% nonfat dry milk, was carried out overnight at 4 °C. After 3 washes with TBS-T, the membranes were incubated with a peroxidase conjugated–linked secondary antibody (1:5 000 in TBS-T with 1% nonfat dry milk) for 2 h at RT. After washing, the membranes were revealed using an ECL kit (Amersham) or Luminata forte substrate (Millipore) and visualized under a fluorescence imaging system (ChemiDoc, Bio-Rad). After the reprobing and stripping, membranes were blocked again with TBS-T with 5% nonfat dry milk, followed by incubation with new primary antibodies, namely anti β-actin. Secondary antibodies incubation, membrane washing and revelation were done as described before. Densities of protein bands were calculated using a ChemiDoc precision digitizer equipped with the Image Lab software version 6.0 (Bio-rad).

Table 6 | Polyacrylamide gel formulation.

Gel formulation (1 Gel)	10% - Resolving Gel	4% - Stacking Gel
Tris-buffer, 1.5 M, pH 8.8	2.5 mL	-
Tris-buffer, 0.5 M, pH 6.8	-	2.5 mL
Acrylamide 30%	3.3 mL	1.3 mL
Water	4.1 mL	6.1 mL
SDS 10%	0.1 mL	0.1 mL
TEMED	5 µL	10 µL
APS 20 % (freshly prepared)	50 µL	50 µL

3.2.11. Western blotting for synaptosomes and gliosomes preparation

Synaptosomes and gliosomes samples were lysed with RIPA buffer supplemented with protease inhibitor cocktail (CLAP), PMSF and DTT. The lysates were assayed for protein content with the BCA protein method. Afterward, the samples were denatured by addition of sample buffer (500 mM Tris, 600 mM DTT, 10.3% SDS, 30% glycerol and 0.012% Bromophenol Blue, pH 6.8) and loaded into the Western blot gels for separation. Equal amounts of protein were then separated by electrophoresis on 10% TGX Stain-Free gels. After electrophoresis, the stain-free compound in gel was activated for stain-free blot imaging for 1 min. After stain-free activation, the proteins on the TGX Stain-Free gels were transferred to a nitrocellulose membrane, during 9 min at 2.3A and 25V, in a semi-dry system (Tans-Blot® Turbo™ blotting system). Then, to verify if the protein transfer from the gel to the membrane, the membranes were instantly observed using Stain-Free technology at the ChemiDoc. The images obtained in this step were used to quantify the total protein levels present in the membrane. Afterward, these membranes were blocked in TBS (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) with 0.1% Tween (TBS-T) and 5% nonfat dry milk, for 1h

at room temperature (RT). Incubation with the primary antibodies, namely anti-GFAP, anti-EAAT1, anti-synaptophysin, anti-Connexin43 and anti-Glutamine synthetase (Table 3) all diluted in TBS-T with 5% nonfat dry milk, was carried out overnight at 4 °C. After 3 washes with TBS-T, the membranes were incubated with a peroxidase conjugated–linked secondary antibody (1:5 000 in TBS-T with 1% nonfat dry milk) for 2 h at RT. After washing, the membranes were revealed using an ECL kit (Amersham) or Luminata forte substrate and visualized under a fluorescence imaging system (ChemiDoc, Bio-Rad). After the reprobing and stripping, membranes were blocked again with TBS-T with 5% nonfat dry milk, followed by incubation with new primary antibodies, namely anti-β-actin (Table 3). Secondary antibodies incubation, membrane washing and revelation were done as described before. Densities of protein bands were calculated using the Image Lab Software (Bio-rad).

Table 7 | TGX Stain-Free gel formulation

	1.5 mm Bio-Rad Glass Plates (n = gels)	
	Stacker	**Resolver**
Resolver A	-	4 mL x n
Resolver B	-	4 mL x n
Stacker A	1.5 mL x n	-
Stacker B	1.5 mL x n	-
Total Volume	**3 mL** x n	**8 mL** x n
TEMED	3 µL x n	4 µL x n
10% APS	15 µL x n	40 µL x n

3.2.12. Sripping and reprobing membranes

To remove the ECL reaction product, the membranes were rinsed twice in TBS-T for 10 min. For antibodies removal, the membranes were submerged twice in stripping solution (15 g/L glycine, 1 g/L SDS, 10 mL/L Tween 20, pH 2.2) for 15 min, and washed once in TBS-T for 10 min. Afterward, the membranes were blocked by immersing them in 5% nonfat dry milk in TBS-T for 1 h at room temperature, and incubated overnight with primary antibody and proceeded as in the Western blot protocol.

3.2.13. Statistical data analysis

The statistical analysis was carried out in GraphPad Prism version 6.01 (GraphPad Software Inc., USA). Data were expressed as percentage of values obtained in control conditions and were presented as mean ± standard error of the mean (SEM) for *n* experiments. Our controls were normalized for a hypothetical value of 100. One sample *t*-test compared with the hypothetical value of 100 was used to measure statistical differences between one independent mean and control. Comparison between two independent means was done by a Student's *t* test. To assess differences between three groups or to determine how a response is affected by two factors, a one-way and a two-way analysis of variance (ANOVA), respectively, was used, followed by a Tukey's Multiple Comparison *post hoc* Tests, to compare all groups. Differences were considered significant at $p<0.05$.

CHAPTER 4

RESULTS

4.1. Dexamethasone exposure increase glutamate and ATP release by astrocytes

First, we performed a set of experiments in order to determine if dexamethasone (DEX) could induce gliotransmitters release, namely glutamate and ATP. Alterations in these two gliotransmitters levels are largely discussed to be associated with depression etiology. Cultured astrocytes were exposed to DEX (100 nM) for 1 h, and significantly (** $p < 0.01$) alterations on the extracellular glutamate levels were observed (183.3 ± 27.6% of control cells), as can be seen in Figure 11A. In the same way, it was observed that ATP levels were also significantly (# # $p < 0.01$) increase upon treatment with DEX (182.2 ± 33.1% of control cells) (Figure 11B).

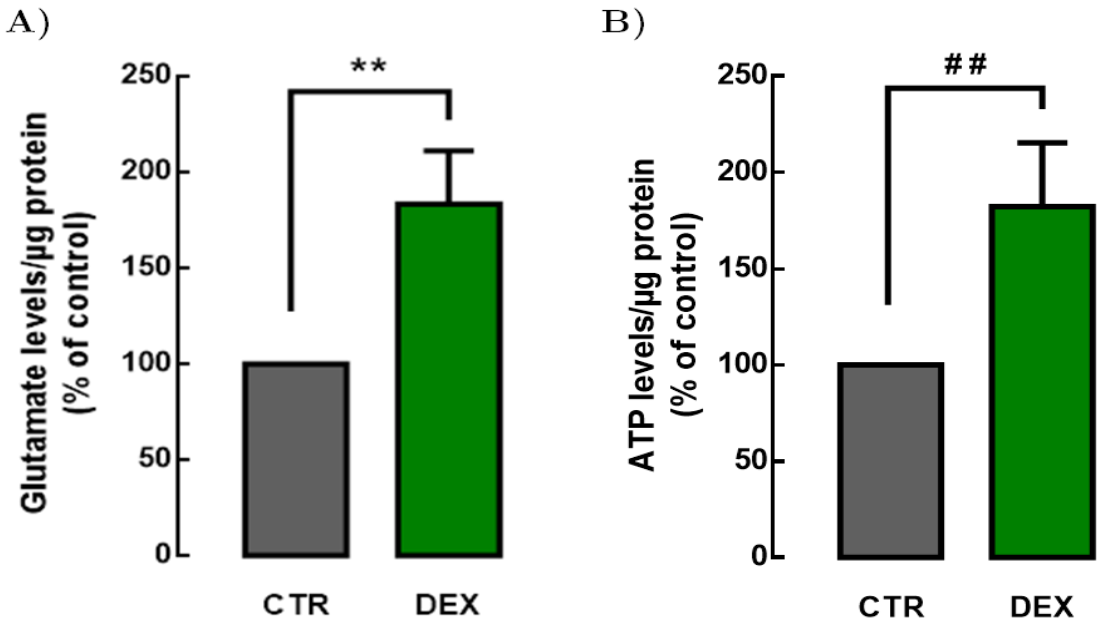

Figure 11 | Dexamethasone increase glutamate and ATP levels. Dexamethasone (100nM) were exposure for 1h in astrocyte primary cell cultures. Glutamate (A) and ATP (B) levels were calculated as percentage of control cells (not exposed to dexamethasone) and normalized by µg of total protein. Data shown as mean ± SEM of 7-9 and 14-15 independent experiments of glutamate and ATP levels, respectively. ** $p < 0.01$, ## $p < 0.01$, one sample *t*-test as compared with a hypothetical value of 100% (control).

4.2. Dexamethasone exposure increase ATP release by astrocytes

In this part of the study we performed a time course with 2 additional time points, to investigated if there are differences in the ATP release induced by DEX (100 nM) according to time exposure to this compound. Since it has been described previously that in depressive like conditions, normally it is observed a decrease in the ATP levels (Cao et al., 2013a). Alterations on the extracellular ATP levels were determined at 5 min, 1h and 24h of incubation periods with DEX (100 nM) (Figure 12). In astrocytes incubated with DEX there is a significant (# $p < 0.05$) increase in the levels of ATP release for 5 min (222.2 ± 37.3%), 1h (182.2 ± 33.1% of control cells) and 24h (172.2 ± 26.3% of control cells) when compared to control cells (non-treated cells).

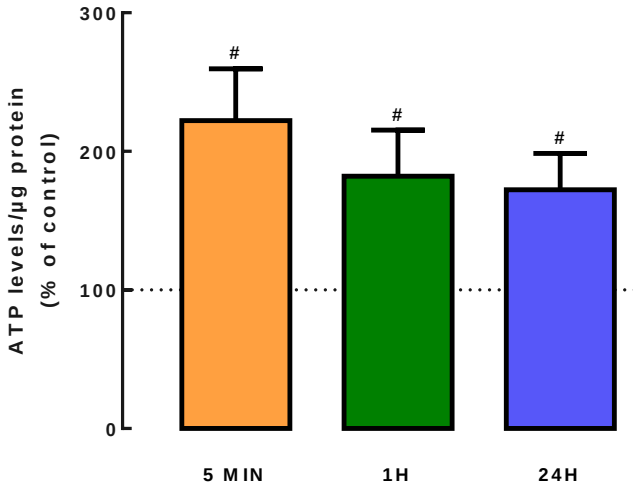

Figure 12 | Time course of ATP release by cultured astrocytes exposed to dexamethasone (100nM). ATP levels were calculated as a percentage of control cells (not exposed to dexamethasone) and normalized by µg of total protein. Data shown as mean ± SEM of 5-14 independent experiments. # $p < 0.05$, one sample *t*-test as compared with control cells (100%).

4.3. Possible release mechanisms of glutamate release

Subsequently, we performed a set of experiments in order to determine if functional HCs, constituted mainly by connexin 43 is involved in glutamate release induced by DEX, after 1h. Cultured astrocytes were exposed to carbenoxolone (CBX, 50 µM, used as inhibitor of hemichannels Cx43), 30 min before the incubation with DEX (100 nM) for 1 h. As can be seen in Figure 13, CBX, *per se*, caused a significant (# # # $p < 0.001$) increase in the glutamate levels (365.0 ± 48.9% of control cells). Furthermore, glutamate levels significantly (*** $p < 0.001$) increase when Cx43 was blocked in the presence of DEX (835.4 ± 107.5% of control cells) when compared with CBX-treated cells. Additionally, it was possible to observe a synergistic effect upon incubation of cells with both CBX and DEX.

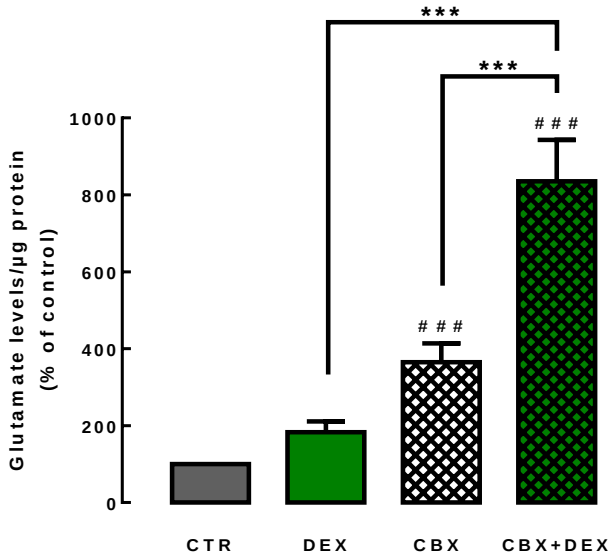

Figure 13 | Inhibition of hemichannels Cx43 increase glutamate released caused by dexamethasone. The inhibitor of hemichannels Cx43, CBX (50 µM) was added to cells 30 min before the dexamethasone (100nM) exposure (1h). Data shown as mean ± SEM of 3-7 independent experiments, # # # $p < 0.001$ as compared with control cells (100%), one sample *t*-test, *** $p < 0.001$ as compared with DEX-treated cells or CBX-treated cells, two-way ANOVA, post-hoc Tukey's test.

Additionally, we also investigate if P2X7 purinergic receptors (P2X7R) controlled the dexamethasone-induced alterations of extracellular glutamate. Cultured astrocytes were exposed to Blue Brilliant G (BBG, 100 nM), a P2X7 purinergic receptors antagonist, 30 min before the incubation with DEX (100 nM) for 1 h. As can be seen in Figure 14, in these preliminary results, extracellular glutamate levels seem to be increased in the presence of BBG when cells were exposed to DEX (462.2 ± 279.0% of control cells) as compared with dexamethasone-treated cells (184.7 ± 32.6% of control cells), but these results remain to be confirmed.

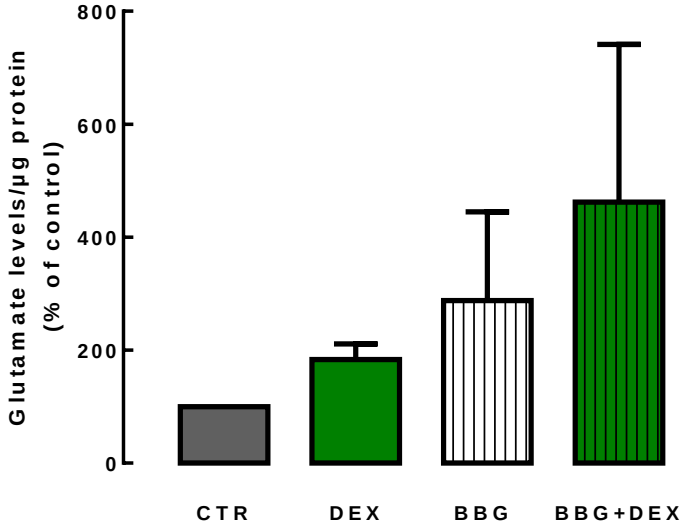

Figure 14 | Block of P2X7 receptors may affect the release of extracellular glutamate. The antagonist of P2X7 receptors, Blue Brilliant G (BBG, 100 nM) was added to cells 30 min before the 1h incubation with DEX (100nM). Data shown as mean ± SEM of 2-6 independent experiments.

4.4. Role of $A_{2A}R$ in DEX-induced alterations in astrocytes

To better understand the long-term alterations induced by DEX in astrocytes, we performed an evaluation, by Western blot analysis, of the density of different astrocytic markers. We selected glial fibrillary acidic protein (GFAP) intermediate filament, that can be a marker of astrogliosis; Connexin43 (Cx43) that is a main component of hemichannels and gap junctions and can have a determinant role in the astrocytic communication; and glutamine synthetase (GS), since we know that it is an important enzyme in the glutamate-glutamine cycle.

Upon comparison with control cells, the cells treated with DEX (100 nM) display a significant (# $p < 0.05$) increase in Cx43 (158.3 ± 17.6% of control) and GS (147.6 ± 14.5% of control) levels and no change for GFAP levels (124.1 ± 23.1% of control) (Figure 15).

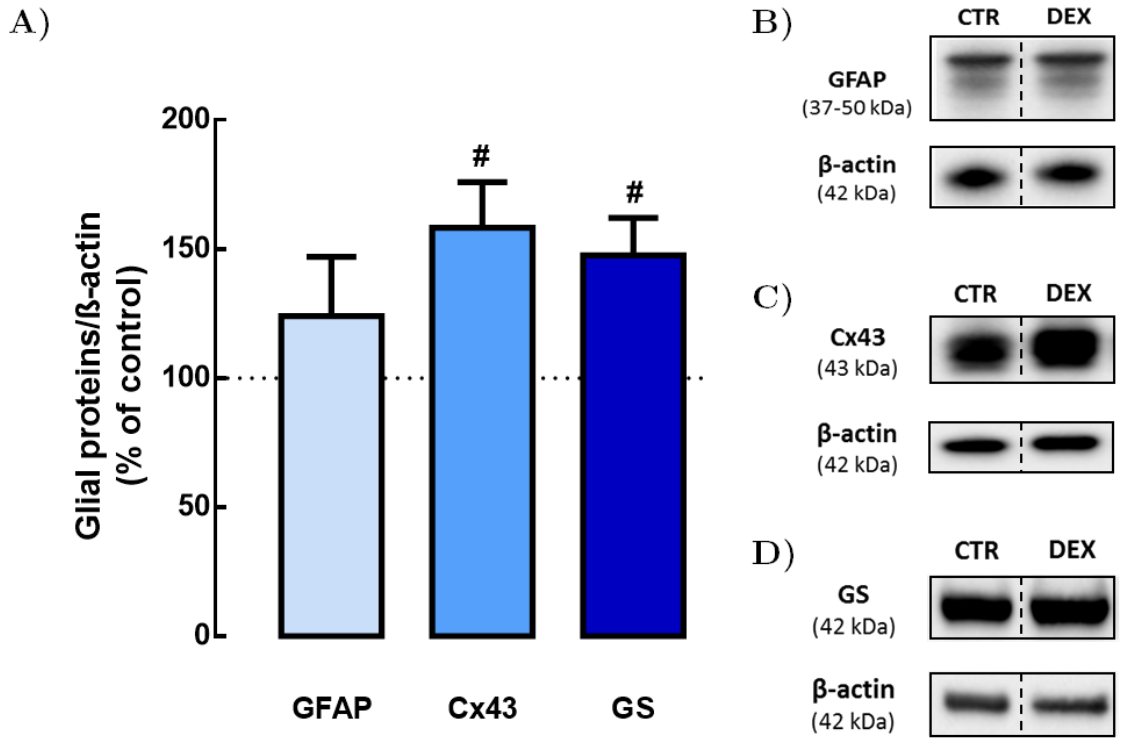

Figure 15 | Effect of dexamethasone (DEX) on GFAP, Connexin43 (Cx43) and glutamine synthetase (GS) levels. Cultured astrocytes were treated or not (control) with DEX (100 nM) for 24h. Cell lysates were examined by immunoblotting with an anti-GFAP and anti-Cx43 or anti-GS. **(A)** Graphical representation of protein densities upon DEX treatment. Representative Western blot of GFAP **(B)**, Cx43 **(C)** and GS **(D)** levels are shown. Data shown as mean ± SEM of n=5-10. # $p < 0.05$, one sample *t*-test compared with the hypothetical value of 100.

In order to try to understand the role of $A_{2A}R$ in DEX-induced alterations, we started by doing an Western blot analysis, to ascertain if there were any alterations in the density of $A_{2A}R$ in astrocytes. As we can see in (Figure 16), there is a tendency to observe an increase in $A_{2A}R$ density in cells treated with DEX (100 nM) when compared to non-treated cells (211.4 ± 43.3% of control).

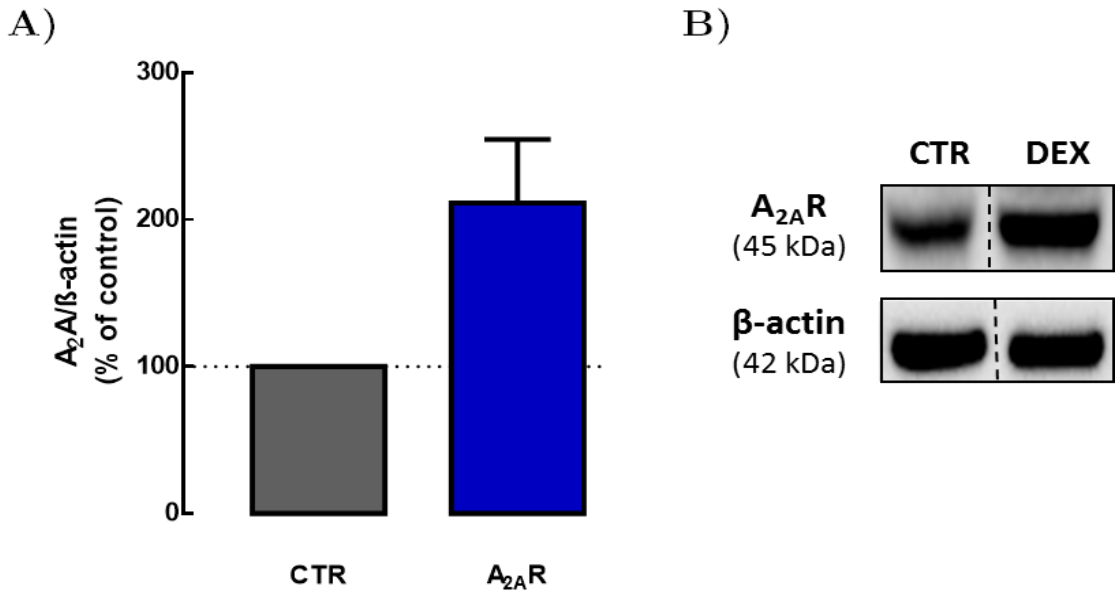

Figure 16 | Effect of dexamethasone (DEX) on $A_{2A}R$ levels. Cultured astrocytes were treated or not (control) with DEX (100 nM) for 24h. Cell lysates were examined by immunoblotting with an anti-$A_{2A}R$. **(A)** Graphical representation of $A_{2A}R$ densities upon DEX treatment. Representative Western blot of $A_{2A}R$ **(B)** levels are shown. Data shown as mean ± SEM of n=2.

Furthermore, we also investigated how dexamethasone (DEX, 100 nM) affected astrocyte reactivity and morphology. Cultured astrocytes were treated with saline or DEX for 24 h. Alterations in the reactive state of astrocytes and in the levels of Cx were assessed by monitoring the immunoreactivities of GFAP and Cx43.

The data obtained by immunocytochemistry (Figure 17) showed that DEX-treated astrocytes exhibited a cellular hypertrophy and developed a compacted and stellate morphology, while control cells maintained the typical flattened and asymmetrical appearance of resting astrocytes. In addition, it was possible to observe an up-regulation of GFAP immunostaining in DEX treated cells, a characteristic of reactive astrocytes (Eng et al., 2000). These data suggest that DEX is able to trigger a series of structural and functional changes in the astrocytes typical of a reactive morphology – astrogliosis. In parallel, we observed an increase in the levels of Cx43 when cells were exposed to DEX.

To understand if the blockade of $A_{2A}R$ was able to prevent the alterations induced by DEX, a pharmacological approach (SCH 58261, 50 nM) was used. Upon blockade of $A_{2A}R$, it was observed a prevention of the alterations induced by dexamethasone.

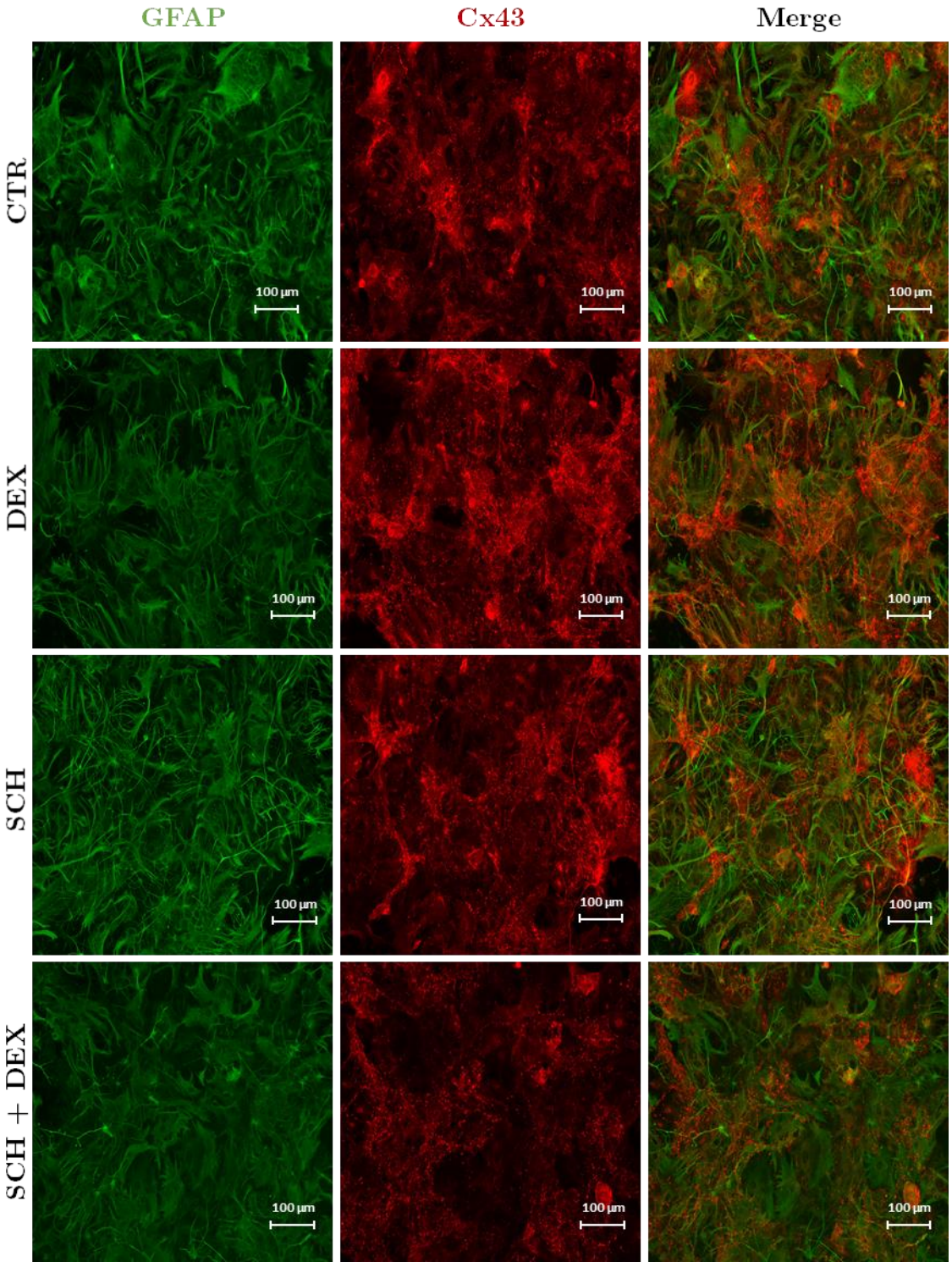

Figure 17 | Immunostaining for GFAP (astrocytes - in green) and Cx43 (hemichannels - in red) in astroglial-enriched cultures. The $A_{2A}R$ selective antagonist, SCH58261, prevented the increase of connexin43 (Cx43) levels caused by dexamethasone (DEX). The selective antagonist SCH58261 (50 nM) was added to cells 30 min before the DEX (100 nM) exposure (24h).

To explore a possible interaction between $A_{2A}R$ and Cx43 we used a technique named *in situ* proximity ligand assay (PLA), that can confirm the existence of a physical interaction between $A_{2A}R$ and Cx43. This approach detects proteins located within a radius of <40 nm. If there is an interaction, it is possible to observe fluorescent dots in the presence of both anti-$A_{2A}R$ and anti-Cx43 antibodies in astrocyte primary cell culture (Figure 18). Cultured astrocytes were exposed to dexamethasone (DEX, 100 nM) for 1 h and 24h (data not shown). The results showed red dots, meaning that physical interaction exist with astrocytic $A_{2A}R$ and Cx43. Upon comparison with control cells, the cells treated with DEX (100 nM) display an increase in the red dots, suggesting that a physically interaction between $A_{2A}R$ and Cx43 increases upon DEX-treated of astrocytes.

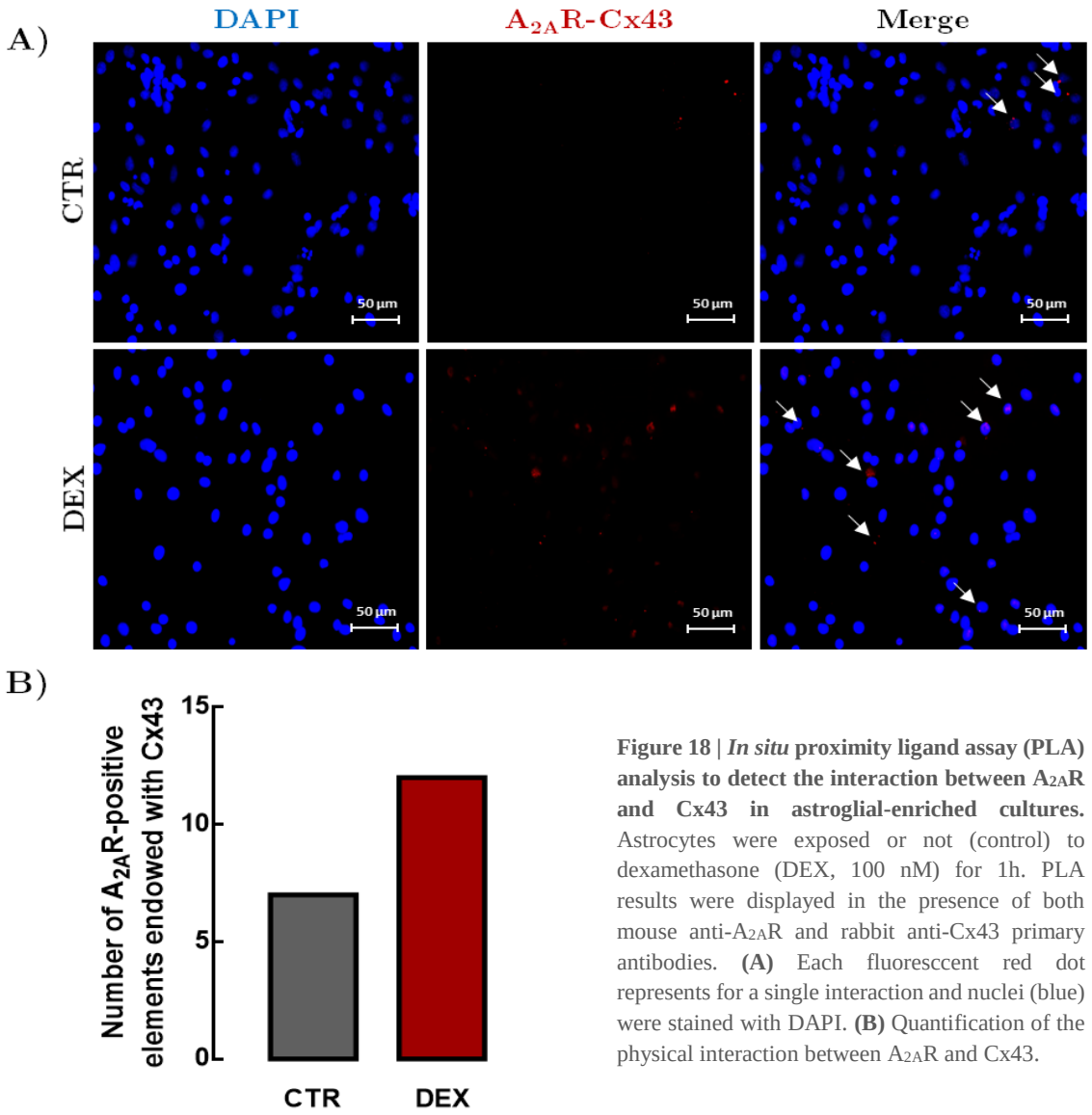

Figure 18 | *In situ* proximity ligand assay (PLA) analysis to detect the interaction between $A_{2A}R$ and Cx43 in astroglial-enriched cultures. Astrocytes were exposed or not (control) to dexamethasone (DEX, 100 nM) for 1h. PLA results were displayed in the presence of both mouse anti-$A_{2A}R$ and rabbit anti-Cx43 primary antibodies. **(A)** Each fluoresccent red dot represents for a single interaction and nuclei (blue) were stained with DAPI. **(B)** Quantification of the physical interaction between $A_{2A}R$ and Cx43.

4.5. Role of $A_{2A}R$ in gliotransmitters release induced by DEX

In this part of the study we investigated if the blockade of $A_{2A}R$ could prevent the increase in glutamate release induced by 1h dexamethasone-exposed (DEX). For that purpose, cultured astrocytes were exposed to a selective $A_{2A}R$ antagonist, SCH 58261 (SCH, 50 nM), 30 min before the incubation with DEX (100 nM) for 1 h. Figure 19 shows a significant (# # $p < 0.01$) increase in the levels of glutamate in DEX-incubated cells, as demonstrated previously. The blockade of $A_{2A}R$ *per se* does not affect glutamate release when compared with control cells. Nevertheless, the blockade of $A_{2A}R$ do not prevent glutamate release induced by DEX (187.9 ± 25.9% of control cells) when compared with dexamethasone-treated cells (183.3 ± 27.6% of control cells).

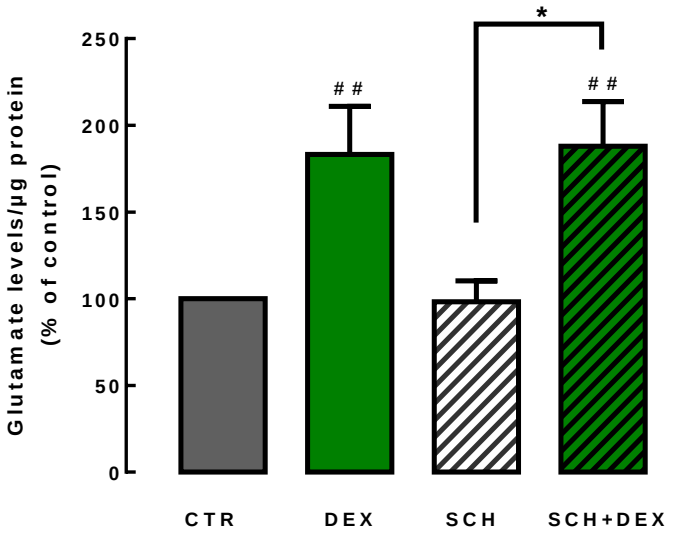

Figure 19 | The $A_{2A}R$ selective antagonist, SCH 58261, did not affected the glutamate release caused by dexamethasone (100 nM). SCH58261 (50 nM) was added to cells 30 min before the dexamethasone exposure (1h). Data shown as mean ± SEM of 5-9 independent experiments, # # $p < 0.01$ as compared with control cells (100%), one sample *t*-test, * $p < 0.05$ as compared with SCH-treated cells, two-way ANOVA, post-hoc Tukey's test.

We also investigated if $A_{2A}R$ could control the release of extracellular ATP levels. Selective $A_{2A}R$ antagonist, SCH 58261 (SCH, 50 nM), were exposed to cultured astrocytes, 30 min before the incubation with DEX (100 nM) for 1 h. The data obtained showed that SCH 58261 does not have an effect *per se*. Yet, the blockade of $A_{2A}R$ when cells were exposed to DEX significantly (*$p < 0.05$) prevent the increase of ATP release induced by DEX (75.4 ± 22.9% of control cells) as compared with dexamethasone-treated cells (182.2 ± 33.1% of control cells) (Figure 20).

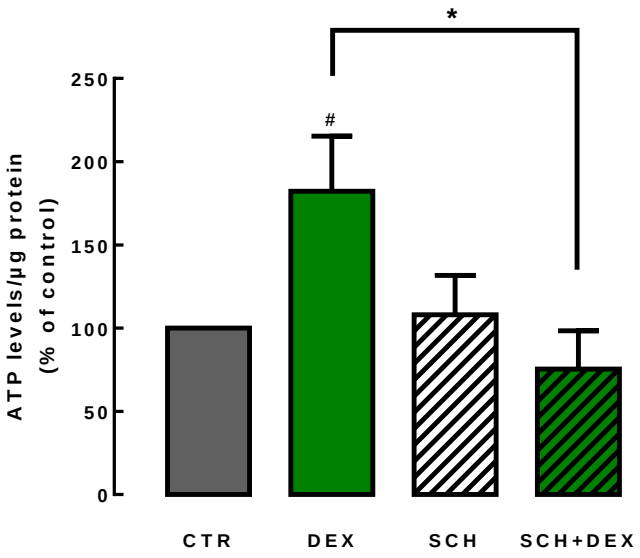

Figure 20 | The $A_{2A}R$ selective antagonist, SCH58261, prevented the increase of ATP release caused by dexamethasone (100 nM). SCH58261 (50 nM) was added to cells 30 min before the dexamethasone exposure (1h). Data shown as mean ± SEM of 7-14 independent experiments, # $p < 0.05$ as compared with control cells (100%), one sample *t*-test, * $p < 0.05$ as compared with DEX-treated cells, two-way ANOVA, post-hoc Tukey's test.

4.6. Synaptic and glial markers validation

In order to validate the gliosomal preparations from human postmortem tissue (hippocampus), the relative amount of synaptic and glial markers was compared between gliosomal and nerve terminals preparation of the same sample, by Western blot analysis.

We observed, as expected, an increase in the density of synaptophysin (184.5% ± 23.8%, n=11-18) and syntaxin (128.2% ± 9.4%, n=11-18) in synaptosomes compared to gliosomes (Figure 21A, C).

In addition, we also observed a decrease in the density of fibrillary acidic protein (GFAP) in synaptosomes (44.5% ± 9.1%, n=9-17) than in gliosomes (Figure 21E), which validates our gliosomal preparation.

The validation of gliosomal preparation with synaptic and glial markers is summarized in Table 8.

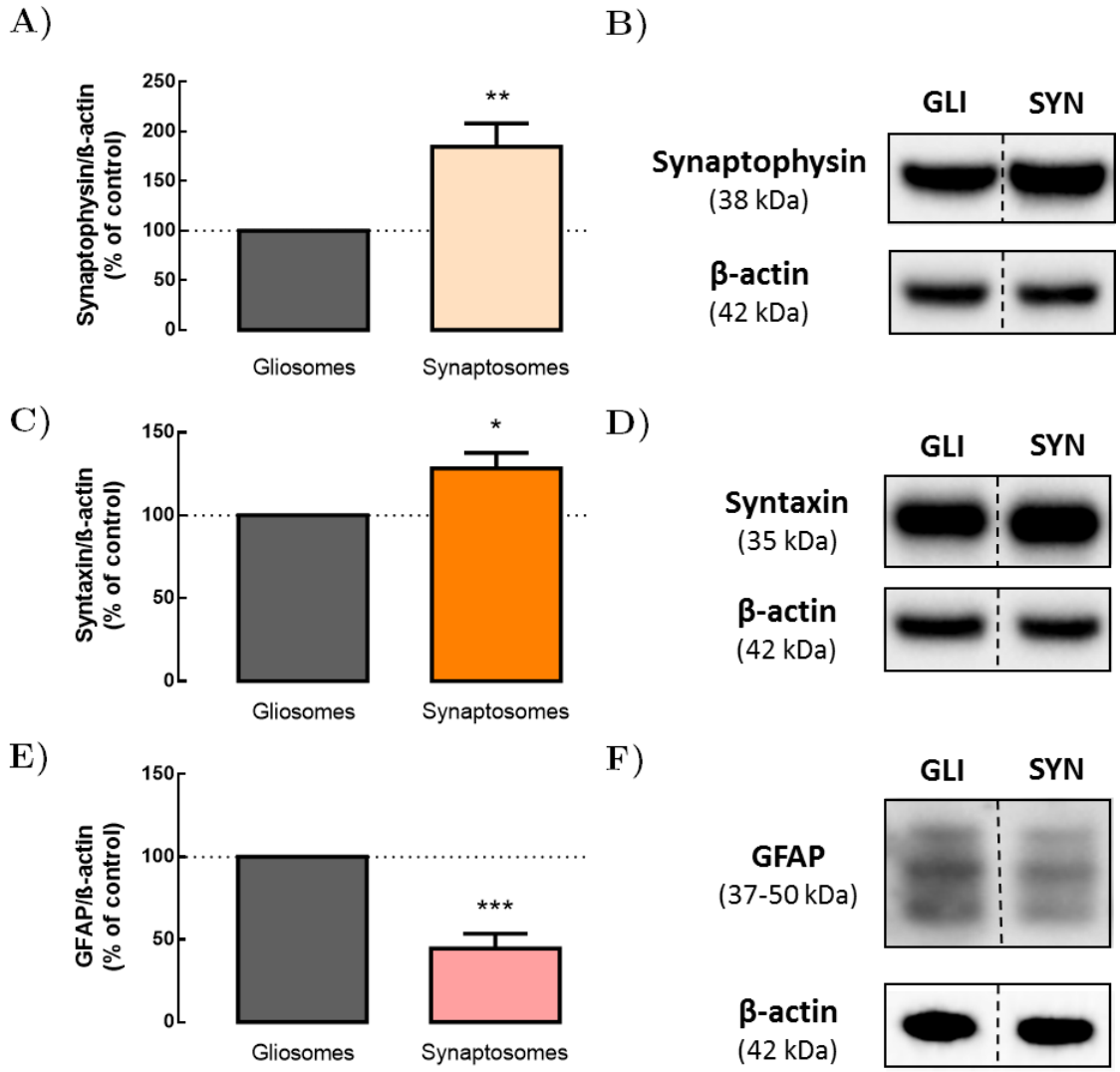

Figure 21| Validation of nerve terminals (synaptosomes (SYN) and gliosomes (GLI)) preparation from hippocampus using two synaptic markers (synaptophysin (A) and syntaxin (C)) and a glial marker (GFAP) (E). Representative Western blot of synaptophysin **(B)**, syntaxin **(D)** and GFAP **(F)** levels are shown. Data shown as mean

± SEM of n=9-18. * p < 0.05, **p < 0.01, *** p < 0.001, one sample t-test compared with the hypothetical value of 100.

Table 8 | Differential presence of synaptic markers (synaptophysin and syntaxin) and glial marker (GFAP) in gliosomes and synaptosomes from hippocampus.

		Gliosomes (control, %)	*Synaptosomes (% of control)*	
Synaptic markers	**Synaptophysin**	100	184.5 ± 23.8	↑
	Syntaxin	100	128.2 ± 9.4	↑
Glial markers	**GFAP**	100	44.5 ± 9.1	↓

4.7. Glial markers in suicide

After validation of the gliosomes, we focus on determining modifications in hippocampus of different astrocytic markers in suicide completers in comparison with age-matched controls, since it is already known that there is a decrease in glial cells in postmortem samples of individuals with MDD or suicide completers (reviewed in (Rial et al., 2015)). We assessed the levels of GFAP, connexin 43, glutamine synthetase and EAAT1 by Western blot analysis, and the immunoreactivity of each band was normalized with β-actin (this loading control does not change with suicide - data not shown).

There were no significant differences observed in GFAP density (267.3% ± 173.6%, n=6) of suicide completers when compared with age-matched controls (Figure 22).

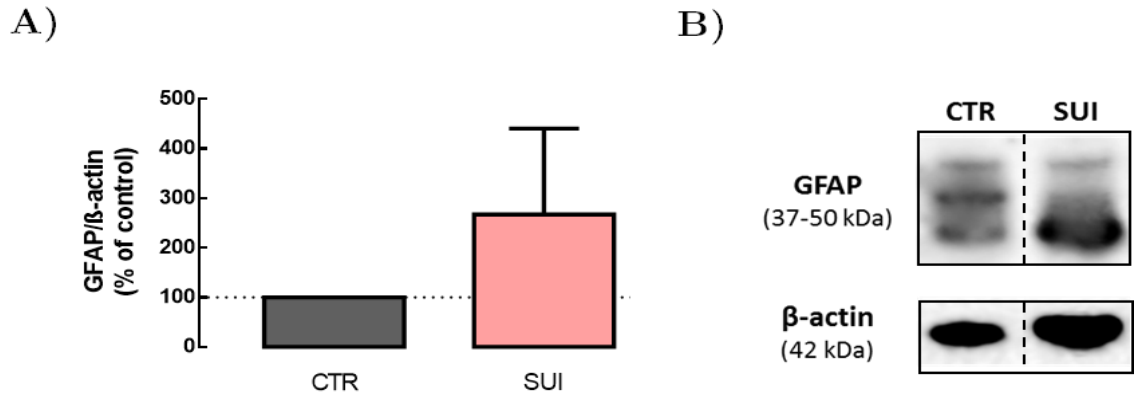

Figure 22 | GFAP density levels do not change with suicide in hippocampus (A, B). Representative Western blot of GFAP and β-actin (loading control) levels in the suicide and control samples **(B)**. The data were calculated as percentage of control and normalized by β-actin. Data shown as mean ± SEM of n=6, one sample *t*-test compared with the hypothetical value of 100.

We also observed that there was a tendency to increase in connexin 43 (Cx43) density (452.2% ± 175.4%, n=5) of suicide completers when compared with age-matched controls (Figure 23).

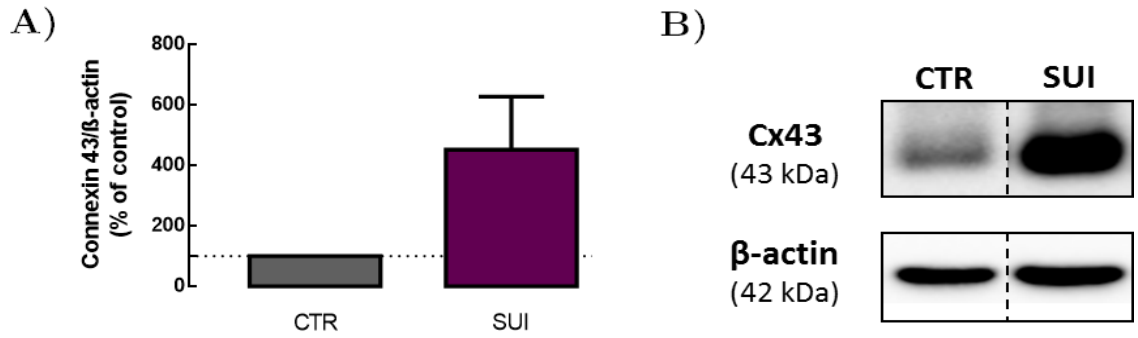

Figure 23 | Connexin43 (Cx43) density levels do not change with suicide in hippocampus (A, B). Representative Western blot of Cx43 and β-actin (loading control) levels in the suicide and control samples **(B)**. Data shown as mean ± SEM of n=5, one sample *t*-test compared with the hypothetical value of 100.

In addition, glutamine synthetase (GS) density do not change with suicide (138.7% ± 34.8%, n=7) (Figure 24).

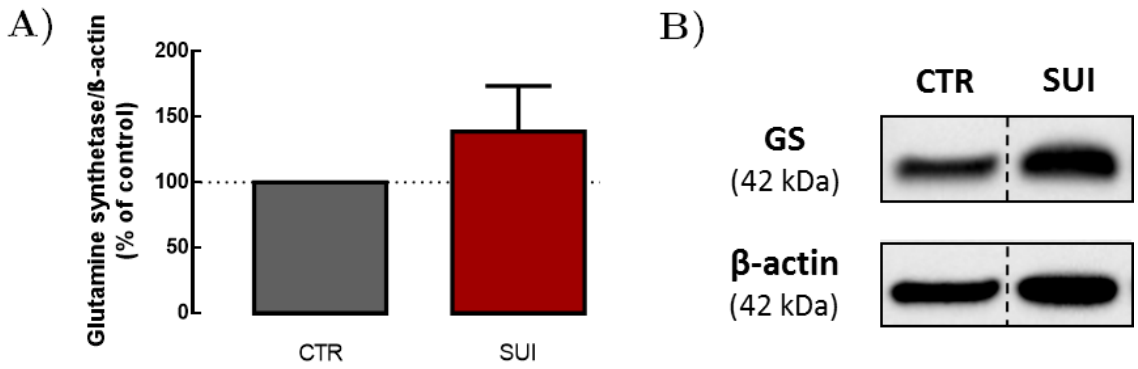

Figure 24 | Glutamine synthetase (GS) density levels do not change with suicide in hippocampus (A, B). Representative Western blot of GS and β-actin (loading control) levels in the suicide and control samples **(B)**. Data shown as mean ± SEM of n=7, one sample t-test compared with the hypothetical value of 100.

Furthermore, we observed an increase in the density of EAAT1 (122.6% ± 4.9%, n=7) of suicide completers when compared with age-matched controls (Figure 25).

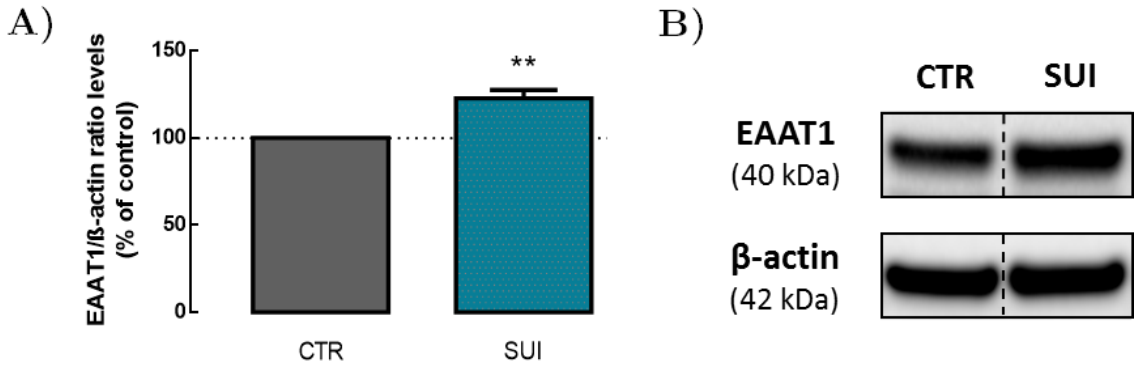

Figure 25 | Up-regulation of EAAT1 density levels with suicide in gliosomes from hippocampus (A, B). EAAT1 density increases with suicide in gliossomes **(A)**. Representative Western blot of EAAT1 and β-actin (control for protein loading) levels in the suicide and control samples **(B)**. Data shown as mean ± SEM of n=7, **p < 0.01, one sample *t*-test compared with the hypothetical value of 100.

In table 2 we present a compilation of the data collected regarding the alterations encountered in astrocytic markers of suicide completers.

Table 9 | Compilation of changes observed in glial proteins in suicide completers in comparison with age-matched controls.

	Age-matched controls (control, %)	*Suicide completers (% of control)*	
GFAP	100	267.3 ± 173.6	ns
Cx43	100	452.2 ± 175.4	ns
GS	100	138.7 ± 34.8	ns
EAAT1	100	122.6 ± 4.9*	↑

* $p < 0.05$, when compared with age-matched controls

4.8. Synaptic and glial markers ontogeny

MDD affects many people of all ages however according to *World Health Organization* the prevalence rates of depression peak among older adults (Secretariat, 2011). For that reason, in this part of the study, we investigated if age may also play an important role in synaptic and glial markers. To answer to that question, we analyzed the levels of different synaptic and glial markers in 3 different age ranges: 20-40 years, 60 years and 70-80 years. Moreover, in this ontogeny study, it was decided to consider the 20-40 years-old as 100%, to normalize results between different membranes. In order to determine these changes Western blot was performed and the immunoreactivity of each band was normalized with total protein, since β-actin changes with age (Figure 26).

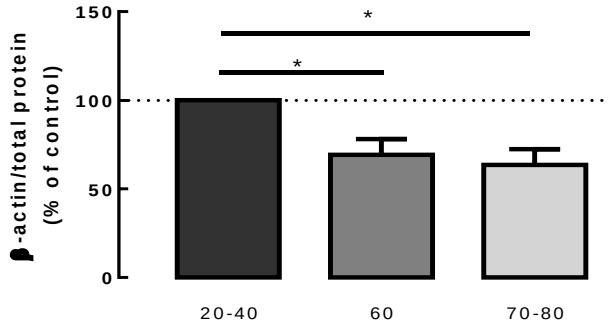

Figure 26 | β-actin levels decrease with age in the hippocampus. Data shown as mean ± SEM of n=3-4, *p < 0.05, one-way analysis of variance (ANOVA), followed by a Tukey's Multiple Comparison post hoc Tests, to compare all groups.

The Figure 27 shows the levels of synaptophysin decreases with aging. The levels of synaptophysin in subjects around 60 years (69.2 ± 4.6%, n=3) were significantly (p < 0.001) lower than levels in subjects between 20-40 years (100%, n=4), and these levels remained lower in subjects between 70-80 years (62.2 ± 4.9%, n=4) comparing to subjects between 20-40 years.

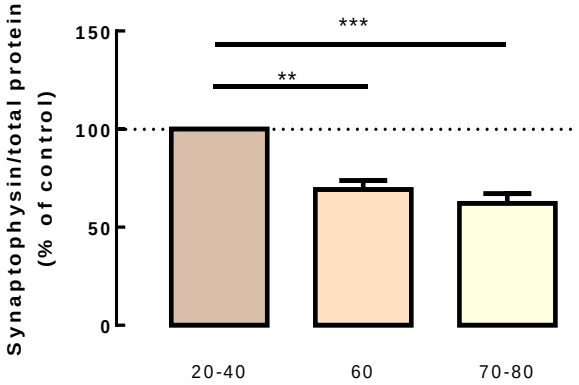

Figure 27 | Synaptophysin levels decrease with age in the hippocampus. Data shown as mean ± SEM of n=3-4, **p< 0.01, ***p < 0.001, one-way analysis of variance (ANOVA), followed by a Tukey's Multiple Comparison post hoc Tests, to compare all groups.

We also observed that there was a tendency to decrease syntaxin levels when compared in different age ranges, however these differences were not significant (Figure 28).

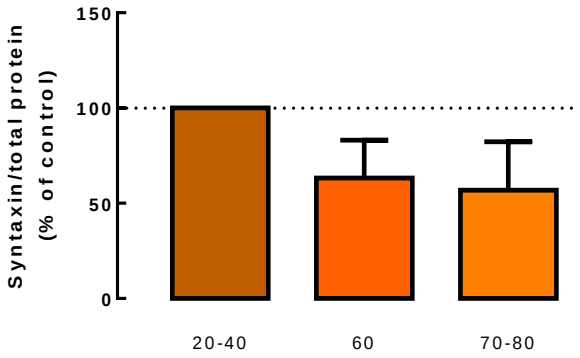

Figure 28 | Distribution of syntaxin levels by age in hippocampus. Data shown as mean ± SEM of n=3-4, one-way analysis of variance (ANOVA).

On the contrary, GFAP density significantly increases in older men when compared with young men. The levels of GFAP in subjects around 60 years (174.8 ± 9.5%, n=3) were increased when compared with subjects between 20-40 years (100%, n=4). Additionally, the levels of GFAP in subjects between 70-80 years (138.9 ± 10.1%, n=3) were also increased when compared with subjects between 20-40 years (Figure 29).

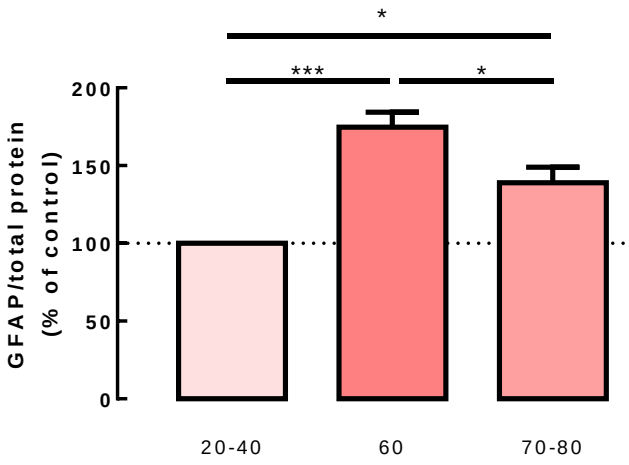

Figure 29 | Distribution of GFAP levels by age in hippocampus. Data shown as mean ± SEM of n=3-4, * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$, one-way analysis of variance (ANOVA), followed by a Tukey's Multiple Comparison post hoc Tests, to compare all groups.

No significant differences were encountered in connexin 43 density when comparing different age intervals (Figure 30).

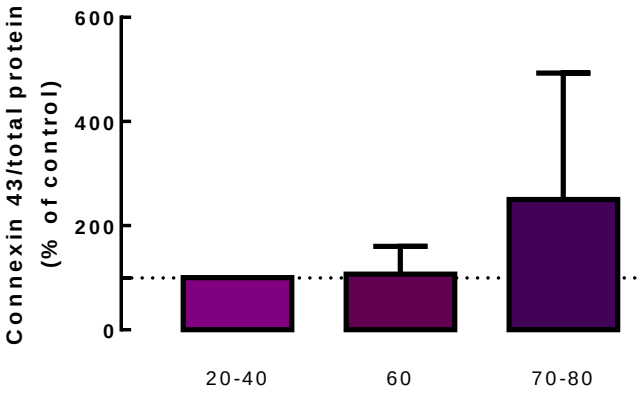

Figure 30 | Distribution of connexin43 levels by age in hippocampus. Data shown as mean ± SEM of n=3-4, one-way analysis of variance (ANOVA), followed by a Tukey's Multiple Comparison post hoc Tests, to compare all groups.

Furthermore, the levels of glutamine synthetase (GS) decrease in gliosomes with the age (Figure 31). The levels of GS in subjects between 70-80 years (63.9 ± 8.7%, n=4) were significantly ($p < 0.05$) lower than levels in subjects between 20-40 years (100%, n=4).

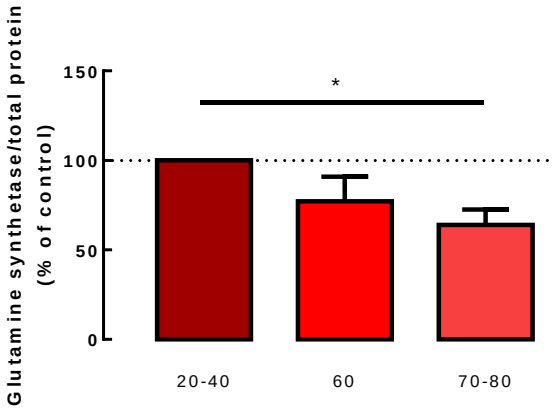

Figure 31 | Distribution of glutamine synthetase levels by age in hippocampus. The glutamine synthetase levels were calculated as percentage of control and normalized by total protein. Data shown as mean ± SEM of n=3-4. *$p < 0.05$, one-way analysis of variance (ANOVA), followed by a Tukey's Multiple Comparison post hoc Tests, to compare all groups.

The EAAT1 levels appear not to be modified with aging (Figure 32).

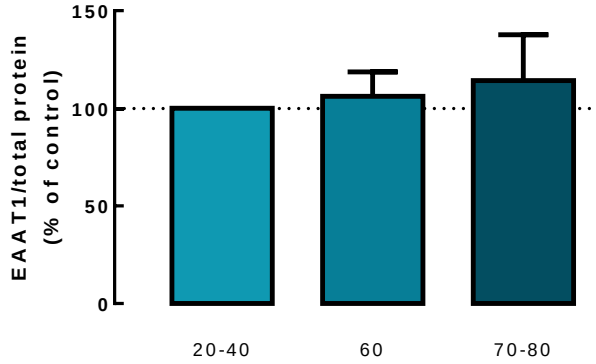

Figure 32 | Distribution of EAAT1 levels by age in hippocampus. The EAAT1 levels were calculated as percentage of control and normalized by total protein. Data shown as mean ± SEM of n=3-4, one-way analysis of variance (ANOVA), followed by a Tukey's Multiple Comparison post hoc Tests, to compare all groups.

The alterations occurred in synaptic and glial markers with aging is summarized in Table 10.

Table 10 | Alterations of hippocampal synaptic markers and glial markers with age.

		20-40 years (control, %)	*60 years (control, %)*	*70-80 years (control, %)*	
Synaptic markers	**Synaptophysin**	100	69.2 ± 4.6*	62.2 ± 4.9*	↓
	Syntaxin	100	63.3 ± 19.8	56.9 ± 25.4	ns
Glial markers	**GFAP**	100	174.8 ± 9.5*	138.9 ± 10.1*	↑
	Cx43	100	106.8 ± 53.5	250.3 ± 242.9	ns
	GS	100	77.2 ± 13.7	63.9 ± 8.7*	↓
	EAAT1	100	106.1 ± 12.6	114.2 ± 23.5	ns

* $p < 0.05$, when compared with 20-40 years

CHAPTER 5

DISCUSSION

5.1. Alterations induced by DEX in astrocytic cultures – role of $A_{2A}R$ receptors

Major depression disorder (MDD) is the most common of all psychiatric disorders (Kessler et al., 2003) and affects many people of all ages and whose incidence seems to be on the rise. This and others mood/anxiety disorders, as well as immunosuppression, diabetes, osteoporosis, reproductive failure, neuronal atrophy (Pardon and Rattray 2008; Sotiropoulos et al. 2008) are some of the many consequences of exposure to environmental chronic stress and its impact on brain tissue morphology (Sanacora et al., 2012). Upon a stress stimuli, endogenous glucocorticoids (GC) increase and glucocorticoids receptors (GR) became active to mediate a plethora of physiological changes in order to restore homeostasis (Sapolsky, 1999). However, under chronic stress conditions, GCs action can change its protective function to harmful function, and the persistently elevated GCs increase the difficulty to maintain homeostasis. Moreover, these high levels of GCs can increase the susceptibility to develop mood alterations observed in humans and in animal models of mood disorders (Charney and Manji, 2004; McEwen, 2000; Oliveira et al., 2006). Since dexamethasone (DEX) is the primary glucocorticoids receptor (GR)-selective agonist and it is commonly used in clinic when a peak of endogenous GCs is need (Crane et al., 2003; Forest et al., 1998; New et al., 2001), we used this synthetic GC to mimic stress conditions, which is risk factor for depression (Nestler et al., 2002).

Recent studies indicate that depression results from maladaptive neuroplastic processes occurring in defined frontolimbic circuits responsible for emotional processing (Rial et al., 2015). The potential involvement of astrocytes in these processes due to their essential role in central nervous system (CNS) development and function has long been suggested. They control homeostasis, regulate neurogenesis and synaptic transmission, interaction with synapses, among other functions (reviewed in: Rial et al., 2015). Furthermore, this involvement of astrocytes is supported by the observations that depressive-like conditions are associated with a decreased density and morphological and functional atrophy of astrocytes in frontolimbic regions (reviewed in: Rial et al., 2015). Due to these evidences and the expression of GCs in astrocytes (Bohn et al., 1991; Crossin et al., 1997; Vielkind et al., 1990), we used an *in vitro* model of depressive-like conditions.

Moreover, astrocytes are responsible for the release of the majority of glutamate released (Danbolt, 2001), therefore, astrocytes are essential in modulating and maintaining glutamatergic transmission in the brain (Schousboe, 2003; Tani et al., 2014). Dysfunction in glutamate-glutamine cycle would result in an excess glutamate in the extracellular space, leading to overactivation of extrasynaptic receptors and, consequently, neuronal excitotoxicity, which may result in neurodegeneration and mood/anxiety disorders (Burnstock, 2007; Hertz and Zielke,

2004; Matos et al., 2008, 2012a, 2012b; Persson and Rönnbäck, 2012; Popoli et al., 2012; Sanacora et al., 2012).

With this in mind, we wanted to analyze the effects on glutamate release in DEX-exposure astrocyte primary cell culture. Our results are in agreement with those showing a marked increase in the level of extracellular glutamate and a trend for decrease plasma glutamine/glutamate ratios in the plasma of depressed patients compared to healthy comparison subjects (Altamura et al., 1995, 1993; Kucukibrahimoglu et al., 2009; Mauri et al., 1998; Mitani et al., 2006; Sanacora et al., 2012). Furthermore, other studies performed in stress animal models showed the same evidence to increase glutamate levels in stress conditions (Bagley and Moghaddam, 1997; Hascup et al., 2010; Musazzi et al., 2010; Reznikov et al., 2007; Satoh and Shimeki, 2010; Venero and Borrell, 1999). These results highlight astrocytic glutamate release as being involved in the pathophysiology of MDD.

Afterwards, we moved on to another gliotransmitter, ATP, that is a source of cellular energy and is also an important signaling molecule that mediate diverse biological effects and allow cells to communicate with one another (Burnstock, 2007; Cao et al., 2013b; Yang et al., 2016; Zhang et al., 2003). Using DEX to treated astrocyte primary cell culture, we expected to observe a decrease in ATP release. Unfortunately, our results showed an increase in ATP levels, which is the opposite effect that we were expecting and what Cao and colleagues (2013) observed in their stress mouse model. One possible explanation is the adaptive processes that occur in the brain during the 6 weeks protocol, that are difficult to mimic in this simple model (Krügel, 2015).

To understand if the dexamethasone induced-ATP release varied with different incubation periods, we analyze the levels of ATP release by astrocytes in three time points. Strikingly, both acute (5 min and 1h) and chronic (24h) incubation with DEX led to the same significant increase of extracellular ATP levels. These results suggest that maybe longer exposure times could mimic what occurs in a stress animal models and depression, but remains to be tested.

Afterwards, we wanted to study which is the mechanism involved in dexamethasone induced-gliotransmitter release. According to previous studies, several different mechanisms have been proposed for astrocytic release of excitatory neurotransmitters. These mechanisms include: (i) release through connexon, constituted mainly by connexin 43 (Cx43) assembles, or pannexin functional hemichannels (HCs) (Chever et al., 2014; Huang et al., 2007; Orellana and Stehberg, 2014; Torres et al., 2012); (ii) through ionotropic purinergic receptors (Duan et al., 2003; Malarkey and Parpura, 2008) and (iii) through volume-regulated anion channels (VRAC), which open cell swelling (Benfenati et al., 2009; Malarkey and Parpura, 2008; Mongin and Orlov, 2001). However, in this work we were limited by time, so it was only possible to study the release of gliotransmitters through Cx43 and P2X7 purinergic receptors.

Therefore, we took advantage of a well-known molecule (carbenoxolone, CBX) that in the CNS is able to inhibit some subtypes of gap-junction (GJ) and HCs, to examine the impact of these HCs and GJ on regulating gliotransmitters release induced by DEX. Our data revealed that CBX by itself significantly increased the levels of glutamate, but moreover when DEX was added to CBX-treated cells, a synergistic effect was observed in the glutamate levels. The reason why DEX and CBX combined displayed a synergistic effect could be because these compounds could act through parallel mechanism that culminate in the same effect (high levels of glutamate release). Furthermore, this hypothesis is in accordance with some studies showed that CBX can interfere with all the released mechanisms above mentioned (reviewed in (Benfenati et al., 2009)) and other studies that indicate that astrocyte-enriched mRNAs associated with glutamate reuptake and metabolism are regulated by GCs (Carter et al., 2013a). Moreover, the role of CBX in pathological conditions is controversial. CBX was shown to have a strong anticonvulsant activity in rodent seizure models (Gajda et al., 2005; Hosseinzadeh and Nassiri Asl, 2003) and a neuroprotective role through modulation of GJ activity in a mouse model of perinatal global ischemia (de Pina-Benabou et al., 2005). However, *in vitro* CBX had opposite effects exacerbating neuronal death in response to various pathological insults (Ozog et al., 2002; Zündorf et al., 2007). Since Cx43 is a crucial player in the neuroglial dialogue promoting synaptic efficacy of excitatory terminals and CBX can interfere with other pathways and its role is controversial, we should use other pharmacological tools able to block HCs and/or gap junction that are more specific for Cx43 HCs (Abudara et al., 2014; Chever et al., 2014).

Furthermore, we also studied another gliotransmitter release mechanism, which is P2X7 purinergic receptors. For this experiment, we used to Blue Brilliant G (BBG), a selective P2X antagonists, and we observed that DEX on BBG-treated cells increased glutamate levels. Unfortunately, our results with BBG are preliminary data, preventing us from statistic analyze and making conclusions. Nevertheless, it seems to suggest that the block of P2X7 affects the levels of extracellular glutamate.

In this work, we also performed Western blot and immunocytochemical analyses to evaluate changes in astrocytic and HCs/GJ markers of DEX-treated cells. We observed increased levels of Cx43 and also on glutamine synthetase (GS) when compared to untreated cells, that are in agreement, with other studies showing that DEX was able to markedly enhance GS activity in astrocytic cultures but not in near pure neuronal cultures (Debroas et al., 2015). In the immunocytochemistry, DEX was sufficient to induce morphological changes in astrocytes (GFAP-positive cells) and in Cx43 immunoreactivity. This increase could be due to an increase in functional Cx43 HCs expression or due an alteration in the organization of GJ coupling.

Moreover, the blockade of $A_{2A}R$ was able to prevent DEX-induced alterations in GFAP and Cx43. The data collected for GFAP is in agreement with a study performed by colleagues, where the administration of caffeine (non-selective antagonist of $A_{2A}R$) in an animal model of stress could prevent gliosis (Kaster et al., 2015). Furthermore, there is almost no information about the relation between $A_{2A}R$ and CX43, for that purpose we carried out an *in situ* proximity ligand assay experiment. Our results show for the first time that astrocytic $A_{2A}R$ have a physical interaction with Cx43. With that evidence, we can conjecture what are the effects of that interaction, so we suggest that $A_{2A}R$ could control the Cx43 structure, rearrangement in the cells, function, the functional number or protein expression of Cx43, or vice versa, which mean that Cx43 can control $A_{2A}R$ effects. This interaction between $A_{2A}R$ and Cx43 opens a plenty of paths that we can follow to understand the mechanisms behind depression.

It is widely discussed the interaction of $A_{2A}R$ with glutamatergic system at several levels in the brain because (i) extra-striatal $A_{2A}Rs$ are mostly synaptically-located (Rebola et al., 2005a), particularly in glutamatergic synapses (Rebola et al., 2005b) and these receptors have been demonstrated to control the release of glutamate in different brain regions (Ciruela et al., 2006; Lopes et al., 2002; Marchi et al., 2002; Popoli et al., 1995); (ii) $A_{2A}Rs$ may also indirectly control the level of extracellular glutamate by modulating the activity of glutamate transporter in astrocytes (Gao et al., 2001; Nishizaki et al., 2002); and (iii) the existence of heteromeric receptor complexes containing $A_{2A}R$ and mGluR5 have been suggested (Ferré et al., 2002; Rodrigues et al., 2005). Moreover, in conditions were astrocytes become reactive, for instance, in emotional disturbances, evidence state that higher levels of $A_{2A}Rs$ are expressed on their surfaces (data showed for us). However, this modification could impair the regular removal of glutamate from the extracellular space, as showed by others (Matos et al., 2008, 2012a, 2012b). If astrocytes fail to reuptake glutamate to the intracellular space, there will be an accumulation of extracellular levels of glutamate, which in turn, lead to excitotoxicity, initiating a complex cascade of events, that are already demonstrated being key aspects in neurodegenerative brain diseases (Hardingham and Bading, 2010). For that reason, we evaluated the effect on glutamate release levels by blocking $A_{2A}R$. What we saw when DEX was applied in SCH-treated cells was that SCH 58261 (SCH), a selective antagonist of $A_{2A}R$, cannot abrogate the effect on glutamate release induced by DEX-exposure. These results obtained demonstrate, that $A_{2A}R$ does not appear to control this gliotransmitter release, but it was already described by others that, astrocytic $A_{2A}R$ can control glutamate uptake through a mechanism that involves their functional interaction with Na^+/K^+ATPase (Matos et al., 2013). They also observed that an acute exposure to the $A_{2A}R$ agonist, CGS 21680, inhibited glutamate uptake, and an effect prevented by the $A_{2A}R$ antagonist, SCH. Furthermore, the prolonged activation of $A_{2A}R$ lead to a cAMP/protein kinase A-dependent reduction of glutamate transporters expression and protein levels, which leads to a sustained

decrease of glutamate uptake (Matos et al., 2012a). However, the increase of extracellular glutamate levels induced by DEX in the presence of SCH could be justified whether a compensatory mechanism has been happening at the same time, which meaning that other purinergic receptors may be over activated leading to inhibited glutamate uptake and increased glutamate release. Other explanation could be reductions in Na^+/K^+ATPase expression and function that were associated with depressive disorders in humans (Hokin-Neaverson and Jefferson, 1989; Naylor et al., 1980; Tochigi et al., 2008; Wood et al., 1991) as well as in animal models of depression (Gamaro et al., 2003; de Vasconcellos et al., 2005) that would affect glutamate transports and consequently the glutamate uptake and release. Furthermore, alterations in the functional GJ and HCs could also lead to an increased in the levels of extracellular glutamate (Ozog et al., 2002). Additionally, how DEX could interfere with glutamate metabolism remains to be studied and could be a reason for the non-effect of A_{2A}R blockade (Carter et al., 2013a). Nevertheless, all these hypotheses remain to be tested.

Later, since adenosine is formed when ATP is degraded outside the area of activated synapses and is released either by astrocytes or neurons (Costenla et al., 2010; Hines and Haydon, 2014; Lopes et al., 2002, 2011), we evaluated the effect of SCH 58261 on ATP release levels. Notably, the blockade of A_{2A}R normalized to basal levels the extracellular ATP levels induced by DEX. The results obtained were expected whereas astrocyte-derived adenosine may participate in modulating depressive-like behaviors, since over-expression of their receptors triggers emotional disturbances (data showed for us and (Coelho et al., 2014)) and their blockade can help in prevention of these behaviors (Batalha et al., 2013; Kaster et al., 2015). Accordingly, epidemiological studies also show an inverse relation between the intake of moderate amounts of caffeine, an AR antagonist, and the incidence of depression (Lucas et al., 2011; Lucas et al., 2014).

Many experiments remained to be done, notwithstanding, with this study we presented strong evidences that GCs could affect gliotransmitters release, for instance glutamate and ATP, and the alterations in morphology of astrocytes, namely, size of cell bodies and processes, could lead to pathological conditions, such as depression. Moreover, the effect of GCs in other astrocytic functions that are altered in major depression, including ion and water homeostasis, GABA and monoamine recycling, BBB integrity, neurotrophic support, energy metabolism, gliogenesis and synaptogenesis continues to be studied. In order to develop new anti-depressant treatments, it is necessary to continue the research in the glial field, due to the importance of these type of cells in the disease. These study and others indicate that astrocytic gap junctions and gliotransmission should be considered as novel potential therapeutic targets for the treatment of depression and mood disorders.

5.2. Glial markers alterations in suicide completers and age

Synaptic dysfunction is a key aspect of depression (Duman and Aghajanian, 2012). Furthermore ARs, as previously demonstrated in animal models (Rebola et al., 2005a), and now in human samples, are mainly synaptic receptor (unpublished data from the group). Besides, we know that $A_{2A}R$ have an impact on cognitive dysfunction only in conditions involving synaptic deterioration, by way of controlling neurodegeneration (Canas et al., 2009b; Cunha et al., 2008a; Silva et al., 2007). Therefore, it is possible that mood-related disorders trigger a synaptic dysfunction, accompanied by a deregulation of ARs. Since astrocytes play an important role in synaptic transmission, the performed evaluation of the loss of glial markers in suicide completers may then be extremely helpful in identifying the molecular mechanisms underlying the depression-associated impairments. As revealed in postmortem studies of patients with MDD or suicide completers, numerical and morphological alterations of astrocytes in the frontolimbic systems are closely associated with depression (Altshuler et al., 2010; Cobb et al., 2016; Rajkowska and Stockmeier, 2013; Rial et al., 2015; Rubinow et al., 2016). For example, the number and density of astrocytic-like components in the frontolimbic regions is consistently decreased (Cotter, 2002; Medina et al., 2016; Nagy et al., 2015; Rajkowska et al., 1999; Torres-Platas et al., 2014). For this reason, we started our study with hippocampus; but it would be interesting to expand the study to other brain regions.

We optimize and validated the gliosomal preparation in human tissue using a similar protocol with few alterations described in rodents, by Marco Matos (Matos et al., 2012a, 2013, 2015), a previous colleague from the group Purine at CNC. Furthermore, we were able to use identical synaptic and glial markers described by Marco Matos. Synaptophysin and syntaxin are membrane proteins that are associated with synaptic vesicles and presynaptic active zones at nerve terminals, respectively (Nag and Wadhwa, 2001) and GFAP, although expressed by several cell types in the CNS, is considered an astrocytic marker (Matos et al., 2012a; Sanacora and Banasr, 2013).

In suicide completers, we did not observe differences in GFAP protein density in comparison with age-matched controls. In one hand, previous postmortem studies of subjects with MDD showed significant reductions in the number and density of astrocytes in hippocampus (Cobb et al., 2016), which is in disagreement with our results. However, in this study, the authors analyzed GFAP alterations in specific sub-regions of hippocampus, such as CA1, whereas we studied alterations in the hippocampus as a whole. Additionally, they analyzed samples from males and females, while we use only samples from male, so this could suggest that gender is important in the pathology of depression. On the other hand, and in agreement with our results, Gos and colleagues (2013) did not report alterations in density of astrocytes immunolabeled for GFAP in the pyramidal cell layer of CA1 in left and right posterior hippocampus of postmortem brain tissue

of subjects with MDD. Recently, another research group examined the cell number and density of the total population of astrocytes in the left and right posterior hippocampus and they observed that there were no significant changes in either astrocytes number or density in CA4 in MDD as compared to control subjects (Malchow et al., 2015).

We also observed no differences in Cx43 protein density in suicide completers in comparison with age-matched controls. According to postmortem studies from patients with MDD and suicide completers, a consistent downregulation of Cx43 in brain regions involved in mood regulation, such as prefrontal cortex (PFC) (Nagy et al., 2015, 2016), orbitofrontal cortex (Miguel-Hidalgo et al., 2014), hippocampus (Medina et al., 2016), locus coeruleus (Bernard et al., 2011), mediodorsal thalamus and caudate nucleus (Nagy et al., 2015) were observed, which are not in line with our results. Additionally, alterations in the Cx43 protein levels have also been observed in the PFC of an animal model of depression (Sun et al., 2012). One possible explanation is, these findings vary possibly due to the use of different experimental paradigms.

Later, we analyzed two components of glutamate-glutamine shuttle, EAAT1 and GS, and whereas we observed a significant increase for EAAT1, for GS we did not observe differences in suicide completers when compared to age-matched controls. Several studies performed in animal models of depression have claimed reductions in GLAST, which correspond to human EAAT1. For example, the GLAST protein levels are significantly reduced in the hippocampus of mouse when they are exposure to chronic corticosteroid or DEX (Gourley et al., 2012; Skupio et al., 2015). In another study, GLAST down-regulation was also observed in a rat model of depression (Gomez-Galan et al., 2013). Moreover, studies of subjects with MDD showed a reduction expression of EAAT1 and GS protein in the orbitofrontal cortex (Miguel-Hidalgo et al., 2010) and in the frontal limbic system (Bernstein et al., 2015; Choudary et al., 2005; Sanacora and Banasr, 2013). Additionally, astrocytic glutamate transporters expression has been hypothesized to undergo a compensatory increase followed by a decrease in response to stressful stimulations (Chen et al., 2014), which could explained the increase in EAAT1 that we observed, however, much work is required to test this hypothesis.

MDD affects many people of all ages however according to *World Health Organization* the prevalence rates of depression peak among older adults (Secretariat, 2011). If aging is, a risk factor to develop depression, it is important to study aging in particular synaptic and glial markers that can provide information about the integrity of the structures. Our results showed that synaptophysin levels decrease throughout adult life, as well as the syntaxin levels (tendency to decrease with no statistical significance). This age-related decrease of presynaptic markers is in accordance with the majority of studies reporting a decrease in synaptophysin mRNA and protein density and in the number of elements immuno-positive for synaptophysin with aging in the hippocampus and various cortical structures (Canas et al., 2009a; Chen et al., 1995; Eastwood et

al., 1994; Frick and Fernandez, 2003; King and Arendash, 2002; Masliah et al., 1993; Rutten et al., 2005; Saito et al., 1994; Vanguilder et al., 2010). Besides this, alterations in synaptic proteins are good markers of synaptotoxicity in animal models disease (Canas et al., 2009b; Kaster et al., 2015). Likewise, a significant decrease in the levels of GS in gliosomes was observed with the age. These results are in disagreement, with a study performed by Olabarria and colleagues (2011) that showed a uniform pattern of GS immunoreactivity in the dentate gyrus and CA1 being constant at all age groups (Olabarria et al., 2011). However, we analyzed alterations in the hippocampus as a whole, and did not distinguish specific regions, so this could be a reason to obtain different results, since alterations could be dependent of brain region or sub-region. Moreover, in our studies EAAT1 levels appear to not modify when compared in different age ranges. Oppositely to the results obtained for synaptic markers, GFAP significantly increase its levels throughout adult life (Cobb et al., 2016), suggesting an increase in astrogliosis in older people. This could mean, that with aging can provoke alterations in shape of the cells, which could limit the number of connections between cells and contribute to MDD (Rajkowska and Stockmeier, 2013).

This model is relevant because it allows the study in particular of astrocytes and better understand the importance of these cells in depression. Moreover, the knowledge of the ways in which age may alter factors associated with the onset and maintenance of depression is essential for effective treatment of depressed older adults.

CHAPTER 6

CONCLUSIONS

The results presented in our work provide a study of gliotransmitters release, namely glutamate and ATP, affected by GCs and some mechanisms that could regulated that release, in astrocyte primary cell cultures. Additionally, this work also compares the levels of glial markers in hippocampal gliosomes of suicide completers when compared with age-matched controls and the alterations in synaptic and glial markers density according to age, in human postmortem samples. We observed that DEX-treated cells increased glutamate and ATP levels. These results are in accordance with glutamatergic hypothesis proposed to the etiology of depression, but not with the purinergic hypothesis. Moreover, we reported that the blockade of $A_{2A}R$, could prevent the increase of Cx43 and ATP release in cells treated with DEX. Besides, we showed for the first time a physical interaction with astrocytic $A_{2A}R$ and Cx43. This might explain the effect of blockade $A_{2A}R$ in controlling Cx43 levels.

Furthermore, we observed MDD pathology-related changes in the EAAT1 density, but not in the others, glial markers studied. The results obtained also showed that some synaptic and glial markers could change with age, for example, synaptophysin, GFAP and glutamine synthetase.

To sum up, our work is extremely helpful, since it might contribute to better understand the gliotransmission process in depression-like conditions and find glial markers that are dysregulated in MDD. This point us in a new direction to further develop new and more effective anti-depressant therapies.

CHAPTER 7

FUTURE PERSPECTIVES

Given the novel focus on astrocytes, our findings have established principles relevant for understanding regulation of gliotransmitters release and alterations in morphology and components of astrocytes by glucocorticoids and have also raised a number of additional questions for consideration in future studies:

- Perform longer incubation time periods of DEX to study ATP release to investigate if we observe a decrease in ATP release levels that are descripted in depression (Cao et al., 2013a).
- Use different pharmacological tools to confirm the effect of Cx43 in astrocytic cultures, since CBX could interfere with various mechanism in addition to Cx43, such as Gap26 and Gap19 peptides that are more specific for Cx43 HCs (Abudara et al., 2014; Chever et al., 2014).
- Assess other gliotransmitters release mechanisms.
- Evaluate the effect of GCs in other astrocytic functions that are altered in MDD, including ion and water homeostasis, GABA and monoamine recycling, blood-brain barrier integrity, neurotrophic support, energy metabolism, gliogenesis and synaptogenesis.
- Perform different approaches to try to understand how physical interaction between $A_{2A}R$ and Cx43 could occur, such as co-immunoprecipitation.
- Evaluate if astrocytic adenosine receptors are altered in suicide completers.
- Assess how age factor interfere with synaptic and glial markers in suicide completers.
- Study other brain regions, that were described as being altered in depressed patients, such as, amygdala, prefrontal cortex, Brodmann area 25 (Gotlib et al., 2005; Lorenzetti et al., 2009; Pizzagalli et al., 2004).

REFERENCES

REFERENCES

Abudara, V., Bechberger, J., Freitas-Andrade, M., De Bock, M., Wang, N., Bultynck, G., Naus, C.C., Leybaert, L., and Giaume, C. (2014). The connexin43 mimetic peptide Gap19 inhibits hemichannels without altering gap junctional communication in astrocytes. Front. Cell. Neurosci. *8*, 306.

Allen, N.J., Bennett, M.L., Foo, L.C., Wang, G.X., Chakraborty, C., Smith, S.J., and Barres, B.A. (2012). Astrocyte glypicans 4 and 6 promote formation of excitatory synapses via GluA1 AMPA receptors. Nature *486*, 410–414.

Altamura, C., Maes, M., Dai, J., and Meltzer, H.Y. (1995). Plasma concentrations of excitatory amino acids, serine, glycine, taurine and histidine in major depression. Eur. Neuropsychopharmacol. *5*, 71–75.

Altamura, C.A., Mauri, M.C., Ferrara, A., Moro, A.R., D'Andrea, G., and Zamberlan, F. (1993). Plasma and platelet excitatory amino acids in psychiatric disorders. Am. J. Psychiatry *150*, 1731–1733.

Altshuler, L.L., Abulseoud, O.A., Foland-Ross, L., Bartzokis, G., Chang, S., Mintz, J., Hellemann, G., and Vinters, H. V. (2010). Amygdala astrocyte reduction in subjects with major depressive disorder but not bipolar disorder. Bipolar Disord. *12*, 541–549.

Anacker, C., Zunszain, P.A., Cattaneo, A., Carvalho, L.A., Garabedian, M.J., Thuret, S., Price, J., and Pariante, C.M. (2011). Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor. Mol. Psychiatry *16*, 738–750.

Anderson, C.M., and Swanson, R.A. (2000). Astrocyte glutamate transport: Review of properties, regulation, and physiological functions. Glia *32*, 1–14.

di Angelantonio, S., Bertollini, C., Piccinin, S., Rosito, M., Trettel, F., Pagani, F., Limatola, C., and Ragozzino, D. (2015). Basal adenosine modulates the functional properties of AMPA receptors in mouse hippocampal neurons through the activation of A1R A2AR and A3R. Front. Cell. Neurosci. *9*, 409.

Araque, A., Parpura, V., Sanzgiri, R.P., and Haydon, P.G. (1999). Tripartite synapses: Glia, the unacknowledged partner. Trends Neurosci. *22*, 208–215.

Attwell, D. (2000). Brain uptake of glutamate: food for thought. J. Nutr. *130*, 1023S–5S.

Auer, D.P., Pütz, B., Kraft, E., Lipinski, B., Schill, J., and Holsboer, F. (2000). Reduced glutamate in the anterior cingulate cortex in depression: an in vivo proton magnetic resonance spectroscopy study. Biol. Psychiatry *47*, 305–313.

Augusto, E., Matos, M., Sévigny, J., El-Tayeb, A., Bynoe, M.S., Müller, C.E., Cunha, R. A., and Chen, J.F. (2013). Ecto-5'-nucleotidase (CD73)-mediated formation of adenosine is critical for the striatal adenosine A_{2A} receptor functions. J. Neurosci. *33*, 11390–11399.

Azdad, K., Gall, D., Woods, A.S., Ledent, C., Ferré, S., and Schiffmann, S.N. (2009). Dopamine D2 and adenosine A_{2A} receptors regulate NMDA-mediated excitation in accumbens neurons through A_{2A}-D2 receptor heteromerization. Neuropsychopharmacology *34*, 972–986.

Bagley, J., and Moghaddam, B. (1997). Temporal dynamics of glutamate efflux in the prefrontal cortex and in the hippocampus following repeated stress: Effects of pretreatment with saline or diazepam. Neuroscience *77*, 65–73.

Bagot, R.C., Labonte, B., Pena, C.J., and Nestler, E.J. (2014). Epigenetic signaling in psychiatric disorders: Stress and depression. Dialogues Clin. Neurosci. *16*, 281–295.

Bai, F., and Witzmann, F.A. (2007). Synaptosome proteomics. Subcell. Biochem. *43*, 77–98.

Bak, L.K., Schousboe, A., and Waagepetersen, H.S. (2006). The glutamate/GABA-glutamine cycle: Aspects of transport, neurotransmitter homeostasis and ammonia transfer. J. Neurochem. *98*, 641–653.

Banasr, M., and Duman, R.S. (2008). Glial Loss in the Prefrontal Cortex Is Sufficient to Induce Depressive-like Behaviors. Biol. Psychiatry *64*, 863–870.

Banasr, M., Chowdhury, G.M.I., Terwilliger, R., Newton, S.S., Duman, R.S., Behar, K.L., and Sanacora, G. (2010). Glial pathology in an animal model of depression: reversal of stress-induced cellular, metabolic and behavioral deficits by the glutamate-modulating drug riluzole. Mol. Psychiatry *15*, 501–511.

Barrett, K., Barman, S., Boitano, S., and Brooks, H. (2016). Ganong's Review of Medical Physiology.

Batalha, V.L., Pego, J.M., Fontinha, B.M., Costenla, A.R., Valadas, J.S., Baqi, Y., Radjainia, H., Müller, C.E., Sebastião, A.M., and Lopes, L. V (2013). Adenosine A_{2A} receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation. Mol. Psychiatry *18*, 320–331.

Benfenati, V., Caprini, M., Nicchia, G.P., Rossi, A., Dovizio, M., Cervetto, C., Nobile, M., and Ferroni, S. (2009). Carbenoxolone inhibits volume-regulated anion conductance in cultured rat cortical astroglia. Channels *3*, 323–336.

Berman, R.M., Cappiello, A., Anand, A., Oren, D.A., Heninger, G.R., Charney, D.S., and Krystal, J.H. (2000). Antidepressant effects of ketamine in depressed patients. Biol. Psychiatry *47*, 351–

354.

Bernard, R., Kerman, I.A., Thompson, R.C., Jones, E.G., Bunney, W.E., Barchas, J.D., Schatzberg, A.F., Myers, R.M., Akil, H., and Watson, S.J. (2011). Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. Mol. Psychiatry *16*, 634–646.

Bernstein, H.G., Meyer-Lotz, G., Dobrowolny, H., Bannier, J., Steiner, J., Walter, M., and Bogerts, B. (2015). Reduced density of glutamine synthetase immunoreactive astrocytes in different cortical areas in major depression but not in bipolar I disorder. Front. Cell. Neurosci. *9*, 273.

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

Biber, K., Neumann, H., Inoue, K., and Boddeke, H.W.G.M. (2007). Neuronal "On" and "Off" signals control microglia. Trends Neurosci. *30*, 596–602.

Bohn, M.C., Howard, E., Vielkind, U., and Krozowski, Z. (1991). Glial cells express both mineralocorticoid and glucocorticoid receptors. J. Steroid Biochem. Mol. Biol. *40*, 105–111.

Boison, D., Chen, J.F., and Fredholm, B.B. (2010). Adenosine signaling and function in glial cells. Cell Death Differ. *17*, 1071–1082.

Bonelli, R.M., Kapfhammer, H.P., Pillay, S.S., and Yurgelun-Todd, D.A. (2006). Basal ganglia volumetric studies in affective disorder: What did we learn in the last 15 years? J. Neural Transm. *113*, 255–268.

Bremner, J.D., Narayan, M., Anderson, E.R., Staib, L.H., Miller, H.L., and Charney, D.S. (2000). Hippocampal volume reduction in major depression. Am. J. Psychiatry *157*, 115–118.

Burnstock, G. (2007). Physiology and pathophysiology of purinergic neurotransmission. Physiol. Rev. *87*, 659–797.

Caetano, S.C., Hatch, J.P., Brambilla, P., Sassi, R.B., Nicoletti, M., Mallinger, A.G., Frank, E., Kupfer, D.J., Keshavan, M.S., and Soares, J.C. (2004). Anatomical MRI study of hippocampus and amygdala in patients with current and remitted major depression. Psychiatry Res. - Neuroimaging *132*, 141–147.

Cajal, S., and Ramon, Y. (1911). Histologie du système nerveux de l'homme et des vertébrés. Paris: Maloine.

Canas, P.M., Duarte, J.M.N., Rodrigues, R.J., Köfalvi, A., and Cunha, R.A. (2009a). Modification upon aging of the density of presynaptic modulation systems in the hippocampus. Neurobiol.

Aging *30*, 1877–1884.

Canas, P.M., Porciúncula, L.O., Cunha, G.M.A., Silva, C.G., Machado, N.J., Oliveira, J.M.A., Oliveira, C.R., and Cunha, R.A. (2009b). Adenosine A_{2A} receptor blockade prevents synaptotoxicity and memory dysfunction caused by beta-amyloid peptides via p38 mitogen-activated protein kinase pathway. J. Neurosci. *29*, 14741–14751.

Cao, X., Li, L.P., Wang, Q., Wu, Q., Hu, H.H., Zhang, M., Fang, Y.Y., Zhang, J., Li, S.J., Xiong, W.C., et al. (2013a). Astrocyte-derived ATP modulates depressive-like behaviors. Nat Med *19*, 773–777.

Cao, X., Li, L.P., Qin, X.H., Li, S.J., Zhang, M., Wang, Q., Hu, H.H., Fang, Y.Y., Gao, Y.B., Li, X.W., et al. (2013b). Astrocytic adenosine 5'-triphosphate release regulates the proliferation of neural stem cells in the adult hippocampus. Stem Cells *31*, 1633–1643.

Carpenter, L.L., Janicak, P.G., Aaronson, S.T., Boyadjis, T., Brock, D.G., Cook, I.A., Dunner, D.L., Lanocha, K., Solvason, H.B., and Demitrack, M.A. (2012). Transcranial magnetic stimulation (TMS) for major depression: A multisite, naturalistic, observational study of acute treatment outcomes in clinical practice. Depress. Anxiety *29*, 587–596.

Carriba, P., Ortiz, O., Patkar, K., Justinova, Z., Stroik, J., Themann, A., Muller, C., Woods, A.S., Hope, B.T., Ciruela, F., et al. (2007). Striatal adenosine A_{2A} and cannabinoid CB_1 receptors form functional heteromeric complexes that mediate the motor effects of cannabinoids. Neuropsychopharmacology *32*, 2249–2259.

Carter, B.S., Hamilton, D.E., and Thompson, R.C. (2013a). Acute and chronic glucocorticoid treatments regulate astrocyte-enriched mRNAs in multiple brain regions in vivo. Front. Neurosci. *7*, 139.

Carter, M.E., Soden, M.E., Zweifel, L.S., and Palmiter, R.D. (2013b). Genetic identification of a neural circuit that suppresses appetite. Nature *503*, 111–114.

de Cesare, D., Fimia, G.M., and Sassone-Corsi, P. (1999). Signaling routes to CREM and CREB: Plasticity in transcriptional activation. Trends Biochem. Sci. *24*, 281–285.

Charney, D.S., and Manji, H.K. (2004). Life stress, genes, and depression: multiple pathways lead to increased risk and new opportunities for intervention. Sci. STKE *2004*, re5.

Chaudhry, F.A., Lehre, K.P., Van Lookeren Campagne, M., Ottersen, O.P., Danbolt, N.C., and Storm-Mathisen, J. (1995). Glutamate transporters in glial plasma membranes: Highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron *15*, 711–720.

Chen, J., Yao, L., Xu, B., Qian, K., Wang, H., Liu, Z., Wang, X., and Wang, G. (2014). Glutamate transporter 1-mediated antidepressant-like effect in a rat model of chronic unpredictable stress. J. Huazhong Univ. Sci. Technol. Med. Sci. = Hua Zhong Ke Ji Da Xue Xue Bao. Yi Xue Ying Wen Ban = Huazhong Keji Daxue Xuebao. Yixue Yingdewen Ban *34*, 838–844.

Chen, J.F., Eltzschig, H.K., and Fredholm, B.B. (2013). Adenosine receptors as drug targets — what are the challenges? Nat. Rev. Drug Discov. *12*, 265–286.

Chen, K.S., Masliah, E., Mallory, M., and Gage, F.H. (1995). Synaptic loss in cognitively impaired aged rats is ameliorated by chronic human nerve growth factor infusion. Neuroscience *68*, 19–27.

Cheng, H.C., Shih, H.M., and Chern, Y. (2002). Essential role of cAMP-response element-binding protein activation by A_{2A} adenosine receptors in rescuing the nerve growth factor-induced neurite outgrowth impaired by blockage of the MAPK cascade. J. Biol. Chem. *277*, 33930–33942.

Chever, O., Lee, C.Y., and Rouach, N. (2014). Astroglial connexin43 hemichannels tune basal excitatory synaptic transmission. J. Neurosci. *34*, 11228–11232.

Choudary, P. V, Molnar, M., Evans, S.J., Tomita, H., Li, J.Z., Vawter, M.P., Myers, R.M., Bunney, W.E., Akil, H., Watson, S.J., et al. (2005). Altered cortical glutamatergic and GABAergic signal transmission with glial involvement in depression. Proc. Natl. Acad. Sci. U. S. A. *102*, 15653–15658.

Chourbaji, S., Zacher, C., Sanchis-Segura, C., Dormann, C., Vollmayr, B., and Gass, P. (2005). Learned helplessness: Validity and reliability of depressive-like states in mice. Brain Res. Protoc. *16*, 70–78.

Christopherson, K.S., Ullian, E.M., Stokes, C.C.A., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P., and Barres, B.A. (2005). Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell *120*, 421–433.

Chung, W.S., Clarke, L.E., Wang, G.X., Stafford, B.K., Sher, A., Chakraborty, C., Joung, J., Foo, L.C., Thompson, A., Chen, C., et al. (2013). Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. Nature *504*, 394–400.

Ciruela, F., Casadó, V., Rodrigues, R.J., Lujan, R., Burgueño, J., Canals, M., Borycz, J., Rebola, N., Goldberg, S.R., Mallol, J., et al. (2006). Presynaptic control of striatal glutamatergic neurotransmission by adenosine A_1-A_{2A} receptor heteromers. J. Neurosci. *26*, 2080–2087.

Cobb, J.A., O'Neill, K., Milner, J., Mahajan, G.J., Lawrence, T.J., May, W.L., Miguel-Hidalgo, J., Rajkowska, G., and Stockmeier, C.A. (2016). Density of GFAP-immunoreactive astrocytes is decreased in left hippocampi in major depressive disorder. Neuroscience *316*, 209–220.

Coelho, J.E., Alves, P., Canas, P.M., Valadas, J.S., Shmidt, T., Batalha, V.L., Ferreira, D.G., Ribeiro, J.A., Bader, M., Cunha, R.A., et al. (2014). Overexpression of adenosine A_{2A} receptors in rats: Effects on depression, locomotion, and anxiety. Front. Psychiatry *5*.

Conrad, C.D. (2008). Chronic stress-induced hippocampal vulnerability: the glucocorticoid vulnerability hypothesis. Rev. Neurosci. *19*, 395–411.

Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S., and Smith, S.J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science *247*, 470–473.

Coryell, W., and Young, E.A. (2005). Clinical predictors of suicide in primary major depressive disorder. J. Clin. Psychiatry *66*, 412–417.

Costenla, A.R., Cunha, R.A., and De Mendonça, A. (2010). Caffeine, adenosine receptors, and synaptic plasticity. Journal of Alzheimer's Disease, pp. S25–S34.

Cotter, D. (2002). Reduced Neuronal Size and Glial Cell Density in Area 9 of the Dorsolateral Prefrontal Cortex in Subjects with Major Depressive Disorder. Cereb. Cortex *12*, 386–394.

Crane, J., Schneider, C., Mb, W., Aerde, J. Van, and Ab, E. (2003). Antenatal Corticosteroid Therapy for Fetal Maturation Maternal-Fetal Medicine Committee. J Obs. Gynaecol Can *25*, 45–52.

Crossin, K.L., Tai, M.H., Krushel, L.A., Mauro, V.P., and Edelman, G.M. (1997). Glucocorticoid receptor pathways are involved in the inhibition of astrocyte proliferation. Proc. Natl. Acad. Sci. U. S. A. *94*, 2687–2692.

Cunha, R.A. (2001). Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals. Neurochem. Res. *26*, 979–991.

Cunha, R.A. (2016). How does adenosine control neuronal dysfunction and neurodegeneration? J. Neurochem. *139*, 1019–1055.

Cunha, R.A. (2005). Neuroprotection by adenosine in the brain: From A1 receptor activation to A_{2A} receptor blockade. Purinergic Signal. *1*, 111–134.

Cunha, R.A., and Ribeiro, J.A. (2000). Adenosine A_{2A} receptor facilitation of synaptic transmission in the CA1 area of the rat hippocampus requires protein kinase C but not protein kinase A activation. Neurosci. Lett. *289*, 127–130.

Cunha, G.M.A., Canas, P.M., Oliveira, C.R., and Cunha, R.A. (2006). Increased density and synapto-protective effect of adenosine A_{2A} receptors upon sub-chronic restraint stress. Neuroscience *141*, 1775–1781.

Cunha, G.M.A., Canas, P.M., Melo, C.S., Hockemeyer, J., Müller, C.E., Oliveira, C.R., and Cunha, R.A. (2008a). Adenosine A_{2A} receptor blockade prevents memory dysfunction caused by β-amyloid peptides but not by scopolamine or MK-801. Exp. Neurol. *210*, 776–781.

Cunha, R.A., Vizi, E.S., Ribeiro, J.A., and Sebastião, A.M. (1996). Preferential release of ATP and its extracellular catabolism as a source of adenosine upon high- but not low-frequency stimulation of rat hippocampal slices. J. Neurochem. *67*, 2180–2187.

Cunha, R.A., Ferre, S., Vaugeois, J.M., and Chen, J.F. (2008b). Potential therapeutic interest of adenosine A_{2A} receptors in psychiatric disorders. Curr. Pharm. Des. *14*, 1512–1524.

Dai, S.-S., Zhou, Y.G., Li, W., An, J.H., Li, P., Yang, N., Chen, X.Y., Xiong, R.-P., Liu, P., Zhao, Y., et al. (2010). Local glutamate level dictates adenosine A_{2A} receptor regulation of neuroinflammation and traumatic brain injury. J. Neurosci. *30*, 5802–5810.

Danbolt, N.C. (2001). Glutamate uptake. Prog. Neurobiol. *65*, 1–105.

Debroas, E., Ali, C., and Duval, D. (2015). Dexamethasone enhances glutamine synthetase activity and reduces N-methyl-D-aspartate neurotoxicity in mixed cultures of neurons and astrocytes. AIMS Mol. Sci. *2*, 175–189.

Decrock, E., De Bock, M., Wang, N., Bultynck, G., Giaume, C., Naus, C.C., Green, C.R., and Leybaert, L. (2015). Connexin and pannexin signaling pathways, an architectural blueprint for CNS physiology and pathology? Cell. Mol. Life Sci. *72*, 2823–2851.

Dias, R.B., Ribeiro, J.A., and Sebastião, A.M. (2012). Enhancement of AMPA currents and GluR1 membrane expression through PKA-coupled adenosine A_{2A} receptors. Hippocampus *22*, 276–291.

Drevets, W.C., Price, J.L., and Furey, M.L. (2008). Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. Brain Struct. Funct. *213*, 93–118.

Du, K., Asahara, H., Jhala, U.S., Wagner, B.L., and Montminy, M. (2000). Characterization of a CREB gain-of-function mutant with constitutive transcriptional activity in vivo. Mol. Cell. Biol. *20*, 4320–4327.

Duan, S., Anderson, C.M., Keung, E.C., Chen, Y., Chen, Y., and Swanson, R.A. (2003). P2X7 receptor-mediated release of excitatory amino acids from astrocytes. J. Neurosci. *23*, 1320–1328.

Duman, R.S., and Aghajanian, G.K. (2012). Synaptic Dysfunction in Depression: Potential Therapeutic Targets. Science. *338*, 68–72.

Dunkley, P.R., Jarvie, P.E., Heath, J.W., Kidd, G.J., and Rostas, J.A.P. (1986). A rapid method

for isolation of synaptosomes on Percoll gradients. Brain Res. *372*, 115–129.

Dunkley, P.R., Jarvie, P.E., and Robinson, P.J. (2008). A rapid Percoll gradient procedure for preparation of synaptosomes. Nat. Protoc. *3*, 1718–1728.

Eastwood, S.L., Burnet, P.W.J., McDonald, B., Clinton, J., and Harrison, P.J. (1994). Synaptophysin gene expression in human brain: A quantitative in situ hybridization and immunocytochemical study. Neuroscience *59*, 881–892.

Eng, L.F., Ghirnikar, R.S., and Lee, Y.L. (2000). Glial Fibrillary Acidic Protein : GFAP-Thirty-One Years (1969-2000). Neurochem. Res. *25*, 1439–1451.

Eulenburg, V., and Gomeza, J. (2010). Neurotransmitter transporters expressed in glial cells as regulators of synapse function. Brain Res. Rev. *63*, 103–112.

Fellin, T., and Carmignoto, G. (2004). Neurone to astrocyte signalling in the brain represents a distinct multifunctional unit. J. Physiol. *559*, 3–15.

Ferré, S., Karcz-Kubicha, M., Hope, B.T., Popoli, P., Burgueño, J., Gutiérrez, M.A., Casadó, V., Fuxe, K., Goldberg, S.R., Lluis, C., et al. (2002). Synergistic interaction between adenosine A_{2A} and glutamate mGlu5 receptors: implications for striatal neuronal function. Proc. Natl. Acad. Sci. U. S. A. *99*, 11940–11945.

Forest, M.G., Morel, Y., and David, M. (1998). Prenatal treatment of congenital adrenal hyperplasia. Trends Endocrinol. Metab. *9*, 284–289.

Fredholm, B.B. (1995). Adenosine, Adenosine Receptors and the Actions of Caffeine. Pharmacol. Toxicol. *76*, 93–101.

Fredholm, B.B. (2007). Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ. *14*, 1315–1323.

Fredholm, B.B., IJzerman, A.P., Jacobson, K.A., Klotz, K.N., and Linden, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev. *53*, 527–552.

Fredholm, B.B., Chen, J.F., Cunha, R.A., Svenningsson, P., and Vaugeois, J.M. (2005). Adenosine and Brain Function. Int. Rev. Neurobiol. *63*, 191–270.

Fredholm, B.B., Chern, Y., Franco, R., and Sitkovsky, M. (2007). Aspects of the general biology of adenosine A_{2A} signaling. Prog. Neurobiol. *83*, 263–276.

Fredholm, B.B., IJzerman, A.P., Jacobson, K.A., Linden, J., Mu, C.E., and Müller, C.E. (2011). International Union of Basic and Clinical Pharmacology . LXXXI . Nomenclature and

Classification of Adenosine Receptors — An Update. Pharmacol. Rev. *63*, 1–34.

Frick, K.M., and Fernandez, S.M. (2003). Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. Neurobiol. Aging *24*, 615–626.

Frodl, T., Schaub, A., Banac, S., Charypar, M., Jäger, M., Kümmler, P., Bottlender, R., Zetzsche, T., Born, C., Leinsinger, G., et al. (2006). Reduced hippocampal volume correlates with executive dysfunctioning in major depression. J. Psychiatry Neurosci. *31*, 316–325.

Furczyk, K., Schutová, B., Michel, T.M., Thome, J., and Büttner, A. (2013). The neurobiology of suicide - A Review of post-mortem studies. J. Mol. Psychiatry *1*, 2.

Gajda, Z., Szupera, Z., Blazsó, G., and Szente, M. (2005). Quinine, a blocker of neuronal Cx36 channels, suppresses seizure activity in rat neocortex in vivo. Epilepsia *46*, 1581–1591.

Gamaro, G.D., Streck, E.L., Matté, C., Prediger, M.E., Wyse, A.T.S., and Dalmaz, C. (2003). Reduction of Hippocampal Na^+/K^+-ATPase Activity in Rats Subjected to an Experimental Model of Depression. Neurochem. Res. *28*, 1339–1344.

Gao, W.J., Krimer, L.S., and Goldman-Rakic, P.S. (2001). Presynaptic regulation of recurrent excitation by D1 receptors in prefrontal circuits. Proc Natl Acad Sci U S A *98*, 295–300.

George, J., Gonçalves, F.Q., Cristóvão, G., Rodrigues, L., Meyer Fernandes, J.R., Gonçalves, T., Cunha, R.A., and Gomes, C.A. (2015). Different danger signals differently impact on microglial proliferation through alterations of ATP release and extracellular metabolism. Glia *63*, 1636–1645.

Giaume, C., and McCarthy, K.D. (1996). Control of gap-junctional communication in astrocytic networks. Trends Neurosci. *19*, 319–325.

Giaume, C., Leybaert, L., Naus, C.C., and Sáez, J.C. (2013). Connexin and pannexin hemichannels in brain glial cells: Properties, pharmacology, and roles. Front. Pharmacol. *4*, 88.

Gittins, R.A., and Harrison, P.J. (2011). A morphometric study of glia and neurons in the anterior cingulate cortex in mood disorder. J. Affect. Disord. *133*, 328–332.

Golden, S.A., Covington, H.E., Berton, O., and Russo, S.J. (2011). A standardized protocol for repeated social defeat stress in mice. Nat Protoc *6*, 1183–1191.

Gomes, C. V, Kaster, M.P., Tomé, A.R., Agostinho, P.M., and Cunha, R.A. (2011). Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochim. Biophys. Acta *1808*, 1380–1399.

Gomez-Galan, M., De Bundel, D., Van Eeckhaut, A., Smolders, I., and Lindskog, M. (2013).

Dysfunctional astrocytic regulation of glutamate transmission in a rat model of depression. Mol. Psychiatry *18*, 582–594.

Gordon, G.R.J., Baimoukhametova, D. V, Hewitt, S.A., Rajapaksha, W.R.A.K.J.S., Fisher, T.E., and Bains, J.S. (2005). Norepinephrine triggers release of glial ATP to increase postsynaptic efficacy. Nat. Neurosci. *8*, 1078–1086.

Gos, T., Schroeter, M.L., Lessel, W., Bernstein, H.G., Dobrowolny, H., Schiltz, K., Bogerts, B., and Steiner, J. (2013). S100B-immunopositive astrocytes and oligodendrocytes in the hippocampus are differentially afflicted in unipolar and bipolar depression: A postmortem study. J. Psychiatr. Res. *47*, 1694–1699.

Gotlib, I.H., Sivers, H., Gabrieli, J.D.E., Whitfield-Gabrieli, S., Goldin, P., Minor, K.L., and Canli, T. (2005). Subgenual anterior cingulate activation to valenced emotional stimuli in major depression. Neuroreport *16*, 1731–1734.

Gourley, S.L., Espitia, J.W., Sanacora, G., and Taylor, J.R. (2012). Antidepressant-like properties of oral riluzole and utility of incentive disengagement models of depression in mice. Psychopharmacology (Berl). *219*, 805–814.

Guntz, E., Dumont, H., Pastijn, E., De Kerchove D'Exaerde, A., Azdad, K., Sosnowski, M., Schiffmann, S.N., and Gall, D. (2008). Expression of adenosine A_{2A} receptors in the rat lumbar spinal cord and implications in the modulation of N-methyl-D-aspartate receptor currents. Anesth. Analg. *106*, 1882–1889.

Guthrie, P.B., Knappenberger, J., Segal, M., Bennett, M. V, Charles, A.C., and Kater, S.B. (1999). ATP released from astrocytes mediates glial calcium waves. J. Neurosci. *19*, 520–528.

Halassa, M.M., Fellin, T., and Haydon, P.G. (2007). The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol. Med. *13*, 54–63.

Halassa, M.M., Fellin, T., and Haydon, P.G. (2009a). Tripartite synapses: Roles for astrocytic purines in the control of synaptic physiology and behavior. Neuropharmacology *57*, 343–346.

Halassa, M.M., Florian, C., Fellin, T., Munoz, J.R., Lee, S.Y., Abel, T., Haydon, P.G., and Frank, M.G. (2009b). Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. Neuron *61*, 213–219.

Hanisch, U.K.K., and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat. Neurosci. *10*, 1387–1394.

Hardingham, G.E., and Bading, H. (2010). Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. Nat. Rev. Neurosci. *11*, 682–696.

Harris, M.E., Wang, Y., Pedigo, N.W., Hensley, K., Butterfield, D.A., and Carney, J.M. (1996). Amyloid beta peptide (25-35) inhibits Na^+-dependent glutamate uptake in rat hippocampal astrocyte cultures. J. Neurochem. *67*, 277–286.

Hascup, E.R., Hascup, K.N., Stephens, M., Pomerleau, F., Huettl, P., Gratton, A., and Gerhardt, G.A. (2010). Rapid microelectrode measurements and the origin and regulation of extracellular glutamate in rat prefrontal cortex. J. Neurochem. *115*, 1608–1620.

Hasler, G. (2010). Pathophysiology of depression: do we have any solid evidence of interest to clinicians? World Psychiatry *9*, 155–161.

Hasler, G., van der Veen, J.W., Tumonis, T., Meyers, N., Shen, J., and Drevets, W.C. (2007). Reduced prefrontal glutamate/glutamine and gamma-aminobutyric acid levels in major depression determined using proton magnetic resonance spectroscopy. Arch. Gen. Psychiatry *64*, 193–200.

Haydon, P.G. (2001). GLIA: listening and talking to the synapse. Nat. Rev. Neurosci. *2*, 185–193.

Haydon, P.G., and Carmignoto, G. (2006). Astrocyte Control of Synaptic Transmission and Neurovascular Coupling. Physiol. Rev. *86*, 1009–1031.

Hazell, A., Itzhak, Y., Liu, H., and Norenberg, M. (1997). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) decreases glutamate uptake in cultured astrocytes. J. Neurochem. *68*, 2216–2219.

Henneberger, C., Papouin, T., Oliet, S.H.R., and Rusakov, D.A. (2010). Long-term potentiation depends on release of D-serine from astrocytes. Nature *463*, 232–236.

Hertz, L., and Zielke, H.R. (2004). Astrocytic control of glutamatergic activity: Astrocytes as stars of the show. Trends Neurosci. *27*, 735–743.

Hickie, I.B., Naismith, S.L., Ward, P.B., Scott, E.M., Mitchell, P.B., Schofield, P.R., Scimone, A., Wilhelm, K., and Parker, G. (2007). Serotonin transporter gene status predicts caudate nucleus but not amygdala or hippocampal volumes in older persons with major depression. J. Affect. Disord. *98*, 137–142.

Higley, M.J., and Sabatini, B.L. (2010). Competitive regulation of synaptic Ca^{2+} influx by D2 dopamine and A_{2A} adenosine receptors. Nat. Neurosci. *13*, 958–966.

Hines, D.J., and Haydon, P.G. (2014). Astrocytic adenosine: from synapses to psychiatric disorders. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *369*, 20130594.

Hokin-Neaverson, M., and Jefferson, J.W. (1989). Deficient erythrocyte Na^+/K^+-ATPase activity

in different affective states in bipolar affective disorder and normalization by lithium therapy. Neuropsychobiology *22*, 18–25.

Holsboer, F. (2000). The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology *23*, 477–501.

Holtzheimer, P.E., and Mayberg, H.S. (2010). Deep brain stimulation for treatment-resistant depression. Am. J. Psychiatry *167*, 1437–1444.

Hosseinzadeh, H., and Nassiri Asl, M. (2003). Anticonvulsant, sedative and muscle relaxant effects of carbenoxolone in mice. BMC Pharmacol. *3*, 3.

Huang, Y.J., Maruyama, Y., Dvoryanchikov, G., Pereira, E., Chaudhari, N., and Roper, S.D. (2007). The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. Proc. Natl. Acad. Sci. U. S. A. *104*, 6436–6441.

Hunter, A., Balleine, B., and Minor, T. (2003). Helplessness and escape performance: Glutamate-adenosine interactions in the frontal cortex. Behav. Neurosci. *117*, 123–135.

Hyman, S.E. (2009). How adversity gets under the skin. Nat. Neurosci. *12*, 241–244.

Janicak, P.G., Dunner, D.L., Aaronson, S.T., Carpenter, L.L., Boyadjis, T.A., Brock, D.G., Cook, I. A., Lanocha, K., Solvason, H.B., Bonneh-Barkay, D., et al. (2013). Transcranial magnetic stimulation (TMS) for major depression: a multisite, naturalistic, observational study of quality of life outcome measures in clinical practice. CNS Spectr. *18*, 322–332.

Jeanson, T., Pondaven, A., Ezan, P., Mouthon, F., Charvériat, M., and Giaume, C. (2015). Antidepressants Impact Connexin 43 Channel Functions in Astrocytes. Front. Cell. Neurosci. *9*, 495.

Jégou, S., El Yacoubi, M., Mounien, L., Ledent, C., Parmentier, M., Costentin, J., Vaugeois, J.M., and Vaudry, H. (2003). Adenosine A_{2A} receptor gene disruption provokes marked changes in melanocortin content and pro-opiomelanocortin gene expression. J. Neuroendocrinol. *15*, 1171–1177.

Jin, S., and Fredholm, B.B. (1997). Adenosine A_1 receptors mediate hypoxia-induced inhibition of electrically evoked transmitter release from rat striatal slices. Eur. J. Pharmacol. *329*, 107–113.

Jin, X., Shepherd, R.K., Duling, B.R., and Linden, J. (1997). Inosine binds to A_3 adenosine receptors and stimulates mast cell degranulation. J. Clin. Invest. *100*, 2849–2857.

Josselyn, S.A., and Nguyen, P. V (2005). CREB, synapses and memory disorders: past progress and future challenges. Curr. Drug Targets. CNS Neurol. Disord. *4*, 481–497.

Jursky, F., Tamura, S., Tamura, A., Mandiyan, S., Nelson, H., and Nelson, N. (1994). Structure, function and brain localization of neurotransmitter transporters. J. Exp. Biol. *196*, 283–295.

Kang, H.J., Voleti, B., Hajszan, T., Rajkowska, G., Stockmeier, C., Licznerski, P., Lepack, A., Majik, M.S., Jeong, L.S., Banasr, M., et al. (2012). Decreased Expression of Synapse-Related Genes and Loss of Synapses in Major Depressive Disorder. Nat. Med. *18*, 1413–1417.

Kaster, M.P., Rosa, A.O., Rosso, M.M., Goulart, E.C., Santos, A.R.S., and Rodrigues, A.L.S. (2004). Adenosine administration produces an antidepressant-like effect in mice: Evidence for the involvement of A_1 and A_{2A} receptors. Neurosci. Lett. *355*, 21–24.

Kaster, M.P., Machado, N.J., Silva, H.B., Nunes, A., Ardais, A.P., Santana, M., Baqi, Y., Müller, C.E., Rodrigues, A.L.S., Porciúncula, L.O., et al. (2015). Caffeine acts through neuronal adenosine A_{2A} receptors to prevent mood and memory dysfunction triggered by chronic stress. Proc. Natl. Acad. Sci. *112*, 7833–7858.

Kawachi, I., Willett, W.C., Colditz, G.A., Stampfer, M.J., and Speizer, F.E. (1996). A prospective study of coffee drinking and suicide in women. Arch Intern Med *156*, 521–525.

Kessler, R.C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K.R., Rush, A.J., Walters, E.E., and Wang, P.S. (2003). The epidemiology of major depressive disorder. Results From the National Comorbidity Survey Replication (NCS-R). JAMA *289*, 3095–3105.

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: New Roles for the Synaptic Stripper. Neuron *77*, 10–18.

Khoa, N.D., Postow, M., Danielsson, J., and Cronstein, B.N. (2006). Tumor necrosis factor-alpha prevents desensitization of Galphas-coupled receptors by regulating GRK2 association with the plasma membrane. Mol. Pharmacol. *69*, 1311–1319.

Kimelberg, H.K., Goderie, S.K., Higman, S., Pang, S., and Waniewski, R.A. (1990). Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. J. Neurosci. *10*, 1583–1591.

King, D.L., and Arendash, G.W. (2002). Maintained synaptophysin immunoreactivity in Tg2576 transgenic mice during aging: Correlations with cognitive impairment. Brain Res. *926*, 58–68.

de Kloet, E.R., Joëls, M., and Holsboer, F. (2005). Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. *6*, 463–475.

Korn, T., Magnus, T., and Jung, S. (2005). Autoantigen specific T cells inhibit glutamate uptake in astrocytes by decreasing expression of astrocytic glutamate transporter GLAST: a mechanism mediated by tumor necrosis factor-alpha. FASEB J. *19*, 1878–1880.

Kreutzberg, G.W., Barron, K.D., and Schubert, P. (1978). Cytochemical localization of 5'-nucleotidase in glial plasma membranes. Brain Res. *158*, 247–257.

Krishnan, V., and Nestler, E.J. (2008). The molecular neurobiology of depression. Nature *455*, 894–902.

Krishnan, V., and Nestler, E.J. (2011). Animal models of depression: Molecular perspectives. Curr. Top. Behav. Neurosci. *7*, 121–147.

Krügel, U. (2015). Purinergic receptors in psychiatric disorders. Neuropharmacology *104*, 212–225.

Kucukibrahimoglu, E., Saygin, M.Z., Çalişkan, M., Kaplan, O.K., Ünsal, C., and Gören, M.Z. (2009). The change in plasma GABA, glutamine and glutamate levels in fluoxetine- or S-citalopram-treated female patients with major depression. Eur. J. Clin. Pharmacol. *65*, 571–577.

Kulkarni, S.K., and Mehta, A.K. (1985). Purine nucleoside-mediated immobility in mice: reversal by antidepressants. Psychopharmacol. *85*, 460–463.

Küst, B.M., Biber, K., Van Calker, D., and Gebicke-Haerter, P.J. (1999). Regulation of K^+ channel mRNA expression by stimulation of adenosine A_{2A}-receptors in cultured rat microglia. Glia *25*, 120–130.

Ladd, C.O., Huot, R.L., Thrivikraman, K. V, Nemeroff, C.B., Meaney, M.J., and Plotsky, P.M. (2000). Long-term behavioral and neuroendocrine adaptations to adverse early experience. Prog. Brain Res. *122*, 81–103.

Lanciotti, A., Brignone, M.S., Bertini, E., Petrucci, T.C., Aloisi, F., and Ambrosini, E. (2013). Astrocytes: Emerging Stars in Leukodystrophy Pathogenesis. Transl. Neurosci. *4*, 144–164.

Lara, D.R. (2010). Caffeine, mental health, and psychiatric disorders. In Journal of Alzheimer's Disease, pp. S239-248.

Lee Martin, J., Brambrink, A.M., Lehmann, C., Portera-Cailliau, C., Koehler, R., Rothstein, J., and Traystman, R.J. (1997). Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum. Ann. Neurol. *42*, 335–348.

Lehre, K.P., Levy, L.M., Ottersen, O.P., Storm-Mathisen, J., and Danbolt, N.C. (1995). Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci *15*, 1835–1853.

Li, X.X., Nomura, T., Aihara, H., and Nishizaki, T. (2001). Adenosine enhances glial glutamate efflux via A_{2A} adenosine receptors. Life Sci. *68*, 1343–1350.

Liévens, J.C., Woodman, B., Mahal, A., Spasic-Boscovic, O., Samuel, D., Kerkerian-Le Goff, L., and Bates, G.P. (2001). Impaired glutamate uptake in the R6 Huntington's disease transgenic mice. Neurobiol. Dis. *8*, 807–821.

Lopes, L. V, Cunha, R.A., and Ribeiro, J.A. (1999). Cross talk between A_1 and A_{2A} adenosine receptors in the hippocampus and cortex of young adult and old rats. J. Neurophysiol. *82*, 3196–3203.

Lopes, L. V, Halldner, L., Rebola, N., Johansson, B., Ledent, C., Chen, J.F., Fredholm, B.B., and Cunha, R.A. (2004). Binding of the prototypical adenosine A_{2A} receptor agonist CGS 21680 to the cerebral cortex of adenosine A_1 and A_{2A} receptor knockout mice. Br. J. Pharmacol. *141*, 1006–1014.

Lopes, L. V., Cunha, R.A., Kull, B., Fredholm, B.B., and Ribeiro, J.A. (2002). Adenosine A_{2A} receptor facilitation of hippocampal synaptic transmission is dependent on tonic A_1 receptor inhibition. Neuroscience *112*, 319–329.

Lopes, L. V., Sebastião, A.M., and Ribeiro, J.A. (2011). Adenosine and related drugs in brain diseases: present and future in clinical trials. Curr. Top. Med. Chem. *11*, 1087–1101.

Lorenzetti, V., Allen, N.B., Fornito, A., and Yücel, M. (2009). Structural brain abnormalities in major depressive disorder: A selective review of recent MRI studies. J. Affect. Disord. *117*, 1–17.

Lucas, M., Mirzaei, F., Pan, A., Okereke, O.I., Willett, W.C., O'Reilly, E.J., Koenen, K., and Ascherio, A. (2011). Coffee, Caffeine, and Risk of Depression Among Women. Arch. Intern. Med. *171*, 1571–1578.

Lucas, M., O'Reilly, E.J., Pan, A., Mirzaei, F., Willett, W.C., Okereke, O.I., and Ascherio, A. (2014). Coffee, caffeine, and risk of completed suicide: results from three prospective cohorts of American adults. World J. Biol. Psychiatry *15*, 377–386.

Lucassen, P.J., Pruessner, J., Sousa, N., Almeida, O.F.X., Van Dam, A.M., Rajkowska, G., Swaab, D.F., and Czéh, B. (2014). Neuropathology of stress. Acta Neuropathol. *127*, 109–135.

Lynn, B.D., Tress, O., May, D., Willecke, K., and Nagy, J.I. (2011). Ablation of connexin30 in transgenic mice alters expression patterns of connexin26 and connexin32 in glial cells and leptomeninges. Eur. J. Neurosci. *34*, 1783–1793.

MacQueen, G., and Frodl, T. (2011). The hippocampus in major depression: evidence for the convergence of the bench and bedside in psychiatric research? Mol. Psychiatry *16*, 252–264.

Madeira, M.H., Elvas, F., Boia, R., Gonçalves, F.Q., Cunha, R.A., Ambrósio, A.F., and Santiago,

A.R. (2015). Adenosine A_{2A}R blockade prevents neuroinflammation-induced death of retinal ganglion cells caused by elevated pressure. J. Neuroinflammation *12*, 115.

Maienschein, V., Marxen, M., Volknandt, W., and Zemmermann, H. (1999). A plethora of presynaptic proteins associated with ATP-storing organelles in cultured astrocytes. Glia *26*, 233–244.

Malarkey, E.B., and Parpura, V. (2008). Mechanisms of glutamate release from astrocytes. Neurochem. Int. *52*, 142–154.

Malchow, B., Strocka, S., Frank, F., Bernstein, H.G., Steiner, J., Schneider-Axmann, T., Hasan, A., Reich-Erkelenz, D., Schmitz, C., Bogerts, B., et al. (2015). Stereological investigation of the posterior hippocampus in affective disorders. J. Neural Transm. *122*, 1019–1033.

Malone, D.A., Dougherty, D.D., Rezai, A.R., Carpenter, L.L., Friehs, G.M., Eskandar, E.N., Rauch, S.L., Rasmussen, S.A., Machado, A.G., Kubu, C.S., et al. (2009). Deep brain stimulation of the ventral capsule/ventral striatum for treatment-resistant depression. Biol. Psychiatry *65*, 267–275.

Manji, H.K., Drevets, W.C., and Charney, D.S. (2001). The cellular neurobiology of depression. Nat Med *7*, 541–547.

Marchi, M., Raiteri, L., Risso, F., Vallarino, A., Bonfanti, A., Monopoli, A., Ongini, E., and Raiteri, M. (2002). Effects of adenosine A_1 and A_{2A} receptor activation on the evoked release of glutamate from rat cerebrocortical synaptosomes. Br J Pharmacol *136*, 434–440.

Masliah, E., Mallory, M., Hansen, L., DeTeresa, R., and Terry, R.D. (1993). Quantitative synaptic alterations in the human neocortex during normal aging. Neurology *43*, 192–197.

Masliah, E., Alford, M., DeTeresa, R., Mallory, M., and Hansen, L. (1996). Deficient glutamate transport is associated with neurodegeneration in Alzheimer's disease. Ann. Neurol. *40*, 759–766.

Matos, M., Augusto, E., Oliveira, C.R., and Agostinho, P. (2008). Amyloid-beta peptide decreases glutamate uptake in cultured astrocytes: Involvement of oxidative stress and mitogen-activated protein kinase cascades. Neuroscience *156*, 898–910.

Matos, M., Augusto, E., Santos-Rodrigues, A. Dos, Schwarzschild, M. a, Chen, J.-F., Cunha, R.A., and Agostinho, P. (2012a). Adenosine A_{2A} receptors modulate glutamate uptake in cultured astrocytes and gliosomes. Glia *60*, 702–716.

Matos, M., Augusto, E., MacHado, N.J., Dos Santos-Rodrigues, A., Cunha, R.A., and Agostinho, P. (2012b). Astrocytic adenosine A_{2A} receptors control the amyloid-β peptide-induced decrease of glutamate uptake. J. Alzheimer's Dis. *31*, 555–567.

Matos, M., Augusto, E., Agostinho, P., Cunha, R.A., and Chen, J.F. (2013). Antagonistic interaction between adenosine A_{2A} receptors and Na^+/K^+-ATPase-α2 controlling glutamate uptake in astrocytes. J. Neurosci. *33*, 18492–18502.

Matos, M., Shen, H.Y., Augusto, E., Wang, Y., Wei, C.J., Wang, Y.T., Agostinho, P., Boison, D., Cunha, R.A., and Chen, J.F. (2015). Deletion of Adenosine A_{2A} Receptors from Astrocytes Disrupts Glutamate Homeostasis Leading to Psychomotor and Cognitive Impairment: Relevance to Schizophrenia. Biol. Psychiatry *78*, 763–774.

Mauri, M.C., Ferrara, A., Boscati, L., Bravin, S., Zamberlan, F., Alecci, M., and Invernizzi, G. (1998). Plasma and platelet amino acid concentrations in patients affected by major depression and under fluvoxamine treatment. Neuropsychobiology *37*, 124–129.

Mayberg, H.S. (2003). Positron emission tomography imaging in depression: A neural systems perspective. Neuroimaging Clin. N. Am. *13*, 805–815.

Mayberg, H.S., Lozano, A.M., Voon, V., McNeely, H.E., Seminowicz, D., Hamani, C., Schwalb, J.M., and Kennedy, S.H. (2005). Deep brain stimulation for treatment-resistant depression. Neuron *45*, 651–660.

McCullumsmith, R., and Meador-Woodruff, J. (2002). Striatal excitatory amino Acid transporter transcript expression in schizophrenia, bipolar disorder, and major depressive disorder. Neuropsychopharmacology *26*, 368–375.

McEwen, B.S. (2000). The neurobiology of stress: From serendipity to clinical relevance. Brain Res. *886*, 172–189.

Medina, A., Watson, S.J., Bunney, W., Myers, R.M., Schatzberg, A., Barchas, J., Akil, H., and Thompson, R.C. (2016). Evidence for alterations of the glial syncytial function in major depressive disorder. J. Psychiatr. Res. *72*, 15–21.

Merighi, S., Borea, P.A., Stefanelli, A., Bencivenni, S., Castillo, C.A., Varani, K., and Gessi, S. (2015). A_{2A} and A_{2B} adenosine receptors affect HIF-1α signaling in activated primary microglial cells. Glia *63*, 1933–1952.

Miguel-Hidalgo, J., Baucom, C., Dilley, G., Overholser, J., Meltzer, H., Stockmeier, C., and Rajkowska, G. (2000). Glial fibrillary acidic protein immunoreactivity in the prefrontal cortex distinguishes younger from older adults in major depressive disorder. Biol. Psychiatry *48*, 861–873.

Miguel-Hidalgo, J.J., Waltzer, R., Whittom, A.A., Austin, M.C., Rajkowska, G., and Stockmeier, C.A. (2010). Glial and glutamatergic markers in depression, alcoholism, and their comorbidity. J. Affect. Disord. *127*, 230–240.

Miguel-Hidalgo, J.J., Wilson, B.A., Hussain, S., Meshram, A., Rajkowska, G., and Stockmeier, C.A. (2014). Reduced connexin 43 immunolabeling in the orbitofrontal cortex in alcohol dependence and depression. J. Psychiatr. Res. *55*, 101–109.

Miller, R.H., and Raff, M.C. (1984). Fibrous and Protoplasmic Astrocytes Are Biochemically and Developmentally Distinct. J. Neurosci. *4*, 585–592.

Min, R., and Nevian, T. (2012). Astrocyte signaling controls spike timing–dependent depression at neocortical synapses. Nat. Neurosci. *15*, 746–753.

Mishra, A., Reynolds, J.P., Chen, Y., Gourine, A. V, Rusakov, D.A., and Attwell, D. (2016). Astrocytes mediate neurovascular signaling to capillary pericytes but not to arterioles. Nat. Neurosci. *19*, 1619–1627.

Mitani, H., Shirayama, Y., Yamada, T., Maeda, K., Ashby, C.R., and Kawahara, R. (2006). Correlation between plasma levels of glutamate, alanine and serine with severity of depression. Prog. Neuro-Psychopharmacology Biol. Psychiatry *30*, 1155–1158.

Mongin, A.A., and Orlov, S.N. (2001). Mechanisms of cell volume regulation and possible nature of the cell volume sensor. Pathophysiology *8*, 77–88.

Moreau, J.L., and Huber, G. (1999). Central adenosine A_{2A} receptors: An overview. Brain Res. Rev. *31*, 65–82.

Moylan, S., Maes, M., Wray, N.R., and Berk, M. (2013). The neuroprogressive nature of major depressive disorder: pathways to disease evolution and resistance, and therapeutic implications. Mol. Psychiatry *18*, 595–606.

Musazzi, L., Milanese, M., Farisello, P., Zappettini, S., Tardito, D., Barbiero, V.S., Bonifacino, T., Mallei, A., Baldelli, P., Racagni, G., et al. (2010). Acute stress increases depolarization-evoked glutamate release in the rat prefrontal/frontal cortex: The dampening action of antidepressants. PLoS One *5*.

Myers, B., McKlveen, J.M., and Herman, J.P. (2014). Glucocorticoid actions on synapses, circuits, and behavior: Implications for the energetics of stress. Front. Neuroendocrinol. *35*, 180–196.

Nag, T.C., and Wadhwa, S. (2001). Differential expression of syntaxin-1 and synaptophysin in the developing and adult human retina. J. Biosci. *26*, 179–191.

Nagy, C., Suderman, M., Yang, J., Szyf, M., Mechawar, N., Ernst, C., and Turecki, G. (2015). Astrocytic abnormalities and global DNA methylation patterns in depression and suicide. Mol. Psychiatry *20*, 320–328.

Nagy, C., Torres-Platas, S.G., Mechawar, N., and Turecki, G. (2016). Repression of astrocytic connexins in cortical and subcortical brain regions and prefrontal enrichment of H3K9me3 in depression and suicide. Int. J. Neuropsychopharmacol. *20*, 50–57.

Nagy, J.I., Dudek, F.E., and Rash, J.E. (2004). Update on connexins and gap junctions in neurons and glia in the mammalian nervous system. In Brain Research Reviews, pp. 191–215.

Naylor, G.J., Smith, A.H., Dick, E.G., Dick, D.A., McHarg, A.M., and Chambers, C.A. (1980). Erythrocyte membrane cation carrier in manic-depressive psychosis. Psychol Med *10*, 521–525.

Nemeroff, C.B., and Owens, M.J. (2002). Treatment of mood disorders. Nat. Neurosci. *5*, 1068–1070.

Nestler, E.J., Barrot, M., DiLeone, R.J., Eisch, A.J., Gold, S.J., and Monteggia, L.M. (2002). Neurobiology of depression. Neuron *34*, 13–25.

New, M.I., Carlson, A., Obeid, J., Marshall, I., Cabrera, M.S., Goseco, A., Lin-Su, K., Putnam, A.S., Wei, J.Q., and Wilson, R.C. (2001). Prenatal diagnosis for congenital adrenal hyperplasia in 532 pregnancies. J. Clin. Endocrinol. Metab. *86*, 5651–5657.

Newman, E.A. (2003a). New roles for astrocytes: Regulation of synaptic transmission. Trends Neurosci. *26*, 536–542.

Newman, E. a (2003b). Glial cell inhibition of neurons by release of ATP. J. Neurosci. *23*, 1659–1666.

Nishizaki, T., Nagai, K., Nomura, T., Tada, H., Kanno, T., Tozaki, H., Li, X.X., Kondoh, T., Kodama, N., Takahashi, E., et al. (2002). A new neuromodulatory pathway with a glial contribution mediated via A_{2A} adenosine receptors. Glia *39*, 133–147.

O'Callaghan, J.P., Brinton, R.E., and McEwen, B.S. (1989). Glucocorticoids regulate the concentration of glial fibrillary acidic protein throughout the brain. Brain Res. *494*, 159–161.

Oberheim, N.A., Goldman, S.A., and Nedergaard, M. (2012). Heterogeneity of astrocytic form and function. Methods Mol. Biol. *814*, 23–45.

Oitzl, M.S., Champagne, D.L., van der Veen, R., and de Kloet, E.R. (2010). Brain development under stress: Hypotheses of glucocorticoid actions revisited. Neurosci. Biobehav. Rev. *34*, 853–866.

Okada, M., Nutt, D.J., Murakami, T., Zhu, G., Kamata, A., Kawata, Y., and Kaneko, S. (2001). Adenosine receptor subtypes modulate two major functional pathways for hippocampal serotonin release. J Neurosci *21*, 628–640.

Olabarria, M., Noristani, H.N., Verkhratsky, A., and Rodríguez, J.J. (2011). Age-dependent decrease in glutamine synthetase expression in the hippocampal astroglia of the triple transgenic Alzheimer's disease mouse model: mechanism for deficient glutamatergic transmission? Mol. Neurodegener. *6*, 55.

Oliveira, M., Bessa, J.M., Mesquita, A., Tavares, H., Carvalho, A., Silva, R., Pêgo, J.M., Cerqueira, J.J., Palha, J.A., Almeida, O.F.X., et al. (2006). Induction of a Hyperanxious State by Antenatal Dexamethasone: A Case for Less Detrimental Natural Corticosteroids. Biol. Psychiatry *59*, 844–852.

Orellana, J.A., and Stehberg, J. (2014). Hemichannels: New roles in astroglial function. Front. Physiol. *5 JUN*.

Orellana, J.A., Retamal, M.A., Moraga-Amaro, R., and Stehberg, J. (2016). Role of Astroglial Hemichannels and Pannexons in Memory and Neurodegenerative Diseases. Front. Integr. Neurosci. *10*, 26.

Ota, Y., Zanetti, A.T., and Hallock, R.M. (2013). The role of astrocytes in the regulation of synaptic plasticity and memory formation. Neural Plast. *2013*.

Ozog, M.A., Siushansian, R., and Naus, C.C. (2002). Blocked gap junctional coupling increases glutamate-induced neurotoxicity in neuron-astrocyte co-cultures. J. Neuropathol. Exp. Neurol. *61*, 132–141.

Palacin, M., Estevez, R., Bertran, J., and Zorzano, A. (1998). Molecular biology of mammalian plasma membrane amino acid transporters. Physiol. Rev. *78*, 969–1054.

Panatier, A., Vallée, J., Haber, M., Murai, K.K., Lacaille, J.C., and Robitaille, R. (2011). Astrocytes are endogenous regulators of basal transmission at central synapses. Cell *146*, 785–798.

Pardon, M.C., and Rattray, I. (2008). What do we know about the long-term consequences of stress on ageing and the progression of age-related neurodegenerative disorders? Neurosci. Biobehav. Rev. *32*, 1103–1120.

Pariante, C.M. (2003). Depression, stress and the adrenal axis. J. Neuroendocrinol. *15*, 811–812.

Pariante, C.M., and Miller, A.H. (2001). Glucocorticoid receptors in major depression: Relevance to pathophysiology and treatment. Biol. Psychiatry *49*, 391–404.

Parkhurst, C.N., Yang, G., Ninan, I., Savas, J.N., Yates, J.R., Lafaille, J.J., Hempstead, B.L., Littman, D.R., and Gan, W.B. (2013). Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. Cell *155*, 1596–1609.

Parpura, V., Basarsky, T.A., Liu, F., Jeftinija, K., Jeftinija, S., and Haydon, P.G. (1994). Glutamate-mediated astrocyte-neuron signalling. Nature *369*, 744–747.

Parpura, V., Heneka, M.T., Montana, V., Oliet, S.H.R., Schousboe, A., Haydon, P.G., Stout, R.F., Spray, D.C., Reichenbach, A., Pannicke, T., et al. (2012). Glial cells in (patho)physiology. J. Neurochem. *121*, 4–27.

Pascual, O., Casper, K.B., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, J.Y., Takano, H., Moss, S.J., McCarthy, K., and Haydon, P.G. (2005). Astrocytic Purinergic Signaling Coordinates Synaptic Networks. Science. *310*, 113–116.

Pascual, O., Ben Achour, S., Rostaing, P., Triller, A., and Bessis, A. (2012). Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. Proc. Natl. Acad. Sci. *109*, 197–205.

Peng, L., Huang, R., Yu, A.C.H., Fung, K.Y., Rathbone, M.P., and Hertz, L. (2005). Nucleoside transporter expression and function in cultured mouse astrocytes. Glia *52*, 25–35.

Perea, G., and Araque, A. (2005). Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes. J. Neurosci. *25*, 2192–2203.

Perez Velazquez, J.L., Frantseva, M., Naus, C.C.G., Bechberger, J.F., Juneja, S.C., Velumian, A., Carlen, P.L., Kidder, G.M., and Mills, L.R. (1996). Development of astrocytes and neurons in cultured brain slices from mice lacking connexin43. Dev. Brain Res. *97*, 293–296.

Persson, M., and Rönnbäck, L. (2012). Microglial self-defence mediated through GLT-1 and glutathione. Amino Acids, 207–219.

Pillay, S.S., Renshaw, P.F., Bonello, C.M., Lafer, B.C., Fava, M., and Yurgelun-Todd, D. (1998). A quantitative magnetic resonance imaging study of caudate and lenticular nucleus gray matter volume in primary unipolar major depression: relationship to treatment response and clinical severity. Psychiatry Res. *84*, 61–74.

de Pina-Benabou, M.H., Szostak, V., Kyrozis, A., Rempe, D., Uziel, D., Urban-Maldonado, M., Benabou, S., Spray, D.C., Federoff, H.J., Stanton, P.K., et al. (2005). Blockade of gap junctions in vivo provides neuroprotection after perinatal global ischemia. Stroke *36*, 2232–2237.

Pizzagalli, D.A., Oakes, T.R., Fox, A.S., Chung, M.K., Larson, C.L., Abercrombie, H.C., Schaefer, S.M., Benca, R.M., and Davidson, R.J. (2004). Functional but not structural subgenual prefrontal cortex abnormalities in melancholia. Mol. Psychiatry *9*, 393–405.

Popoli, M., Yan, Z., McEwen, B.S., and Sanacora, G. (2012). The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. Nat. Rev. Neurosci. *13*, 22–37.

Popoli, P., Betto, P., Reggio, R., and Ricciarello, G. (1995). Adenosine A_{2A} receptor stimulation enhances striatal extracellular glutamate levels in rats. Eur. J. Pharmacol. *287*, 215–217.

Purves, D., Augustine, G., Fitzpatrick, D., Katz, L., LaMantia, A.S., McNamara, J.O., and Williams, S.M. (2001). Neuroscience. Sunderl. Sinauer Assoc. 1–773.

Quesseveur, G., Portal, B., Basile, J.A., Ezan, P., Mathou, A., Halley, H., Leloup, C., Fioramonti, X., Déglon, N., Giaume, C., et al. (2015). Attenuated Levels of Hippocampal Connexin 43 and its Phosphorylation Correlate with Antidepressant- and Anxiolytic-Like Activities in Mice. Front. Cell. Neurosci. *9*, 490.

Raivich, G. (2005). Like cops on the beat: The active role of resting microglia. Trends Neurosci. *28*, 571–573.

Rajkowska, G., and Stockmeier, C.A. (2013). Astrocyte pathology in major depressive disorder: insights from human postmortem brain tissue. Curr. Drug Targets *14*, 1225–1236.

Rajkowska, G., Miguel-Hidalgo, J.J., Wei, J., Dilley, G., Pittman, S.D., Meltzer, H.Y., Overholser, J.C., Roth, B.L., and Stockmeier, C.A. (1999). Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression. Biol. Psychiatry *45*, 1085–1098.

Rebola, N., Oliveira, C.R., and Cunha, R.A. (2002). Transducing system operated by adenosine A_{2A} receptors to facilitate acetylcholine release in the rat hippocampus. Eur. J. Pharmacol. *454*, 31–38.

Rebola, N., Canas, P.M., Oliveira, C.R., and Cunha, R.A. (2005a). Different synaptic and subsynaptic localization of adenosine A_{2A} receptors in the hippocampus and striatum of the rat. Neuroscience *132*, 893–903.

Rebola, N., Rodrigues, R.J., Lopes, L. V., Richardson, P.J., Oliveira, C.R., and Cunha, R.A. (2005b). Adenosine A_1 and A_{2A} receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. Neuroscience *133*, 79–83.

Rebola, N., Lujan, R., Cunha, R.A., and Mulle, C. (2008). Adenosine A_{2A} Receptors Are Essential for Long-Term Potentiation of NMDA-EPSCs at Hippocampal Mossy Fiber Synapses. Neuron *57*, 121–134.

Rebola, N., Simões, A.P., Canas, P.M., Tomé, A.R., Andrade, G.M., Barry, C.E., Agostinho, P.M., Lynch, M.A., and Cunha, R.A. (2011). Adenosine A_{2A} receptors control neuroinflammation and consequent hippocampal neuronal dysfunction. J. Neurochem. *117*, 100–111.

Reznikov, L.R., Grillo, C.A., Piroli, G.G., Pasumarthi, R.K., Reagan, L.P., and Fadel, J. (2007). Acute stress-mediated increases in extracellular glutamate levels in the rat amygdala: Differential

effects of antidepressant treatment. Eur. J. Neurosci. *25*, 3109–3114.

Rial, D., Lemos, C., Pinheiro, H., Duarte, J.M., Gonçalves, F.Q., Real, J.I., Prediger, R.D., Gonçalves, N., Gomes, C.A., Canas, P.M., et al. (2015). Depression as a Glial-Based Synaptic Dysfunction. Front. Cell. Neurosci. *9*, 521.

Ribeiro, J.A. (1999). Adenosine A2A receptor interactions with receptors for other neurotransmitters and neuromodulators. Eur. J. Pharmacol. *375*, 101–113.

Rihmer, Z. (2007). Suicide risk in mood disorders. Curr. Opin. Psychiatry *20*, 17–22.

Rodrigues, R.J., Alfaro, T.M., Rebola, N., Oliveira, C.R., and Cunha, R.A. (2005). Co-localization and functional interaction between adenosine A_{2A} and metabotropic group 5 receptors in glutamatergic nerve terminals of the rat striatum. J. Neurochem. *92*, 433–441.

Rubinow, M.J., Mahajan, G., May, W., Overholser, J.C., Jurjus, G.J., Dieter, L., Herbst, N., Steffens, D.C., Miguel-Hidalgo, J.J., Rajkowska, G., et al. (2016). Basolateral amygdala volume and cell numbers in major depressive disorder: a postmortem stereological study. Brain Struct. Funct. *221*, 171–184.

Russo, S.J., and Nestler, E.J. (2013). The brain reward circuitry in mood disorders. Nat. Rev. Neurosci. *14*, 609–625.

Rutten, B.P.F., Van der Kolk, N.M., Schafer, S., van Zandvoort, M.A.M.J., Bayer, T.A., Steinbusch, H.W.M., and Schmitz, C. (2005). Age-related loss of synaptophysin immunoreactive presynaptic boutons within the hippocampus of APP751SL, PS1M146L, and APP751SL/PS1M146L transgenic mice. Am. J. Pathol. *167*, 161–173.

Saito, S., Kobayashi, S., Ohashi, Y., Igarashi, M., Komiya, Y., and Ando, S. (1994). Decreased synaptic density in aged brains and its prevention by rearing under enriched environment as revealed by synaptophysin contents. J. Neurosci. Res. *39*, 57–62.

Sanacora, G., and Banasr, M. (2013). From pathophysiology to novel antidepressant drugs: Glial contributions to the pathology and treatment of mood disorders. Biol. Psychiatry *73*, 1172–1179.

Sanacora, G., Mason, G.F., Rothman, D.L., Behar, K.L., Hyder, F., Petroff, O.A., Berman, R.M., Charney, D.S., and Krystal, J.H. (1999). Reduced cortical gamma-aminobutyric acid levels in depressed patients determined by proton magnetic resonance spectroscopy. Arch. Gen. Psychiatry *56*, 1043–1047.

Sanacora, G., Treccani, G., and Popoli, M. (2012). Towards a glutamate hypothesis of depression: An emerging frontier of neuropsychopharmacology for mood disorders. In Neuropharmacology, 63–77.

Sapolsky, R.M. (1996). Why stress is bad for your brain. Science *273*, 749–750.

Sapolsky, R.M. (1999). Glucocorticoids, stress, and their adverse neurological effects: Relevance to aging. Exp. Gerontol. *34*, 721–732.

Sarantis, K., Tsiamaki, E., Kouvaros, S., Papatheodoropoulos, C., and Angelatou, F. (2015). Adenosine A_{2A} receptors permit mGluR5-evoked tyrosine phosphorylation of NR2B (Tyr1472) in rat hippocampus: A possible key mechanism in NMDA receptor modulation. J. Neurochem. *135*, 714–726.

Satoh, E., and Shimeki, S. (2010). Acute restraint stress enhances calcium mobilization and glutamate exocytosis in cerebrocortical synaptosomes from mice. Neurochem. Res. *35*, 693–701.

Schafer, D.P., Lehrman, E.K., Kautzman, A.G., Koyama, R., Mardinly, A.R., Yamasaki, R., Ransohoff, R.M., Greenberg, M.E., Barres, B.A., and Stevens, B. (2012). Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. Neuron *74*, 691–705.

Schiffmann, S.N., Libert, F., Vassart, G., and Vanderhaeghen, J.J. (1991). Distribution of adenosine A_2 receptor mRNA in the human brain. Neurosci. Lett. *130*, 177–181.

Schousboe, A. (2003). Role of astrocytes in the maintenance and modulation of glutamatergic and GABAergic neurotransmission. Neurochem. Res. *28*, 347–352.

Scianni, M., Antonilli, L., Chece, G., Cristalli, G., Di Castro, M.A., Limatola, C., and Maggi, L. (2013). Fractalkine (CX3CL1) enhances hippocampal N-methyl-D-aspartate receptor (NMDAR) function via D-serine and adenosine receptor type A_2 (A_{2A}R) activity. J. Neuroinflammation *10*, 108.

Secretariat, T. (2011). Global burden of mental disorders and the need for a comprehensive, coordinated response from health and social sectors at the country level. 130Th Sess. *EB130*, 1–6.

Sequeira, A., Mamdani, F., Ernst, C., Vawter, M.P., Bunney, W.E., Lebel, V., Rehal, S., Klempan, T., Gratton, A., Benkelfat, C., et al. (2009). Global brain gene expression analysis links glutamatergic and GABAergic alterations to suicide and major depression. PLoS One *4*, e6585.

Sheline, Y.I., Gado, M.H., and Kraemer, H.C. (2003). Untreated depression and hippocampal volume loss. Am. J. Psychiatry *160*, 1516–1518.

Silva, C.G., Porciúncula, L.O., Canas, P.M., Oliveira, C.R., and Cunha, R.A. (2007). Blockade of adenosine A_{2A} receptors prevents staurosporine-induced apoptosis of rat hippocampal neurons. Neurobiol. Dis. *27*, 182–189.

Simard, M., and Nedergaard, M. (2004). The neurobiology of glia in the context of water and ion homeostasis. Neuroscience *129*, 877–896.

Simpson, M.D.C., Slater, P., Royston, M.C., and Deakin, J.F.W. (1992). Regionally selective deficits in uptake sites for glutamate and gamma-aminobutyric acid in the basal ganglia in schizophrenia. Psychiatry Res. *42*, 273–282.

Singh, S.K., Stogsdill, J.A., Pulimood, N.S., Dingsdale, H., Kim, Y.H., Pilaz, L.J., Kim, I.H., Manhaes, A.C., Rodrigues, W.S., Pamukcu, A., et al. (2016). Astrocytes Assemble Thalamocortical Synapses by Bridging NRX1α and NL1 via Hevin. Cell *164*, 183–196.

Skupio, U., Tertil, M., Sikora, M., Golda, S., Wawrzczak-Bargiela, A., and Przewlocki, R. (2015). Behavioral and molecular alterations in mice resulting from chronic treatment with dexamethasone: Relevance to depression. Neuroscience *286*, 141–150.

Smith, A.P. (2009). Caffeine, cognitive failures and health in a non-working community sample. Hum. Psychopharmacol. *24*, 29–34.

Söderberg, O., Gullberg, M., Jarvius, M., Ridderstråle, K., Leuchowius, K.J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L.G., et al. (2006). Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat. Methods *3*, 995–1000.

Sofroniew, M. V (2015). Astrocyte barriers to neurotoxic inflammation. Nat. Rev. Neurosci. *16*, 249–263.

Sofroniew, M. V., and Vinters, H. V. (2010). Astrocytes: Biology and pathology. Acta Neuropathol. *119*, 7–35.

Sotiropoulos, I., Cerqueira, J.J., Catania, C., Takashima, A., Sousa, N., and Almeida, O.F.X. (2008). Stress and glucocorticoid footprints in the brain-The path from depression to Alzheimer's disease. Neurosci. Biobehav. Rev. *32*, 1161–1173.

Stigliani, S., Zappettini, S., Raiteri, L., Passalacqua, M., Melloni, E., Venturi, C., Tacchetti, C., Diaspro, A., Usai, C., and Bonanno, G. (2006). Glia re-sealed particles freshly prepared from adult rat brain are competent for exocytotic release of glutamate. J. Neurochem. *96*, 656–668.

Stockmeier, C.A., and Rajkowska, G. (2004). Cellular abnormalities in depression: Evidence from postmortem brain tissue. Dialogues Clin. Neurosci. *6*, 185–197.

Stockmeier, C.A., Mahajan, G.J., Konick, L.C., Overholser, J.C., Jurjus, G.J., Meltzer, H.Y., Uylings, H.B.M., Friedman, L., and Rajkowska, G. (2004). Cellular changes in the postmortem hippocampus in major depression. Biol. Psychiatry *56*, 640–650.

Stone, T.W., Ceruti, S., and Abbracchio, M.P. (2009). Adenosine receptors and neurological

disease: Neuroprotection and neurodegeneration. Handb. Exp. Pharmacol. *193*, 535–587.

Sun, J.D., Liu, Y., Yuan, Y.H., Li, J., and Chen, N.H. (2012). Gap junction dysfunction in the prefrontal cortex induces depressive-like behaviors in rats. Neuropsychopharmacology *37*, 1305–1320.

Svenningsson, P., Le Moine, C., Fisone, G., and Fredholm, B.B. (1999). Distribution, biochemistry and function of striatal adenosine A_{2A} receptors. Prog. Neurobiol. *59*, 355–396.

Szatkowski, M., Barbour, B., and Attwell, D. (1990). Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. Nature *348*, 443–446.

Takahashi, R.N., Pamplona, F.A., and Prediger, R.D.S. (2008). Adenosine receptor antagonists for cognitive dysfunction: a review of animal studies. Front. Biosci. *13*, 2614–2632.

Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., Iwama, H., Nishikawa, T., Ichihara, N., Kikuchi, T., et al. (1997). Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science. *276*, 1699–1702.

Tani, H., Dulla, C.G., Farzampour, Z., Taylor-Weiner, A., Huguenard, J.R., and Reimer, R.J. (2014). A local glutamate-glutamine cycle sustains synaptic excitatory transmitter release. Neuron *81*, 888–900.

Tebano, M.T., Martire, A., Rebola, N., Pepponi, R., Domenici, M.R., Grò, M.C., Schwarzschild, M.A., Chen, J.F., Cunhat, R.A., and Popoli, P. (2005). Adenosine A_{2A} receptors and metabotropic glutamate 5 receptors are co-localized and functionally interact in the hippocampus: A possible key mechanism in the modulation of N-methyl-D-aspartate effects. J. Neurochem. *95*, 1188–1200.

Theis, M., and Giaume, C. (2012). Connexin-based intercellular communication and astrocyte heterogeneity. Brain Res. *1487*, 88–98.

Tochigi, M., Iwamoto, K., Bundo, M., Sasaki, T., Kato, N., and Kato, T. (2008). Gene expression profiling of major depression and suicide in the prefrontal cortex of postmortem brains. Neurosci. Res. *60*, 184–191.

Torres, A., Wang, F., Xu, Q., Fujita, T., Dobrowolski, R., Willecke, K., Takano, T., and Nedergaard, M. (2012). Extracellular Ca^{2+} Acts as a Mediator of Communication from Neurons to Glia. Sci. Signal. *5*, ra8-ra8.

Torres-Platas, S.G., Cruceanu, C., Chen, G.G., Turecki, G., and Mechawar, N. (2014). Evidence for increased microglial priming and macrophage recruitment in the dorsal anterior cingulate white matter of depressed suicides. Brain. Behav. Immun. *42*, 50–59.

Trullas, R., and Skolnick, P. (1990). Functional antagonists at the NMDA receptor complex exhibit antidepressant actions. Eur. J. Pharmacol. *185*, 1–10.

Vanguilder, H.D., Yan, H., Farley, J.A., Sonntag, W.E., and Freeman, W.M. (2010). Aging alters the expression of neurotransmission-regulating proteins in the hippocampal synaptoproteome. J. Neurochem. *113*, 1577–1588.

de Vasconcellos, A.P., Zugno, A.I., Dos Santos, A.H., Nietto, F.B., Crema, L.M., Goncalves, M., Franzon, R., de Souza Wyse, A.T., da Rocha, E.R., and Dalmaz, C. (2005). Na^+/K^+-ATPase activity is reduced in hippocampus of rats submitted to an experimental model of depression: effect of chronic lithium treatment and possible involvement in learning deficits. Neurobiol. Learn. Mem. *84*, 102–110.

Venero, C., and Borrell, J. (1999). Rapid glucocorticoid effects on excitatory amino acid levels in the hippocampus: A microdialysis study in freely moving rats. Eur. J. Neurosci. *11*, 2465–2473.

Vielkind, U., Walencewicz, A., Levine, J.M., and Bohn, M.C. (1990). Type II glucocorticoid receptors are expressed in oligodendrocytes and astrocytes. J. Neurosci. Res. *27*, 360–373.

Virgin, C.E., Ha, T.P., Packan, D.R., Tombaugh, G.C., Yang, S.H., Homer, H.C., and Sapolsky, R.M. (1991). Glucocorticoids inhibit glucose transport and glutamate uptake in hippocampal astrocytes: implications for glucocorticoid neurotoxicity. J. Neurochem. *57*, 1422–1428.

Volterra, A., and Meldolesi, J. (2005). Astrocytes, from brain glue to communication elements: the revolution continues. Nat. Rev. Neurosci. *6*, 626–640.

Wake, H., Moorhouse, A.J., Miyamoto, A., and Nabekura, J. (2013). Microglia: Actively surveying and shaping neuronal circuit structure and function. Trends Neurosci. *36*, 209–217.

Wallraff, A., Odermatt, B., Willecke, K., and Steinhäuser, C. (2004). Distinct types of astroglial cells in the hippocampus differ in gap junction coupling. Glia *48*, 36–43.

Wang, D.D., and Bordey, A. (2008). The astrocyte odyssey. Prog. Neurobiol. *86*, 342–367.

Wasseff, S.K., and Scherer, S.S. (2011). Cx32 and Cx47 mediate oligodendrocyte:astrocyte and oligodendrocyte:oligodendrocyte gap junction coupling. Neurobiol. Dis. *42*, 506–513.

Watase, K., Hashimoto, K., Kano, M., Yamada, K., Watanabe, M., Inoue, Y., Okuyama, S., Sakagawa, T., Ogawa, S.I., Kawashima, N., et al. (1998). Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. Eur. J. Neurosci. *10*, 976–988.

Welberg, L. (2013). Neuroendocrinology: Hypothalamic self-tuning to stress. Nat. Rev. Neurosci. *14*, 377.

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

Wirkner, K., Gerevich, Z., Krause, T., Günther, A., Köles, L., Schneider, D., Nörenberg, W., and Illes, P. (2004). Adenosine A2A receptor-induced inhibition of NMDA and GABAA receptor-mediated synaptic currents in a subpopulation of rat striatal neurons. Neuropharmacology *46*, 994–1007.

Wong, M.L., Kling, M.A., Munson, P.J., Listwak, S., Licinio, J., Prolo, P., Karp, B., McCutcheon, I.E., Geracioti, T.D., DeBellis, M.D., et al. (2000). Pronounced and sustained central hypernoradrenergic function in major depression with melancholic features: relation to hypercortisolism and corticotropin-releasing hormone. Proc. Natl. Acad. Sci. *97*, 325–330.

Wood, A.J., Smith, C.E., Clarke, E.E., Cowen, P.J., Aronson, J.K., and Grahame-Smith, D.G. (1991). Altered in vitro adaptive responses of lymphocyte Na+/K+-ATPase in patients with manic depressive psychosis. J. Affect. Disord. *21*, 199–206.

el Yacoubi, M., Ledent, C., Parmentier, M., Bertorelli, R., Ongini, E., Costentin, J., and Vaugeois, J.M. (2001). Adenosine A_{2A} receptor antagonists are potential antidepressants: evidence based on pharmacology and A2A receptor knockout mice. Br. J. Pharmacol. *134*, 68–77.

el Yacoubi, M., Costentin, J., and Vaugeois, J.M. (2003). Adenosine A_{2A} receptors and depression. Neurology *61*, S82-87.

Yan, H.-C., Cao, X., Das, M., Zhu, X.-H., and Gao, T.-M. (2010). Behavioral animal models of depression. Neurosci. Bull. *26*, 327–337.

Yang, J., Yang, H., Liu, Y., Li, X., Qin, L., Lou, H., Duan, S., and Wang, H. (2016). Astrocytes contribute to synapse elimination Via type 2 inositol 1,4,5-trisphosphate receptor-dependent release of ATP. Elife *5*, e15043.

Yang, Y., Ge, W., Chen, Y., Zhang, Z., Shen, W., Wu, C., Poo, M., and Duan, S. (2003). Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. Proc. Natl. Acad. Sci. U. S. A. *100*, 15194–15199.

Yin, Y.Q., Zhang, C., Wang, J.X., Hou, J., Yang, X., and Qin, J. (2015). Chronic caffeine treatment enhances the resilience to social defeat stress in mice. Food Funct. *6*, 479–491.

Young, E.A., Haskett, R.F., Murphy-Weinberg, V., Watson, S.J., and Akil, H. (1991). Loss of glucocorticoid fast feedback in depression. Arch. Gen. Psychiatry *48*, 693–699.

Zhan, Y., Paolicelli, R.C., Sforazzini, F., Weinhard, L., Bolasco, G., Pagani, F., Vyssotski, A.L., Bifone, A., Gozzi, A., Ragozzino, D., et al. (2014). Deficient neuron-microglia signaling results

in impaired functional brain connectivity and social behavior. Nat. Neurosci. *17*, 400–406.

Zhang, J.M., Wang, H.K., Ye, C.Q., Ge, W., Chen, Y., Jiang, Z.L., Wu, C.P., Poo, M.M., and Duan, S. (2003). ATP Released by Astrocytes Mediates Glutamatergic Activity-Dependent Heterosynaptic Suppression. Neuron *40*, 971–982.

Zündorf, G., Kahlert, S., and Reiser, G. (2007). Gap-junction blocker carbenoxolone differentially enhances NMDA-induced cell death in hippocampal neurons and astrocytes in co-culture. J. Neurochem. *102*, 508–521.