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Role of the adenosinergic system in animal models of chronic stress and depression

Tese de doutoramento em Biociências, ramo de especialização em Neurociências, orientada por Rodrigo Pinto Santos Antunes Cunha e Rui de Albuquerque Carvalho e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Universidade de Coimbra
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Role of the adenosinergic system in animal models of chronic stress and depression

O papel do sistema adenosinérgico em modelos animais de estresse crónico e depressão

A tese e o trabalho foram elaborados sob orientação de **Rodrigo Pinto Santos Antunes Cunha**, professor da Faculdade de Medicina da Universidade de Coimbra, e **Rui de Albuquerque Carvalho**, professor da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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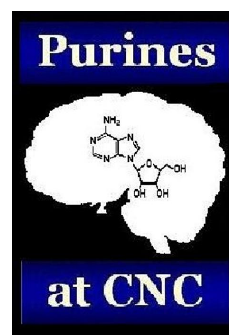

Considerations

The work presented in this thesis is the result of a highly collaborative group and the direct collaboration and participation of some key colleagues and friends who ought to be referred. This section highlights the roles of whom I had the pleasure to work with and who had a major participationon the final data presented. Individual participation is detailed below in chronological order.

Manella P Kaster PhD

Manuella established the chronic unpredictable stress (CUS) model in the group. As a postdoc fellow, she led most experiments published with the CUS model (chapter 4.2), participated in the behavioral evaluation of H/Rouen mice (chapter 4.1) and started the experiments on the 3 stress models with Wistar rats (chapter 4.4).

Ana Nunes, PhD

Ana was the postdoc in charge of the stress project after Manuella changed roles and participated in behavioral experiments presented in chapter 4.4, as well as Western blot and immunohistochemistry experiments not presented in this thesis, among others.

Ana Paula Ardais, MSc

Ana Paula was a visiting PhD student and together we planned and, with more lab members, conducted the behavioral experiments on the H/Rouen animals (chapter 4.1). Ana Paula also performed Western blot analysis on the same animals.

Nélio Gonçalves, PhD

Nélio was a postdoc working with the repeated immobilization stress model (Chapter 4.5). Nélio was responsible for the production of the lentivirus silencing $A_{2A}R$ and for the injection into the BLA. Nélio was co-responsile for planning experiments and tissue collection.

Carolina Melo de Souza, PhD

Carolina was a visiting postdoc fellow who participated in most of the work with the immobilization stress model (Chapter 4.5). Among work in other projects, and during Carolina's stay, we shared responsibility for the behavioral planning, evaluation, and analysis.

Paula Canas, PhD

Paula trained and taught me the kainate injections and the Racine scale evaluation, as well as the usage of the microscope and the slices preparation (chapter 4.3). We also optimized the kainate injection procedure.

List of Publications

The author of this thesis has participated in different projects that resulted in the publication and acceptance for publication of 11 papers in international peer-reviewed journals.

The publications listed below include data from chapters 4.1 (H/Rouen model) and 4.2 (chronic unpredictable stress model) of this thesis, respectively.

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The characterization of the lentiviral injection of short hairpin interference RNA effects on amygdalar function, which is used for the experiments in chapter 4.5, is included in the publication below. Data from this publication was collected during the the doctorate period and are referred in the text but not shown.

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* authors contributed equally in each publication

Abbreviations list

2TYM	two-trial Y maze test
^{3}H-DPCPX	^{3}H-1,3-dipropyl-8-cyclopentylxanthine
A_1R	A1 receptors
$A_{2A}R$	A_{2A} receptors
$A_{2B}R$	A2B receptors
A_3R	A3 receptors
Ab	antibodies
AChE	acetylcholinesterase
ACTH	adrenocorticotrophic hormone
AD	Alzheimer's disease
ADA	adenosine deaminase
ADP	adenosine diphosphate
AK	adenosine kinase
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP	antero-posterior
AP	dorso-ventral
APS	ammonium persulfate
ATP	adenosine triphosphate
BA	basal amygdala
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
BL	basolateral nuclei of the amygdala
BLA	basolateral amygdala
BM	basomedial nuclei of the amygdala
BNST	bed nucleus of the stria terminalis
CA1	*Cornu Amonnis*, subfield 1
CA3	*Cornu Amonnis*, subfield 3
cAMP	cyclic adenosine monophosphate
CAPS	3-(cyclohexylamino)-1-propane-sulfonic acid
CD11b	cluster of differentiation molecule 11B
CeA	central amygdala
CeL	lateral subnucleus of central amygdala
CeM	medial subnucleus of central amygdala
CNS	central nervous system
CORT	corticosterone/cortisol
CRH	corticotrophin-releasing hormone
CUS	chronic unpredictable stress
DPX	distyrene, plasticizer, xylene
DRI	dopamine reuptake inhibitor
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5

ECF	Enhanced Chemi-Fluorescence substrate
fb-A_{2A}R-KO	CaMKII-α gene promoter-driven forebrain A_{2A}R KO
FJ-C	Fluoro-Jade C
FST	forced swimming test
g-A_{2A}R-KO	global A_{2A}R knockout mice
GFAP	glial fibrillary acidic protein
HM	helpless mice
HPA	hypothalamus-pituitary-adrenal
IMAO	irreversible monoamine oxidase (inhibitor)
ITC	intercalated cell masses
KA	kainate
KO	knock out
LA	lateral amygdala
LTD	long-term depression
LTP	long-term potentiation
M	medial nuclei of the amygdala
ML	medial-lateral
mPFC	medial prefrontal cortex
MWM	Morris water maze
NHM	non-helpless mice
NMDA	N-methyl-D-aspartate
NRI	noradrenalin reuptake inhibitor
OD	object displacement test
OF	open field
OR	object recognition test
PBS	phosphate buffer saline
PD	Parkinon’s disease
PFC	prefrontal cortex
PSD95	postsynaptic density protein 95
PTSD	post-traumatic stress disorder
PVDF	polyvinylidene difluoride
s.c.	subcutaneous
S.E.M.	standard error of the mean
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
siRNA	small interference RNA
SNAP25	Synaptosomal-Associated Protein, 25kDa
SNARE	Soluble NSF Attachment Protein Receptor
SNRI	serotonin and noradrenalin reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline/Tween-20
TCA	tricyclic antidepressant
TeCA	tetracyclic antidepressant
TEMED	tetramethylethylenediamine
TST	tail suspension test

vGAT	vesicular GABA transporter
vGluT1	vesicular glutamate transporter type 1
VTA	ventral tegmental area
WT	wild type
XAC	8-{4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl} xanthine
YM	Y maze

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ABSTRACT

Adenosine is a nucleoside able to modulate neurotransmission, in particular glutamatergic transmission, and it has been shown to be important in the processing of memory. Caffeine, a non-selective antagonist of adenosine receptors, alleviates memory impairment in neurodegenerative diseases and selective antagonists of adenosine A_{2A} receptors ($A_{2A}R$), in particular, have proved to be beneficial in animal models, modulating LTP, rescuing synaptic markers and improving memory-related behavior. This ability of caffeine and $A_{2A}R$ antagonists to normalize memory paved a growing interest in other brain dysfunctions associated with synaptic impairment, such as mood disorders.

Depression will become the leading cause of disability and it substantially impairs the life of patients and of their families. Moreover, it represents a high socio-economical burden worldwide. Although an array of antidepressants is available, their efficacy is limited, and they have side effects. Epidemiologic studies show that chronic coffee consumption (and not decaffeinated beverages) is inversely related to the incidence of depression and suicide, which prompts further exploring if there is a causal effect and what mechanisms underlie the beneficial effects of caffeine. In pre-clinical studies, the chronic antagonism selectively of $A_{2A}R$ yields beneficial effects more consistently than the antagonism of other adenosine receptors.

In this work, we tested the impact of caffeine and genetic depletion of $A_{2A}R$ (knock out (KO) mice) in genetic and behavioral (stress) animal models of mood disorders.

The H/Rouen mice ('helpless mice') were selectively bred to present higher immobility (despair behavior) in the forced swimming test, a test that assesses antidepressant effects. These animals presented depressive-like behavior in the forced swimming test (FST) and tail suspension test (TST), and memory impairment in the object recognition test (OR). The chronic intake of caffeine (0.3 g/L in drinking water during 4 weeks) did not revert behavior differences in the FST and TST, but did recover memory function in the OR. Accordingly, caffeine intake also reversed the lower density of synaptic markers observed in the hippocampus of 'helpless' mice.

Chronic unpredictable stress (CUS), applied daily, for 3 weeks, to C57/Bl6 mice, caused both memory impairment in the two-trial Y-maze (YM), increased anxiety- (elevated plus maze, EPM) and depressive-like behavior (FST). In this behavioral model of depression, $A_{2A}R$ levels in synaptic membranes of the hippocampus were higher than controls, while A_1 receptor density was unchanged, which suggests a prominent role of $A_{2A}R$. The genetic KO of $A_{2A}R$ prevented both memory-related and despair-related behavioral changes, as well as the decrease of synaptic markers in the hippocampus. Notably, knocking down $A_{2A}R$ selectively in neurons of the forebrain (which includes the cortex, the

hippocampus and the amygdala) (fb-A_{2A}R-KO mice) also afforded the same beneficial effects, showing that the blockade of neuronal A_{2A}R in the forebrain is sufficient to mimic the effects of the global A_{2A}R blockade.

One of the strongest hypotheses for the hippocampal dysfunction in depressed individuals and animal models is excitotoxicity caused by over-activation of glutamate receptors in excitatory neurons. Since A_{2A}R facilitate glutamate transmission, we injected kainate (s.c.) in fb-A_{2A}R-KO mice. Kainate triggered convulsions and a subsequent cell death through glutamate-mediated excitotoxicity. Notably, fb-A_{2A}R-KO mice presented a similar intensity of convulsions but a lower occurrence of cell death and lower glial reactivity in the hippocampus; this suggests that the absence of neuronal A_{2A}R prevents memory deficits in depressive states through protection against glutamate-mediated excitotoxicity.

Finally, the effects of blocking A_{2A}R selectively in neurons of the basolateral nuclei of the amygdala (BLA) were assessed. The amygdala is pivotal in processing emotional responses to external stimuli and is reported to be over-active in depressed individuals and to have increased dendritic arborization in animal chronic stress models. We selected 3 stress models widely used (physical stress, social defeat and immobilization; male Wistar rats) and ran behavioral tests for memory (YM), anxiety-like (EPM) and depressive-like (FST) behavior. Immobilization stress showed the most consistent behavioral changes and was thus selected for the next step. In that step, we injected, into the BLA of stressed and control animals, a lentiviral vector enconding for a short hairpin interference RNA that decreases A_{2A}R availability in neurons (shA_{2A}R), or a control virus (shCTR). Stressed animals displayed memory deficits in the YM, increased anxiety-like behavior in the EPM and increased depressive-like behavior in the FST. The stressed animals treated with shA_{2A}R presented anxiety and depressive-like behavior similar to controls (i.e., non-stressed), but memory impairment was not rescued by the ablation of A_{2A}R in amygdalar neurons.

Overall, the data collected in this work show that A_{2A}R play an important role in the progression of two features of depression: impaired memory, related to hippocampal function, and “anxious” and “depressed” mood, more closely related to amygdalar function. Blocking A_{2A}R, especially in neurons, showed beneficial effects in both brain structures and their behavioral outcomes, probably in independent ways.

The relevance of adenosine receptors in other brain structures involved in the progression of depression should be studied in order to clarify how the adenosinergic system can modulate the neural networks involved in mood disorders.

RESUMO

A adenosina é um nucleosídeo capaz de modular a neurotransmissão, principalmente a transmissão glutamatérgica, com impacto no processamento da memória. A cafeína, um antagonista não seletivo de recetores de adenosina, melhora a performance mnemónica em doenças neurodegenerativas e, em particular, os antagonistas dos recetores de adenosina A_{2A} ($A_{2A}R$) mostraram benefícios em modelos animais, modulando a plasticidade sináptica (LTP), resgatando marcadores sinápticos e melhorando o comportamento. Recentemente, tem havido um crescente interesse em compreender o papel da adenosina em distúrbios de humor.

A depressão tornar-se-á a maior causa de incapacidade e representa um custo financeiro elevado para muitas economias. Apesar de existir uma gama de antidepressivos disponíveis, a sua eficácia é limitada e os seus efeitos secundários restritivos. Estudos epidemiológicos mostram que o consumo de café (mas não o de bebidas descafeinadas) está inversamente relacionado com a incidência de depressão e suicídio, o que motiva mais estudos que explorem se existe causalidade e que mecanismos subjazem os efeitos benéficos da cafeína. Em estudos pré-clínicos, o antagonismo crónico dos $A_{2A}R$ produz efeitos benéficos mais consistentementes que antagonistas dos outros recetores de adenosina.

Neste trabalho, testámos a cafeína e a depleção genética dos $A_{2A}R$ (murganhos *knock out* (KO)) em modelos animais genéticos e comportamentais (estresse) de distúrbios de humor. Os murganhos H/Rouen (‘*helpless mice*’) foram gerados seletivamente para alta imobilidade (comportamento de desamparo) no teste do nado forçado, um teste que avalia efeitos antidepressivos. Estes animais apresentaram comportamento tipo depressivo no teste do nado forçado (FST) e no teste da suspensão pela cauda (TST), e prejuízo de memória teste de reconhecimento de objetos (OR). A administração crónica de cafeína (0,3 g/L na bebida, 4 semanas) não reverteu as diferenças comportamentais nos testes FST e TST, mas recuperou a função mnemónica no OR. Coerentemente, os murganhos H/Rouen apresentaram menor densidade de marcadores sinápticos no hipocampo, facto que foi revertido pela toma de cafeína.

O estresse crónico imprevisível (CUS), diário, por 3 semanas, em murganhos C57/Bl6, causou prejuízo de memória no teste modificado do labirinto em Y (YM), um aumento do comportamento tipo ansioso (teste do labirinto em cruz elevado, EPM) e do tipo depressivo (FST). Neste modelo comportamental de depressão, os níveis dos recetores $A_{2A}R$ em membranas sinápticas do hipocampo encontrava-se mais elevado do que nos controlos, enquanto os dos recetores A_1 se encontravam inalterados, sugerindo um papel relevante dos $A_{2A}R$ na depressão. A supressão genética global dos $A_{2A}R$ (animais KO)

preveniu tanto as alterações comportamentais relacionadas com a memória como as relacionadas com o desamparo. É importante notar também que a supressão seletiva de $A_{2A}R$ em neurónios do encéfalo frontal (que inclui o córtex, o hipocampo, e a amígdala) (fb-$A_{2A}R$-KO) conferiu os mesmos efeitos neuroprotectores e comportamentais, ou seja, bloquear apenas os $A_{2A}R$ neuronais no encéfalo frontal é suficiente para repetir os efeitos do bloqueio global.

Uma das hipóteses mais fortes para explicar a disfunção hipocampal é a excitotoxicidade causada pela sobre-activação de recetores de glutamato em neurónios excitatórios. Como os $A_{2A}R$ facilitam a transmissão glutamatérgica, injetámos cainato em murganhos fb-$A_{2A}R$-KO, causando um estado epilético e morte celular através de excitotoxicidade mediada pelo glutamato. Os murganhos KO apresentaram menor ocorrência de morte celular e menor reatividade glial no hipocampo, o que sugere que a ausência de $A_{2A}R$ em neurónios pode prevenir os défices de memória em estados depressivos através da proteção contra a excitotoxicidade mediada pelo glutamato.

Finalmente, foram estudados os efeitos do bloqueio dos $A_{2A}R$ em neurónios dos núcleos basolaterais da amígdala (BLA). A amígdala é fundamental no processamento de respostas emocionais a estímulos externos e encontra-se sobre ativada em indivíduos deprimidos e apresenta arborização dendrítica aumentada em modelos animais de estresse crónico. Nós escolhemos 3 modelos de estresse (estresse físico, derrota social e imobilização; ratos Wistar machos) e testámos memória (YM), comportamento tipo ansioso (EPM) e tipo depressivo (FST). O estresse de imobilização causou alterações comportamentais de forma mais consistente e foi selecionado para a fase seguinte. Nessa fase, injetámos, no BLA de animais posteriormente estressados ou controlos, um vetor lentiviral que codifica RNA de interferência "*short hairpin*" que diminui a densidade de $A_{2A}R$ em neurónios (shA2AR), ou vírus controlo (shCTR). Animais estressados mostraram défices de memória no YM, comportamento tipo ansioso no EPM, e tipo depressivo no FST. Os animais estressados injetados com shA2AR apresentaram comportamento tipo ansioso e depressivo ao nível dos controlos (i.e., não deprimidos) mas o défice de memória não foi resgatado pela remoção dos $A_{2A}R$ neuronais da amígdala.

Os resultados obtidos neste trabalho mostram que os recetores A_{2A} desempenham um importante papel na progressão de duas características da depressão: défice de memória, relacionado com a função do hipocampo, e humor ansioso e deprimido, em grande parte relacionado com a função da amídala. Bloquear os recetores A_{2A}, especialmente em neurónios, mostrou ter efeitos benéficos em ambas as estruturas cerebrais e nos seus efeitos comportamentais, provavelmente de forma independente.

A relevância dos recetores de adenosina em outras estruturas cerebrais envolvidas na progressão da depressão deverá também ser estudada de forma a clarificar como o sistema adenosinérgico pode modular as redes neuronais envolvidas em distúrbios de humor.

1. INTRODUCTION

1.1 The adenosinergic system

1.1.1 Historical notes

Adenosine, a purine nucleoside, is a ubiquitous molecule involved in many important cellular processes such as nucleic acid synthesis, amino acid metabolism and cellular energetic status (Stone, 1985). It can act both as a homeostatic modulator in all eukaryotic cells and as a neuromodulator controlling the flow of information through neuronal circuits (Gomes et al., 2011). Drury and Szent-Gyorgyi (1929) first suggested a physiological role for extracellular adenosine, reporting its ability to decrease arterial blood pressure, dilate coronary arteries and increase sleep-like behavior. The study of purines was long focused on coronary function until Holton and Holton (1953) suggested a role in neuronal transmission by a purine nucleotide, adenosine 5'-triphosphate, ATP. This hypothesis was confirmed by Burnstock and colleagues (1970), who later proposed that specific receptors would modulate the effects observed (Burnstock et al. 1978). At about the same time, it was shown that electrical stimulation could release adenosine from brain tissue (Pull & McIlwayn, 1972) and that exogenous addition of adenosine could modulate neuromuscular transmission (Ginsborg & Hirst, 1972). It was subsequently found that adenosine could decrease the release of numerous neurotransmitters such as acetylcholine, dopamine and serotonin (Fredholm & Hedqvist, 1980; Harms et al., 1978; 1979; Ribeiro & Walker, 1975; Vizi & Knoll, 1976). Notably, adenosine controls the release of glutamate, the main excitatory neurotransmitter in the brain (Corradetti et al., 1984; Dunwiddie & Masino, 2001).

1.1.2 Adenosine production and metabolism

Adenosine can be produced both inside and outside cells, mostly due to the activity of nucleotidases. In the extracellular medium, the release of ATP is the trigger of adenosine formation. This involves the activity of NTPDases (nucleoside triphosphate diphosphohydrolases) promoting the extracellular hydrolysis of nucleotides triphosphate and diphosphate to nucleotides monophosphate, e.g., ATP and ADP (adenosine diphosphate) to AMP (adenosine monophosphate); subsequently, the enzyme ecto-5'-nucleotidase catalyzes the hydrolysis of monophosphate nucleotides to their respective nucleosides, e.g., AMP to adenosine (Fig.1) (Zimmerman & Braun 1999). The rate of production of intracellular adenosine is controlled by the endo-5'-nucleotidase and depends on AMP concentration and the metabolic state of the cell. Adenosine can also be produced through the cleavage of S-adenosylhomocysteine by the enzyme S-adenosylhomocysteine hydrolase (SAH). This reaction occurs intracellularly and its balance depends on the concentrations of adenosine, homocysteine and S-adenosihomocysteine (Lloyd & Schrader, 1993).

Adenosine can also be directly released by crossing the membrane through nucleoside transporters. The main transporters have high affinity for adenosine

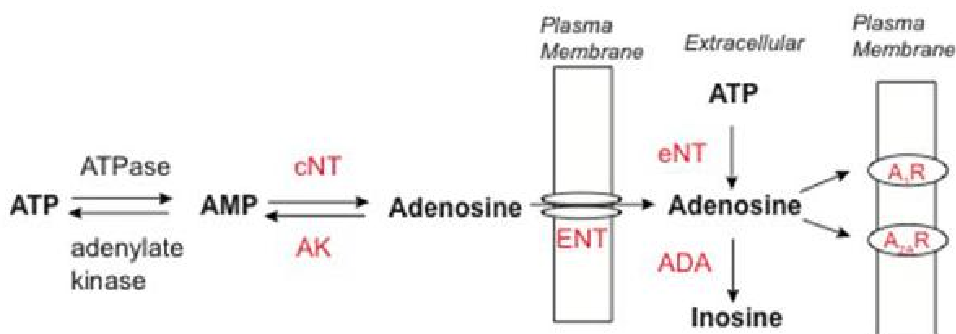

Figure 1: Scheme showing adenosine production. Abbreviations: cNT: cytosolic endo-nucleotidase, AK: adenosine kinase, ENT, equilibrative nucleoside transporters, eNT: ecto-nucleotidase, ADA: adenosine deaminase, A_1R and $A_{2A}R$: adeonsine A_1 and A_{2A} receptors.
in: Ruby et al., 2011

(100 μM) and are equilibrative (Gu et al., 1999), thus transporting adenosine

according to the gradient of concentration across the plasma membrane. These nucleoside transporters are abundant in the brain, both in neurons and astrocytes (Alanko et al. 2006). Some transporters participate in the uptake, transporting adenosine to the intracellular medium along with Na^{2+} (Smith et al., 2004; Smith et al., 2007)). The typical intracellular concentration of adenosine is approximately 50 nM (Cunha, 2001).

Adenosine can be phosphorylated to AMP by adenosine kinase (AK) or metabolized to inosine by adenosine deaminase (ADA). Phosphorylation of adenosine occurs mostly intracellularly but AK has also been found in cell membranes (Brundege et al., 1997; Ralevic & Burnstock, 1998). ADA is found inside most cells and is most active when adenosine is present in high concentrations (Fredholm et al., 1982). In the brain, AK is found mostly in astrocytes, regulating the basal levels of extracellular adenosine (Studer et al., 2006). Thus, there is a need for astrocyte-neuron coupling in the control of adenosine metabolism.

This entire enzymatic cascade regulates the availability of several nucleosides (adenosine, AMP, ADP and ATP) intra- and extracellularly, controlling the purinergic transmission in the brain and the activation of purinergic receptors (Zimmermann, 2000).

1.1.3 Adenosine receptors

Purinergic receptors can be divided in 3 separate classes: P1, P2X and P2Y.

P2X receptors are ligand-gated ion channels that bind to ATP. P2Y receptors are metabotropic G protein-coupled receptors that bind different nucleotides, e.g., ATP, ADP and UTP. P1 receptors are adenosine receptors.

Adenosine receptors are G protein-coupled receptors, with seven transmembrane domains, and divided in 4 subtypes: A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2001). A_1, A_{2A} and A_3 receptors can be activated by physiologic concentrations of adenosine (EC_{50}: A_1, 0.31 μM; A_{2A}, 0.7 μM; A_3, 0.29 μM) while A_{2B} require pathophysiological concentrations (EC_{50}: 24 μM) (Fredholm et al., 2001). Classically, the primary effector of all 4 subtypes of

receptors is adenyl cyclase, but the signaling mechanisms operated by the different adenosine receptors are still unclear (Fredholm et al., 2005, 2007).

A_1 receptors (A_1R) are the most abundant adenosine receptors in the central nervous system (CNS) where they are mainly localized in the cortex and limbic system but also in other regions, like cerebellum, Raphe nuclei, diencephalon and brainstem (Fastbom et al. 1987). These receptors are most abundant in neurons, both pre- and post-synaptically (Tetzlaff et al., 1987; Rebola et al., 2003). A_1R mediate inhibitory signals via $G_{i/o}$ proteins, inhibiting adenyl cyclase and increasing phospholipase C activity, resulting in decreased levels of cAMP (Abbracchio et al., 1995; Jacobson & Gao, 2006).

A_{2A} receptors ($A_{2A}R$) are widely distributed in the periphery and in the CNS. In the CNS, they are mainly found in the striatum, nucleus accumbens and olfactory tubercle but also in the cortex and limbic system (Cunha, et al., 1994; Lopes, et al., 2002). $A_{2A}R$ activate adenyl cyclase via G_s/G_{olf} proteins with the resulting increase in cAMP (Kull et al., 2000; Jacobson & Gao, 2006).

A_{2B} and A_3 receptors ($A_{2B}R$ and A_3R) are present in much lower density and the manipulation of their function has little impact in the brain, which led to the idea that adenosine modulates brain function mostly through A_1R and $A_{2A}R$ (Gomes et al., 2011). $A_{2B}R$ increase cAMP levels through G_s proteins while A_3R decrease those levels through G_{i3}/G_q proteins (Abbracchio et al., 1995b; Linden et al., 1999).

1.1.4 Adenosine in the central nervous system

Adenosine has a modulatory role on nerve activity by fine-tuning the release of neurotransmitters, the post-synaptic responsiveness and the action of other receptors. The overall modulatory role of adenosine in the CNS depends on the balance between A_1R and $A_{2A}R$ actions (Fredholm et al., 2005). Both receptors can be present in the same synapse: in the hippocampus, for example, 80% of nerve terminals with $A_{2A}R$ are also endowed with A_1R (Rebola et al., 2005), and $A_{2A}R$ play a major role in controlling A_1R through intracellular transducing

systems (Dixon et al., 1997, Lopes et al., 1999) or through receptor heteromerization (Ciruela et al., 2006a).

A_1R inhibit synaptic transmission by decreasing the release of several neurotransmitters: glutamate, acetylcholine, serotonin, dopamine and others (Ambrósio et al., 1997; Cunha et al., 2002; Okada et al., 1996; Okada et al., 2001; Proctor & Dunwiddie, 1987). Postsynaptically, A_1R inhibit NMDA-mediated currents, voltage sensitive calcium channels and increase potassium conductance (Ambrósio et al., 1997; de Mendonça et al., 1995; Trussell & Jackson, 1985)

On the contrary, $A_{2A}R$ facilitate the release of several neurotransmitters, including glutamate (Cunha & Ribeiro, 2000; Lopes et al., 2002; Shindou et al., 2002), acetylcholine (Rebola et al., 2002), dopamine (Navarro et al., 2009), serotonin (Okada et al., 2001) and GABA (Cunha & Ribeiro, 2000). Pre-synaptically, $A_{2A}R$ also play an additional role of shutting down A_1R- and cannabinoid CB1-mediated inhibition (Ciruela et al., 2006a; Ferreira et al., 2015; Lopes et al., 1999a). Postsynaptic $A_{2A}R$ can control glutamate NMDA (Rebola et al., 2008; Wirkner et al., 2004) and mGluR5 (Ferré et al., 2002) receptors, dopamine D2 (Ferré et al., 2008) and cannabinoid CB1 receptors (Andersson et al., 2005). Additionally, $A_{2A}R$ interacts with growth factors, particularly facilitating synaptic transmission mediated by brain derived neurotrophic factor (BDNF) (Diogenes et al., 2004). Adenosine can also modulate non-neuronal functions, both astrocytic and microglia-related, in brain tissue (Haselkorn et al., 2010; van Calker & Biber, 2005).

1.1.5 Adenosine and brain disorders

Extracellular levels of adenosine increase with neuronal activity (Mitchell et al., 1993), and increase to even higher levels when brain damage occurs (Latini & Pedata, 2001). This is probably a consequence of the increased usage of intracellular ATP, in an attempt to preserve cell viability, which leads to the formation of adenosine. The sources of extracellular adenosine are not yet clear,

however, data consistently show that noxious brain stimuli enhance the extracellular levels of adenosine (reviewed in Gomes et al., 2011).

A_1R control several events that are associated with ischemic damage, namely calcium influx, glutamate release, changes in membrane potential and cell metabolism (de Mendonça et al., 2000; Fredholm, 1997; Lubitz et al., 1995; Rudolphi et al., 1992). Indeed, A_1R agonists, inhibitors of adenosine re-uptake or metabolization (inhibiting adenosine kinase or adenosine deaminase) generally tend to decrease the extent of brain damage (Fowler, 1993; Kobayashi et al., 1998; Phillis et al., 1988). In contrast, the blockade of A_1R tends to exacerbate ischemic brain damage (reviewed in de Mendonça et al., 2000). Importantly, these manipulations are effective only if made shortly in the temporal vicinity of the ischemic insult. The chronic activation of A_1R causes the opposite effect (Jacobson et al., 1996; von Lubitz et al., 1995), probably due to desensitization of the A_1R. Indeed, oxygen deprivation, together with high levels of adenosine and A_1R activation cause a rapid down-regulation of A_1R (Coelho et al., 2006; Hettinger et al., 1998). These studies indicate that A_1R play a key role as a gate-keeper of neuronal damage, acting as barrier to the initiation of brain damage upon noxious insults (Tomé et al., 2010).

In accordance with the opposite signaling of $A_{2A}R$, the pharmacological or genetic blockade of $A_{2A}R$ affords a robust protection against ischemic damage (von Lubitz et al., 1995; Monopoli et al., 1998; Phillis, 1995). The mechanisms underlying this protection, however, are not clear, and it is possible that only the chronic blockade offers a preventive and prophylactic protection against neuronal damage (de Mendonça & Cunha, 2010). The mechanisms proposed include the control of central inflammatory processes and glial reactivity (Gui et al., 2009; Melani et al., 2009), the permeability of the blood-brain barrier (Chen et al., 2008a; Chen et al., 2008b), the infiltration and fixation of peripheral myeloid cells (Yu et al., 2004) and the control of glutamate release (Carta et al., 2009; Dai et al., 2010). In fact, the involvement of glutamate-associated excitotoxicity in the etiology of different brain disorders (e.g., Dong et al., 2009) and the ability of A_1R and $A_{2A}R$ to control excitatory transmission prompts considering this neuromodulation system as a putative therapeutic target to manage different brain disorders.

In epilepsy, seizures have traditionally been viewed as an imbalance between excitatory and inhibitory transmission in brain circuits, where hyper-excitation or hypo-inhibition would result in an abnormal repetitive firing of the affected brain circuits (Meldrum & Rogawski, 2007). The levels of endogenous extracellular adenosine rise upon seizure activity and A_1R are enriched in excitatory synapses, where they inhibit glutamate release, decrease glutamatergic responsiveness and hyperpolarize neurons (Berman et al., 2000; During & Spencer, 1992). Accordingly, A_1R agonists attenuate seizure and/or convulsive activity in different animal models, whilst the acute administration of either non-selective antagonists of adenosine receptors (such as caffeine or theophylline) or selective A_1R antagonists enhance the duration and severity of seizures and/or convulsions (reviewed by Malva et al., 2003; Tomé et al., 2010). However, some studies have identified a decreased density and efficiency of synaptic A_1R in models of epilepsy (Ekonomou et al., 2000; Glass et al., 1996; Rebola et al., 2003).

Regarding $A_{2A}R$, they seem to be up-regulated upon noxious brain conditions (Cunha, 2005), and so does the particular source of adenosine activating $A_{2A}R$ (ATP-derived adenosine formed through the ecto-nucleotidase pathway) (Augusto et al., 2013; Cunha et al., 1996; Rebola et al., 2005). Moreover, $A_{2A}R$ blockade seems to afford beneficial effects in animal models of epilepsy: thus, genetic deletion of $A_{2A}R$, selective $A_{2A}R$ antagonists (D'Alimonte et al., 2009; El Yacoubi et al., 2008) or non-selective antagonists such as chronic caffeine administration (El Yacoubi et al., 2008) afford a robust protection against the evolution and severity of seizures. Furthermore, chronic caffeine administration or $A_{2A}R$ blockade effectively prevents neuronal damage following convulsions (El Yacoubi et al., 2008; Georgiev et al., 1993; Rigoulot et al., 2003).

The role of adenosine has also been widely studied in neurodegenerative disorders, like Parkinson's and Alzheimer's diseases. Particularly in Alzheimer's disease (AD), caffeine, has been shown to protect against the development of the disorder (e.g., Maia & de Mendonça, 2002; Ritchie et al., 2007). Post-mortem analysis of the frontal cortex of AD patients showed that A_1R and $A_{2A}R$ are significantly increased in either early or advanced stages (Albasanz et al., 2008). In a transgenic mice model of AD carrying the APP Swedish mutation (APPSw), it was also reported that the levels of A_1R and $A_{2A}R$ are augmented

as compared with non-transgenic mice (Angulo et al., 2003), the same occurring in animal models of sporadic dementia (Espinosa et al., 2013). Although data regarding A_1R are not as consistent, a significant reduction of A_1R binding in the temporal cortex and thalamus of AD patients was observed in comparison with elderly normal subjects (Fukumitsu et al., 2008). This reduction in the density of A_1R is in agreement with the decrease of A_1R density and efficiency in other neurodegenerative disorders (reviewed by Cunha, 2005). The up-regulation of $A_{2A}R$ prompts the hypothesis that the manipulation of these excitatory receptors may control neurodegeneration (Cunha, 2008). Accordingly, numerous studies show that the modulation of $A_{2A}R$ could have neuroprotective effects in AD. Studies *in vitro* showed that $A_{2A}R$ antagonism prevents synaptic loss and neuronal death triggered by Aβ synthetic peptides (Canas et al., 2009; Dall'Igna et al., 2003), as well as in transgenic mouse models of dementia (Laurent et al., 2014). Benefits for cognitive deficits in AD patients and AD animal models might be achieved by manipulating $A_{2A}R$, since these receptors facilitate the synaptic mechanisms associated with learning and memory (Cunha & Agostinho, 2010; Takahashi et al., 2008). Moreover, the activation of hippocampal $A_{2A}R$ is sufficient to trigger memory deficits (Li et al., 2015; Pagnussat et al., 2015).

The incidence of AD is inversely associated with the consumption of coffee in the previous two decades of life (Maia & de Mendonça, 2002) and an inverse relation was found between caffeine intake and age-related cognitive impairment and dementia (Eskelinen et al., 2009; Ritchie et al., 2007; Santos et al., 2010). In laboratory studies, either caffeine or selective $A_{2A}R$ antagonists prevented or delay memory deficits caused by intra-cerebral administration of Aβ (Canas et al., 2009; Cunha et al., 2008a). The protective effects of caffeine in AD animal models are not mimicked by antagonists of A_1R but rather by antagonists of $A_{2A}R$ (Dall'Igna et al., 2003; Dall'Igna et al., 2007).

The ability of caffeine and $A_{2A}R$ antagonists to control memory dysfunction under different pathological conditions (Cunha & Agostinho, 2010) indicates that $A_{2A}R$ may affect memory preservation in general rather than affecting selectively pathogenic mechanisms of AD.

1.1.6 Adenosine and glutamate excitotoxicity

Glutamate is an amino acid that, as a neurotransmitter, binds to three families of ligand-gated ion channels that are classified according to their exogenous selective agonist: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate, and NMDA (N-methyl-D-aspartate). In the brain, glutamate can play a key role in pathophysiology in a process known as “excitotoxicity”, whereby the over-activation of excitatory neurons leads to neuronal cell death (Olney, 1969).

The major initiators of the excitotoxic process are these glutamate ionotropic receptors, which are widely distributed in the brain and are highly permeable to calcium (Bonfoco et al., 1995). Glutamate excitotoxicity is implicated in several acute brain injuries and chronic neurodegenerative diseases like Parkinson’s disease and AD (Lipton & Rosenberg, 1994). In Parkinson’s disease, the loss of nigrostriatal dopamine leads to the dishinibition of striatal neurons, thus initiating glutamatergic over-reactivity (Reichmann et al., 2005). In AD, glutamate excitotoxicity seems to play a major role in neuronal damage, but how that relates to Aβ or tau protein, hallmarks of the disease, is unknown (Koutsilieri & Riederer, 2007). The over-activation of excitatory pathways caused by excessive glutamate release is also observed in other brain disorders like epilepsy (Villmann & Becker, 2007).

As referred above, A_1R inhibit whereas $A_{2A}R$ facilitate glutamate release. Thus, the activation of A_1R and/or blockade of $A_{2A}R$ can potentially counteract the excitotoxic effects of excessive glutamate release. Indeed, several studies have shown that $A_{2A}R$ are responsible for the release of glutamate in noxious conditions (Melani et al., 2003; O’Regan et al., 1992, Popoli et al., 2002).

1.2 The hippocampus

The hippocampus is one of the most studied brain structures. It is part of the limbic system and is located inside the medial temporal lobe of the cerebral

cortex, being therefore a part of the forebrain. The hippocampus and its associated medial temporal lobe structures are essential for the formation, consolidation and retrieval of episodic memories (Eichenbaum et al., 2000; Kim & Diamond, 2002; Morris et al., 1982; Squire & Zola, 1997). Rodent models of lesions to the hippocampus or adjacent structures (septum, fimbria/fornix or parahippocampus) have been described to present impaired spatial and working memory (O'Keefe & Conway, 1978).

The hippocampal formation is a bi-lateral limbic structure. The top is known as the “dorsal hippocampus” and the dorsal tip of the hippocampus is called the “septal pole”. The cross-section taken perpendicularly to the long axis (septal-temporal) reveals two interlocking “C”-shapes. These “C”-shaped cell layers interlock, leaning together at the top and spread apart at the base. One of the “C”s is the Ammon’s Horn of *Cornu Ammonis*, also known as “hippocampus proper”. The principal cell layer of the Ammon’s Horn is the *stratum pyramidale*, or the pyramidal cell layer. The other “C” is constituted by the dentate gyrus, of which the *stratum granulosum*, or granule cell layer, is the main cell layer. This cross section cut of the hippocampus also exhibits a strong afferent set of three connected pathways known as “trisynaptic circuit” or loop (Andersen et al., 1966; Swanson et al., 1978; Witter et al., 1989), which is represented by three subdivisions of the hippocampus: CA1, CA2 and CA3 areas. These areas represent a tri-synaptic excitatory circuit processing information. The connections within the hippocampus generally follow a laminar format and are generally uni-directional. They form well-characterized closed loops from and to the adjacent entorhinal cortex (Teyler & DiScenna, 1984). First, layers II and III or “surface layers” of the entorhinal cortex project to the granule cells of the dentate-gyrus, via the perforant path, the major input to the hippocampus, with minor contributions from the deeper layers IV and V. Second, the granule cells of the dentate gyrus project to the large pyramidal cells of *Cornu Amonnis*, subfield 3 (CA3), via the mossy fibers pathway. Third, CA3 pyramidal cells project to the pyramidal cells of the CA1 subfield, via the Schaffer collateral pathway (Amaral, 1978; Amaral & Witter, 1989; Blackstad, 1956; Blackstad, 1958; Witter et al., 1989).

This “trisynaptic circuit” is considered to be the main functional network of the hippocampus and is believed to be involved in neuronal information processing (Amaral & Witter, 1989). This information processing primarily results from a physiologic balance between a complex network of inhibitory GABAergic interneurons and excitatory glutamatergic neurotransmission. However, other

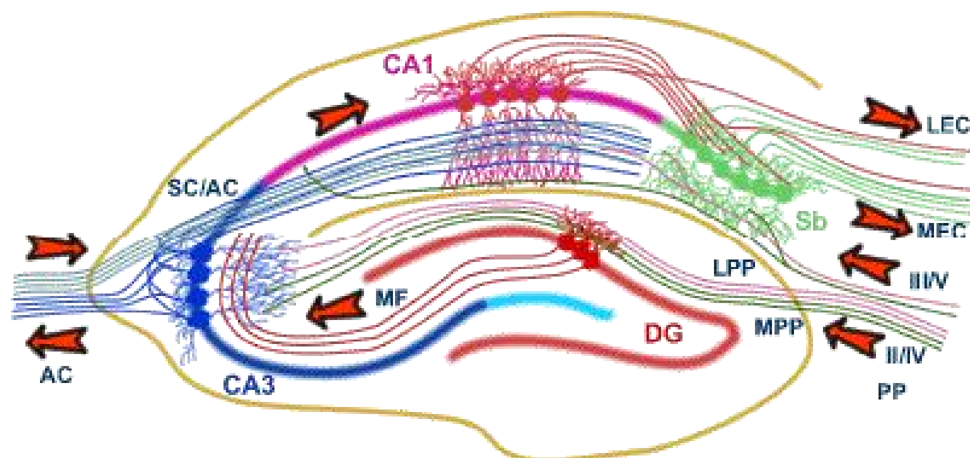

Figure 2: Anatomical representation of a dorso-septal section of the hippocampus and its main neural projections.
Legend: AC, associational commisural pathway; DG, dentate gyrus; EC, entorhinal cortex (L, lateral; M, medial); MF, mossy fibres; PP, perforant path (L, lateral; M, medial); Sb, subiculum; SC, Schaffer collateral pathway.
From: Bristol University (http://www.bristol.ac.uk/synaptic/pathways/)

neurotransmitters might play a major contribution in fine-tuning neurotransmission including acetylcholine, noradrenaline and dopamine (Kullmann, 2007).

Cholinergic afferents, mainly from the medial septum, facilitate the activity of both glutamatergic principal neurons and GABAergic interneurons in the hippocampus through the metabotropic muscarinic receptors and ionotropic nicotinic receptors (Cobb & Davies, 2005). In the hippocampus, noradrenaline, released from neurons projected from the locus coereleus, acts on α1 and α2 adrenergic receptors (Samuels & Szabadi, 2008) facilitating synaptic plasticity (Izumi & Zorunski, 1999). Dopaminergic inputs to the hippocampus originate primarily from the ventral tegmental area (VTA) and substantia nigra (Gasbarri et al., 1997; Scatton et al., 1980) and dopamine activates its D1/D5 receptors that modulate protein synthesis-dependent synaptic plasticity (Lisman & Grace, 2005).

1.2.1 Hippocampus, memory and adenosine modulation

The hippocampus is one of the main regions involved in memory formation (O'Keefe & Conway 1978). The involvement of the hippocampus in memory processes was discovered in 1957 when patient H.M. presented severe memory impairment after the removal of both hippocampi for the treatment of epilepsy (Scoville & Milner, 1957). Causing hippocampal lesions in rodents confirmed this conclusion, as animals presented space and context memory impairments (O'Keefe & Conway 1978).

Activity-dependent synaptic plasticity, e.g. long-term potentiation (LTP) and long-term depression (LTD), is regarded as a substrate for memory formation and provides a cellular basis for information storage in the CNS. Classically, in the hippocampus, LTP formation requires a sustained post-synaptic depolarization and glutamate NMDA receptors activation with increased intracellular calcium levels, causing the strengthening of synaptic transmission. LTD, on the contrary, is the process through which synaptic transmission is weakened and results from the activation of glutamate receptors in lower frequencies. Interestingly, NMDA receptors are also involved in LTD: lower activation of NMDA receptors results in lower influx of calcium, consequently activating intracellular cascades that make synapses less efficient (Martin & Morris, 2002; Mayford et al., 2012).

In the living organism, the storage of new information seems to require a balance between LTP and LTD, although different kinds of memories depend on LTP and LTD differently. However, the sufficiency and necessity of these processes to occur for new memories to be formed has not been clearly demonstrated yet (Martin & Morris, 2002; Martin & Shapiro, 2000).

Recently, Navabi and colleagues (2014) induced the extinction and recall of conditioned memory *in vivo* solely with the optogenetic delivery of LTD and LTP, respectively. Although this was shown in the amygdala, an equivalent process probably occurs in the hippocampus (see Liu et al., 2014). Other processes have also been proposed to be involved in memory formation as neurogenesis and neuronal excitability (Martin & Morris, 2002; Neves et al., 2008).

Regarding the adenosinergic system, it was first shown that A_1R inhibit NMDA receptor-mediated currents (de Mendonça et al., 1995). Accordingly, A_1R hamper long-term plastic changes, inhibiting LTP formation (de Mendonça & Ribeiro, 1994) and attenuating LTD and the related depotentiation in the hippocampus (de Mendonça et al., 1997). On the contrary, postsynaptic $A_{2A}R$ are necessary for the co-activation of NMDA receptors (Rebola et al., 2008), thus are necessary for the implementation of LTP in the hippocampus (Fontinha et al., 2008; Fujii et al., 1999).

1.3 The amygdala

The amygdala has been classically implicated in emotion; a large body of work has identified the amygdala as a critical component of the neural circuitry mediating emotional responses (reviewed in Davis & Shi, 2000; Janak & Tye, 2015; LeDoux, 1996; 2000; McGaugh, 2004; Sah et al., 2003). The amygdala serves two purposes. Firstly, it modulates memory formation by emotions (Janak & Tye, 2015). Emotionally charged experiences are often better remembered (McGaugh, 2004; Paré, 2003), suggesting that the emotional responses that accompany an event determine how strong the memory for that event will be. There is much evidence suggesting that peripheral stress hormones released during emotional arousal are important for the facilitation of memory formation and that the amygdala mediates the enhancing effects of stress hormones on memory (McGaugh, 2000). Secondly, the amygdala is a crucial site of synaptic plasticity for the formation of Pavlovian fear memories, in which animals learn to predict danger from cues in their environment (LeDoux, 2000). In recent years, the amygdala has been implicated in several non-classical roles including reward-related behaviors, feeding, anxiety and social behavior (reviewed by Janak & Tye, 2015).

The circuitry and function of amygdala are well-conserved across evolution (Medina et al., 2011). Even non-mammalian species such as reptiles and birds have an amygdala-like brain region with similar circuits and functions to the amygdala in mammals (Jarvis et al., 2005; Kappers et al., 1936).

Anatomical organization of the amygdala

The amygdala is a structurally heterogeneous group of nuclei (around 12 different regions) located in the medial temporal lobe (LeDoux 2000; Sah et al., 2003). Based on morphology, histochemistry and connectivity analyses, the amygdala can be divided into three main groups of nuclei: 1) the basolateral complex (BLA), comprised of the lateral (LA) and basal amygdala (BA: basolateral (BL) and basomedial (BM)); 2) the centro-medial group (central (CeA) and medial (M) nuclei); 3) the cortical-like group (composed of 4 several cortical nuclei and the nucleus of the lateral olfactory tract). There are also components of the amygdala that do not fall in any of these groups such as the anterior amygdaloid area and the intercalated cell masses (ITC) (Davis & Shi, 2000; McDonald, 1992; Paré et al., 2003; Pitkänen et al., 2000; Sah et al., 2003). Because part of the work presented in this thesis focuses on the BLA the following will be restricted to the BLA and its projections to the central nuclei.

The amygdala receives inputs from the thalamus (primary sensory information), cerebral cortex (sensory and polymodal information), various modulatory cell groups of the basal forebrain and brainstem (Pitkänen et al., 2000; Sah et al., 2003). On the reverse, the amygdala has widespread projections to the cortex, striatum, thalamus, hypothalamus, and brainstem. The BLA in paticular has considerable projections to the medial temporal lobe memory system (including perirhinal cortex, enthorinal cortex, and the hippocampus), the prefrontal cortex and the striatum (McDonald, 1998). On the other hand, it has little brainstem projections. In contrast, CeA does not project to the cerebral cortex or striatum but projects extensively to the brainstem (Medina et al., 2011).

There is a widespread intra-amygdalar connectivity. Extensive inter-nuclear connectivity determines the flow of information within the amygdala that generally follows the direction of the inter-nuclear projections. The LA is the main recipient of inputs from the thalamus and cortex. The sensory information is processed locally and then relayed ventrally (to BL and BM) and medially (to CeA). Classically, the BLA is considered as the input station for sensory inputs whereas the CeA is considered as the output station of the amygdala for fear responses. Consistent with this, the CeA does not project back to the BLA. The CeA can be divided into two subregions: the lateral subnucleus (CeL) and the medial subnucleus (CeM). The flow of information within the CeA follows a lateral to medial direction, as well, with the CeM being the output nucleus to downstream brain regions. Besides the direct projections from the BLA to CeA, the information also flows indirectly via the intercalated cells (ITC) that act as an inhibitory interface between BLA and CeA (Paré et al., 2003).

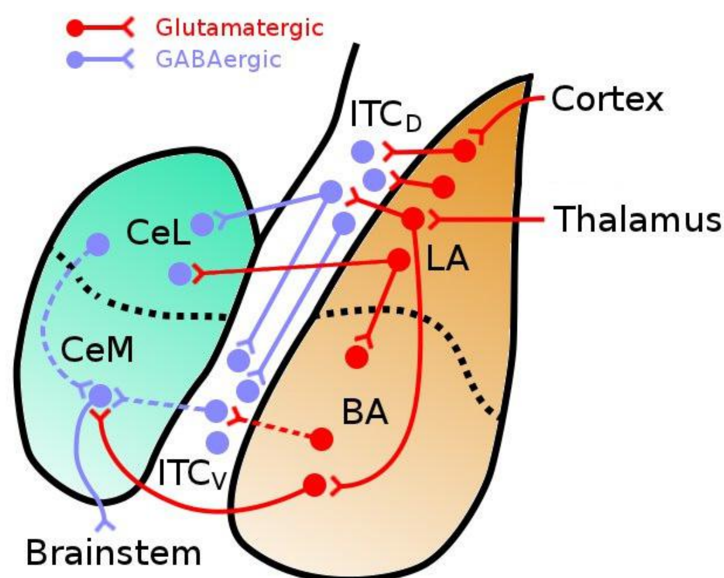

Figure 3: Representation of the amygdala in rats and partial internal and external connectivity (relevant in classical fear conditioning). Nuclei: CeL, lateral central; CeM, medial central; ITC, intercalated cell masses (D, dorsal; V, ventral); BA; basal amygdala; LA, lateral amygdala.
(image adapted from Nair, 2012)

1.4 Depression

Mood disorders are severe, chronic and frequent, and result in serious alterations to the emotional state of the individual. They include unipolar disorders, like major depression and dysthymia (or melancholic depression), and bipolar disorders, which alternate depressive phases with manic phases (American Psychiatric Association, 2006).

Depression is a known illness in many historical civilizations, but usually attributed to evil spirits or supernatural phenomena. Hippocrates, in the fifth century B.C., was the first to propose that depression, at the time termed “melancholia”, was an illness of the body. His description of melancholia was as follows: “aversion to food, despondency, sleeplessness, irritability, restlessness” and “fear or depression that is prolonged” (Jackson, 1990). These symptoms are used nowadays in the diagnosis of depression.

Major depressive disorder is one of the most frequent neuropsychiatric disorders in the world and is expected to be the main cause of disability by 2030 for both men and women (World Health Organization, 2004).

Depressive disorders present a high recurrence rate. After the first depressive episode, the probability of a second episode increases by 50% and a third by 80% (American Psychiatric Association, 1994). Patients with major depression have on average 5 to 9 episodes during their lifetime (Kessler et al., 1997; Kessler & Walters, 1998). The disability caused by this disorder represents not only great suffering to the individual and family (Saarni et al., 2007; Ustun, 2000), but also a high cost to governments (Andlin-Sobocki & Wittchen, 2005; Berto et al., 2000; Smit et al., 2006).

The symptoms of depression include psychological, behavioral and somatic aspects that present high comorbidity with other psychiatric disorders, like anxiety, cause lower productivity, and represent the main risk for coronary diseases and cerebral ischemia; moreover, depressive disorders are associated with clinical conditions like obesity and neurodegenerative diseases (Bornstein et al., 2006; Lemke, 2008). Additionally, the incidence of suicide is 20 times greater in depressed individuals when compared to the rest of the population (Harris & Barraclough, 1997).

According to the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5), which is used worldwide, the following criteria apply to major depression. Firstly, five or more of the following symptoms have been present during the same 2-week period and represent a change in the person's condition:

1. Depressed mood most of the day, nearly every day, as indicated by either a subjective report (e.g. feeling sad or empty) or observation made by others (e.g., appears tearful).

2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day, as indicated by either subjective account or observation made by others.

3. Significant weight loss (when not dieting) or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day.

4. Sleeping disturbance (insomnia or hypersomnia nearly every night).

5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).

6. Feeling of fatigue or loss of energy nearly every day.

7. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).

8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (as indicated either by subjective account or as observed by others).

9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or have a specific plan for committing suicide.

The symptoms are not better explained by other psychiatric conditions and must cause clinically significant suffering or impaired function at work, in social situations or in other important respects. At least one of symptoms 1 or 2 must be present (Nestler & Hyman, 2010).

Clearly, the diagnosis of depression depends on subjective criteria, which are highly variable and sometimes contrasting.

1.4.1 Etiology of depression

The etiology of the disorder is still not well understood. Genetic epidemiological studies have revealed that heritability of major depression is between 31% and 42% (Sullivan et al., 2000), but gene loci that contribute significantly to the disease have not been identified (Bosker et al., 2011). Furthermore, the heritability of major depression is considerably lower compared with other psychiatric disorders such as schizophrenia and bipolar disorder, which show heritability rates of 80% (Cardno et al., 1999). Epidemiological evidence also links environmental factors, especially exposure to stressful life events with an increased risk for depression (Hammen et al., 2005; Keller et al., 2007). However, there is a remarkable individual variability in the vulnerability to environmental stress, with on the one hand, many psychiatric disorders associated with a history of stress and, on the other, most individuals showing normal outcomes despite stress exposure (Dudley et al., 2011). Recent work has indicated that stress exposure may interact with genetic risk factors to increase susceptibility to depression (Caspi et al., 2003; Kaufman et al., 2006).

1.4.2 Depression and stress

Stress is a mechanism that allows the body to react and adapt to a challenging or potentially noxious stimulus in order to maintain homeostasis. These stimuli (stressors) can be external, physical or psychological and initiate actions in the body named by Hans Selye as “general adaptation syndrome” (Seltzer, 1952).
Stress is, however, a diverse entity that can be classified in different manners: experienced once or repetitively, acute or chronic, predictable or unpredictable, mild or severe, isolated or in context, etc. (Lucassen et al., 2014).
The physiological response to a stressful stimulus is divided in two time frames: fast response and delayed response. The quick response, often referred to “fight-or-flight” response, involves the response of the autonomous nervous system, the release of epinephrine and norepinephrine into the bloodstream, and the consequent elevation of heart rate, blood pressure, respiration and

basal metabolic rate (Lucassen et al., 2014). The delayed response involves the additional activation of the hypothalamus-pituitary-adrenal (HPA) axis. The HPA axis is a primary regulation system in stress response, which integrates neurological functions and sensory stimuli with an endocrine function (Smith & Vale, 2006): after a limbic and cortical stimulus, the hypothalamus releases corticotrophin-releasing hormone (CRH) into the pituitary microcirculation; CRH induces the pituitary gland to release adrenocorticotrophic hormone (ACTH) into the blood stream that acts on the adrenal glands' cortex promoting the synthesis and release of glucocorticoids; both glucocorticoids and catecholamines, produced in the adrenal glands and released in response to stress, can induce the increase of blood pressure, heart rate and activation of the immune system, among others (de Kloet et al., 2005). The adrenal glucocorticoids produce a negative feedback response mainly through the hypothalamus, ultimately suppressing the release of CRH and ACTH, which leads to the termination of the stress response once the stressor is not present (Aguilera et al., 2007; Swaab et al., 2005).

Among brain structures contributing to the stress response, the limbic system plays a pivotal role, in particular, the amygdala, the hippocampus and prefrontal cortex (PFC). These structures are interconnected and exhibit bi-directional feedback that can be altered by stress and has been reported to be changed in depressed individuals (Davidson et al., 2002).

The amygdala plays an important role modulating autonomic and neuroendocrine systems. Lesions in the BLA and the medial amygdala dampen the HPA axis responses to psychological stressors, e.g. restraint stress (Bhatnagar et al., 2004). The hippocampus, on the contrary, inhibits the HPA axis (Herman et al., 2003). Lesions in the hippocampus increase the stress-induced glucocorticoid secretion and are more evident in the recovery phase of stress (Herman et al., 2003) and after psychogenic, but not systemic, stressors (Herman & Mueller, 2006). The control of the hippocampus on the autonomic system (i.e. decreasing heart rate, blood pressure and respiratory rate) is mediated by the medial prefrontal cortex (mPFC) (Ruit & Neafsey, 1988). Like the hippocampus, the mPFC, especially the prelimbic region, inhibits the HPA axis and the autonomic responses to psychogenic stressors, in particular in the recovery phase (Radley et al., 2006). It is important to note that these limbic

regions have little anatomical interaction with the primary peripheral stress effectors and information is relayed through other brain centers as the bed nucleus of the stria terminalis (BNST) and the hypothalamus, which integrate both stress excitatory (amygdala) and inhibitory (pre-limbic mPFC and hippocampus) information (Ulrich-Lai et al., 2009).

Crucially, after prolonged or intermittent exposure to stress (chronic or repeated stress, respectively) the neuronal control of the stress response changes, and the response to individual (acute) exposures does not result in a normal physiological response, a process known as allostatic load (McEwen and Stellar, 1993).

In the hippocampus and the mPFC, chronic stress causes a dendritic retraction and a reduction of spine density of apical neurons (Magariños & McEwen, 1995; Radley et al., 2008). In contrast, dendritic branching is increased in the BLA (Vyas et al., 2006). Taken together, these changes mean that the response to stress is altered after prolonged exposure to stressors, and indeed, there is a sensitization to stress responses that results in enhanced excitability of the HPA axis (Akana et al., 1992), partially explained by the over-activation of the BLA (Vyas et al., 2006) and the reduced action of the hippocampus and the mPFC on the negative feedback loop after exposure to stressors (Herman & Mueller, 2006).

Additionally, changes in the synaptic rearrangement of these structures imply behavioral changes beyond the HPA axis. For example, chronic stress causes hippocampus-related memory deficits (Kim & Diamond, 2002), amygdala-related fear-motivated behavior (Conrad et al., 1999) and PFC-dependent attention deficits (Liston et al., 2006).

The hallmarks of maladaptation of stress responses are largely overlapping with those of depressed individuals, including higher basal levels of cortisol in the plasma (Swaab et al., 2005), increased size of pituitary and adrenal glands, impaired negative feedback signaling of the HPA axis (Gold et al., 1986), and memory impairment (Ellwart et al., 2003).

1.4.3 Antidepressants

Pharmacological treatment with modern antidepressant drugs and several forms of psychotherapy, including cognitive therapy and interpersonal therapy, have a documented beneficial effect. The efficacy of each treatment depends greatly on the type of antidepressant class or cognitive therapy used (e.g., interpersonal, psychodynamic) and its interaction with different types of depression (Cuijpers et al., 2013). This thesis will mostly focus on pharmacological approaches to manage depressive-like emotional dysfunction.

Table 1 – List of some of the most prescribed antidepressants and their classes.

<table>
<tr><th>Antidepressant</th><th>Class</th><th>Notes</th></tr>
<tr><td>Tranylcypromine</td><td rowspan="2">IMAO inhibitor</td><td rowspan="2">First generation</td></tr>
<tr><td>Phenelzine</td></tr>
<tr><td>Imipramine</td><td rowspan="3">TCA</td><td rowspan="3">Inhibitors of serotonin and noradrenaline reuptake</td></tr>
<tr><td>Amitriptyline</td></tr>
<tr><td>Clomipramine</td></tr>
<tr><td>Fluoxetine</td><td rowspan="3">SSRI</td><td rowspan="3">Also useful in treating anxiety, PTSD, obesity and aggression</td></tr>
<tr><td>Paroxetine</td></tr>
<tr><td>Sertraline</td></tr>
<tr><td>Reboxetine</td><td>NRI</td><td></td></tr>
<tr><td>Venlafaxine</td><td>SNRI</td><td></td></tr>
<tr><td>Bupropion</td><td>DRI</td><td></td></tr>
<tr><td>Mirtazapine</td><td>TeCA</td><td rowspan="2">Agonists of serotonin receptors</td></tr>
<tr><td>Nefazodone</td><td></td></tr>
</table>

Legend: DRI; dopamine reuptake inhibitor; IMAO, irreversible monoamine oxidase (inhibitor); NRI, noradrenalin reuptake inhibitor; PTSD; post-traumatic stress disorder; SNRI, serotonin and noradrenalin reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor TCA, tricyclic antidepressant; TeCA, tetracyclic antidepressant;

Most antidepressants used nowadays act by changing the availability of monoamines. In the brain, monoamines (serotonin, dopamine and noradrenaline) are an important group of neurotransmitters, which are mainly

synthetized in neurons from the aminoacids thyptophan and tyrosine. The neurons synthesizing biogenic amines project from particular nuclei (e.g., Raphe nuclei, substantia nigra, ventral tegmental area and locus coereulus) and the neurotransmitter release occurs throughout most brain regions modulating the neuronal responses and ultimately several brain functions, as mood and behavior (Harmer, 2008; Kurian et al., 2011).

Monoamines have been related to depressive disorders for more than four decades since iproniazide, a drug used for treating tuberculosis, raised humor and euphoria in patients. On the other hand, reserpine, used in the treatment of hypertension, was reported to cause depressive mood in 25% of patients. Almost simultaneously, the research for anti-histaminic drugs led to the production of imipramine (López-Muñoz & Alamo, 2009). All these drugs were able to change the synaptic levels of monoamines, giving rise to the monoaminergic hypothesis for the etiology and treatment of depression, especially noradrenaline and serotonin (Coppen et al., 1972; Schildkraut, 1965). Examples of antidepressants and their mechanism of action are listed in table 1.

The monoaminergic hypothesis has some flaws. For example, it does not explain why some drugs yield an antidepressant effect without acting on monoaminergic systems, like amphetamine. Even more relevant is the fact that the rapid changes in the metabolism of monoamines caused by monoaminergic antidepressants are not compatible with the slow, chronic effect on the symptoms of the disorder (Baldessarini, 1984).

These discrepant data might explain why less than half the patients respond to antidepressant treatment and only 35-40% of drug-responsive patients achieve full remission of symptoms (Cassano & Fava, 2004). Not surprisingly, the approval for novel antidepressant drugs has been declining since the 1960s (Papakostas & Ionescu, 2014) and the search for new molecular targets is of great interest in the field.

1.5 Adenosine in stress and depression

Adenosine is involved in the regulation of important central mechanisms like memory and cognition, circadian cycle and anxiety (reviewed in Ribeiro et al., 2003). Thus, there is evidence of the adenosinergic system being involved in the etiology and treatment of some neurodegenerative and psychiatric conditions, like Parkinson’s disease, Huntington’s disease and schizophrenia (Blum et al., 2003; Lara et al., 2006; Simola et al., 2006). Moreover, pre-clinical and clinical studies showed that adenosine is also involved in the modulation of mood (reviewed in Cunha et al., 2008b).

Studies in humans have explored the relation between coffee intake and mood changes. Importantly, most of the effects of coffee on brain-related functions are mostly due to the effects of caffeine, since they are not mimicked by decaffeinated coffee or other drinks such as fruit juice (Smith, 2002). Moreover, the only known molecular targets of caffeine at physiological (i.e., nontoxic) doses are the A_1R and $A_{2A}R$, in which caffeine acts as a competitive antagonist (Ferré, 2008; Fredholm et al., 1999).

A role of adenosine receptors in the control of mood is suggested by observations that different therapeutic strategies used to control mood disorders have effects related to the adenosine modulation system (van Calker & Biber, 2005). In fact, electroconvulsive therapy and sleep deprivation are two types of treatments of mood disorders, both of which cause short-term and long-term adaptations of the adenosine neuromodulation system. There are short-term adaptive neuronal responses that are operated through inhibitory A_1R, namely in terms of the slow wave sleep (Basheer et al., 2001) and cerebral metabolic activity (Phillis & O’Regan, 2003). There are also more long-term adaptive changes, such as up-regulation of A_1R (Basheer et al., 2004; Yanik & Radulovacki, 1987) and possibly of $A_{2A}R$ (reviewed in Cunha, 2005).

The reason for the changes observed in the adenosine receptors’ expression are likely related to their control over synaptic plasticity (Dias et al., 2013), which is altered upon chronic stress and in depressive states (McEwen, 2007). Transient mild stress can enhance learning and memory (Luine et al., 1996); conversely, repeated stress impairs memory, reduces LTP and enhances LTD in the hippocampus and PFC of rodents, and causes a remodeling of synaptic

connectivity (de Kloet et al., 2005). The process is probably caused by the abnornomal excitatory transmission through a process called excitotoxicity. Excitotoxicity is a pathological process in which excessive glutamate transmission causes excessive depolarization of the postsynaptic neurons, in particular through excessive calcium influx, which leads to increased metabolic load, oxidative stress and ultimately apoptosis and autophagy (Dong et al., 2009). After stress, glutamate AMPA receptors are increased in the CA3 region of the hippocampus and when NMDA receptors are blocked, no dendritic atrophy is observed (de Kloet et al., 2005). This mechanism is common in archetypal neurodegenerative diseases as Alzheimer’s disease (AD) and Parkinon’s disease (PD) in which excitotoxicity plays an important role (Koutsilieri & Riederer, 2007). It has been shown that adenosine receptors are up-regulated in both AD and PD, and accordingly, the antagonism of these receptors, especially $A_{2A}R$, have beneficial effects on cognition and motor deficits and their respective neurological markers (Canas et al., 2009; Schwarzschild et al., 2006). Thus the antagonism of adenosine receptors in chronic stress and depression may yield a positive outcome through similar processes.

Looking at depression in particular, the consumption of low to moderate doses of caffeine (less than 6 cups of coffee per day) is well known to increase energy and attention and to decrease cognitive failures (reviewed by Takahashi et al., 2008), depressive symptoms and risk of suicide (Baethge et al., 2009; Smith, 2009). However, in large amounts, caffeine can act as a trigger of psychiatric symptoms, from anxiety to depression and even psychosis in both normal and vulnerable subjects (Broderick & Benjamin, 2004). Conversely, a variety of mood related symptoms are also triggered upon withdrawal from regular caffeine consumption, including anxiety, irritability, sleepiness, dysphoria, nervousness or restlessness (Juliano & Griffiths, 2004; Nehlig et al., 1992; Strain et al., 1994). More recently, compromised adenosine transport due to variation in nucleoside transporter gene SLC29A3 was suggested to enhance susceptibility to depression in women (Gass et al., 2010).

The effect of the adenosine neuromodulation system in depression is complex, especially due to its ability to modulate several other neurotransmission systems, such as dopaminergic, glutamatergic and serotoninergic as well as the

corticotropin system (Jégou et al., 2003; Okada et al., 2001; Scaccianoce et al., 1989).

Adenosine and its analogues were shown to cause a depressant-like response in behavioral despair models (Hunter et al., 2003; Minor et al., 1994; Woodson et al., 1998), an effect that was prevented by the administration of classical antidepressants (Kulkarni & Mehta, 1985). In contrast, other studies showed an antidepressant effect associated with adenosine administrated both systemically and centrally (Kaster et al., 2004; Kaster et al., 2005).

The strongest evidence supporting the relation between adenosine and depression in preclinical models came from manipulation of $A_{2A}R$. Available data suggests that $A_{2A}R$ antagonists might be novel antidepressants compounds (reviewed in Cunha, 2008b). The main evidence sustaining this hypothesis relies on the observation that the genetic depletion of $A_{2A}R$ results in an antidepressant-like phenotype in animal models (El Yacoubi et al., 2001; El Yacoubi et al., 2003). In addition, $A_{2A}R$ blockade relieves the early hippocampal modifications induced by stress (Cunha et al., 2006), one of the major environmental factors favoring the implementation of depressive states (de Kloet, et al., 2005).

Moreover, electroconvulsive treatment and tricyclic antidepressants (TCA) such as nortriptiline, chlorimipramine or desipramine can bind to adenosine receptors (Deckert & Gleiter, 1990) and dose-dependently reduce the activity of ecto-nucleotidases and, expectedly, the levels of extracellular adenosine in cortical synapses (Barcellos et al., 1998).

The contrasting anxiogenic and anxiolytic effects of the adenosinergic system is not surprising for the fact that A_1R and $A_{2A}R$ play opposite roles and have different relevance in acute and chronic situations (Cunha, 2005). However, it seems evident that the adenosinergic system is able to modulate mood states by functioning as a normalizing system and both too high and too low levels of activation can cause a failure in the organism to adapt and a predisposition to disease (Cunha et al., 2008b).

2. AIMS

- Analyze how the density of membrane adenosine A_1 and A_{2A} receptors changes in different animal models of emotional dysfunction
- Study the effects of inhibiting adenosine receptors on hippocampal function and related behaviors in animal models of mood disorders
- Study the effects of inhibiting adenosine receptors on amygdalar function and related behaviors in animal models of mood disorders
- Probe the role of neuronal A_{2A} receptors in neuroprotection in animal models

3. ANIMAL MODELS AND METHODS

3.1 Animals

Male Wistar adult (9-10 weeks old) rats were obtained from Charles River (Barcelona, Spain).

Male 'Helpless' and 'non-helpless' adult (11-12 weeks old) mice from the H/Rouen lineage were provided by Malika El Yacoubi and Jean-Marie Veaugois (University of Rouen, Mont-Saint-Aignan, France).

Global $A_{2A}R$ knockout mice (g-$A_{2A}R$-KO) were raised in-house and CaMKII-α gene promoter-driven forebrain $A_{2A}R$ KO (fb-$A_{2A}R$-KO) male adult (10-12 weeks old) mice were provided by Jiang-Fan Chen (Boston University School of Medicine, Boston, MA, USA). Both KO lines were raised based on mating of heterozygotes, in a C57BL/6 background (Shen et al., 2008, 2013)

The animals were maintained under a controlled environment (23 ± 2ºC, 12 h light/dark cycle, free access to food and water) in accordance with the EU (2010/63/EU) and FELASA guidelines, and they were approved by the *Direção Geral de Alimentação e Veterenária* and the Ethical Committee of the Center for Neuroscience and Cell Biology of the University of Coimbra (DGVA 0420/2011 and ORBEA 78-2013). All experiments were conducted between 9 a.m. and 5 p.m. Male Wistar rats were kept in pairs. g-$A_{2A}R$-KO mice and fb-$A_{2A}R$-KO mice (and respective wild type animals) were kept one per cage once experimental manipulations started.

3.2 Animal models of depression

3.2.1 'Helpless mice'

Jean-Marie Vaugeois, Malika El Yacoubi and colleagues selectively bred CD1 mice for high and low immobility in the tail suspension test (TST, detailed below). After 10 generations, 'helpless' mice (H/Rouen) spent more than 200 s immobile in the TST (a depression-related, 'despair'-like behavior) while 'non-helpless' mice (NH/Rouen) spent less than 7 s immobile in a 6-minute session. 'Helpless' mice also showed other behavioral patterns related to depression, like higher immobility in the forced swimming test (FST, described below),

reduced consumption of sucrose solution and changes in the sleep-wakefulness pattern (El Yacoubi et al., 2013). ‘Helpless’ mice responded to noradrenergic and serotonergic anti-depressants in the TST and FST (tests created for assessment of anti-depressant activity) (El Yacoubi et al., 2011).
Biochemically, ‘helpless’ mice display lower serotonergic tone, and higher corticosterone plasma levels, similar to what is observed in depressed patients (El Yacoubi & Vaugeois, 2007). The similarities between this model and features of depression in humans make this a suitable model of depression, which we exploited in chapter 4.1.

3.2.2 Chronic unpredictable stress

It has been reported that depressive disorders have an influence of around 25% from genetic factors and 75% from environment factors (Henn & Vollmayr, 2004). For that reason, stressful events during someone’s life have been identified as the main risk factor in the etiology of depression (Charney & Manji, 2004; Henn & Vollmayr 2004).
Chronic unpredictable stress is one of the most widely used animal models to mimic depressive-like symptoms. The exposure of rodents to varied stressors, both physical and psychological, induces a range of behavioral and physiological alterations like: anhedonia (Moreau et al., 1995), decreased exploratory and sexual behavior (D’Aquila et al., 1994), increased corticosterone secretion (Joels & Van Riel, 2004), and increased apoptosis and decreased neurogenesis in the hippocampus (Joels et al., 2004). Moreover, most of these changes are reverted by the chronic treatment with different classes of antidepressants (Bondi et al., 2008; D’Aquila et al., 1994; Willner, 1997).

Experimental procedure

The protocol used in this work is based on Lu et al. (2006) and was recently published by our group (Kaster et al., 2015). It consists in applying different kinds of stressors, physical and psychological, daily, for a total of 21 days. Those stressors are shown in Table 1. All animals were kept in individual cages.

Table 2 – Chronic unpredictable stress protocol (CUS)

Day	Stressor	Time
1	Damp bedding (24 h)	9 a.m. – 9 a.m.
2	Paired housing (1 h)	5 p.m. – 6 p.m.
3	Restraint stress (2 h)	10 a.m. – 12 p.m.
4	Cold bath (15ºC, 20 min)	10 a.m. – 10:20 p.m.
5	Inescapable shock (in a passive avoidance apparatus, 0.7 mA, 3 s duration, 5 x in 5 min)	4 p.m.
6	Apparatus exposure, no shock (1 h)	10 a.m.
7	Light/dark cycle inverted (24 h)	9 a.m. – 9 a.m.
8	Cage tilt (45º)	2 p.m. – 2 p.m.
9	Food and water deprivation (24 h)	2 p.m. – 2 p.m.
10	Access to empty water bottle (1h)	2 p.m. – 3 p.m.
11	Damp bedding (24 h)	9 a.m. – 9 a.m.
12	Paired housing (1 h)	10 a.m. – 11 a.m.
13	Restraint stress (3 h)	2 p.m. – 5 p.m.
14	Cold bath (15ºC, 20 min)	2 p.m.
15	Inescapable shock (in a passive avoidance apparatus, 0.7 mA, 3 s duration, 5 x in 5 min)	2 p.m.
16	Apparatus exposure, no shock (1 h)	9 a.m. – 10 a.m.
17	Food and water deprivation (24 h)	9 a.m. – 9 a.m.
18	Access to empty water bottle (1 h)	9 a.m. – 10 a.m.
19	Light/dark cycle inverted (24 h)	9 a.m. – 9 a.m.
20	Tilted cage (45º)	9 a.m. – 9 a.m.
21	Restraint stress (4 h)	9 a.m. – 1 p.m.

3.2.3 Social defeat

Social conflicts in humans are known to cause severe stress leading to serious psychological problems (Bjorkqvist, 2001; Brousse et al., 2008) and are widely regarded as a risk factor for the development of psychiatric pathologies such as depression and anxiety disorders (Chrousos, 2009). The findings that mice exposed to repeated social defeat showed reduced sucrose preference and social interaction has been interpreted as supporting the use of chronic social defeat in mice as an animal model for depression (Malatynska & Knapp, 2005; Tsankova et al., 2006). Moreover, in this model, social stress induces long-lasting, adverse physiological, behavioral and neuronal deficits, which resemble certain human psychopathologic features of depression and anxiety, including cognitive impairment (Yu et al., 2011).

Experimental procedure

The protocol is adapted from Miczek et al. (1979). Animals were housed for 10 min each day for 14 consecutive days with male aggressors, which were retired breeders; on each day, the experimental animal and aggressor animal were placed in a clean cage, and physical contact was permitted for 10 min. Following this period of contact, the experimental animal was housed in the home cage of the aggressor, physically separated by a metallic mesh that permits visual, olfactory and auditory contact. Every two days, the male aggressor was changed to avoid habituation.

3.2.4 Forced exercise

Physical stressors, like foot shock, have been widely used to mimic depressive-like symptoms in animals. While exercise has been widely correlated with improvement of cognitive function and rescue of depressive symptoms in both animals and humans (e.g., Carek et al., 2011; Patki et al., 2014), on the contrary, forcing animals to run on a treadmill decreases brain-derived neurotrophic factor (BDNF) levels in the hippocampus and increases basal

levels of corticosterone, two features related to depression, that are not observed in chronic voluntary exercise animals (Ke et al., 2011).

Experimental procedure

Animals were placed in a commercial apparatus (Med-Associates) consisting of a treadmill with a small electric grid located behind the treadmill. Animals were forced to run on the treadmill for 1 h each day for 14 days. The speed of the treadmill was controlled by a computer, and changed every day. If animals did not keep pace with the treadmill, they were pulled towards the electric grid, where they received a small electric shock. This stressor has psychological, physical, and painful components. It is both unpredictable and uncontrollable, both properties which are believed to enhance stress.

3.2.5 Immobilization stress

Some studies reported that the restraint stress model causes depressive-like behavior, impairment of learning and memory, down-regulation of BDNF (Haenisch et al., 2009), nerve growth factor and neurotrophin-3, alterations in synaptic plasticity markers (Bravo et al., 2009), and decrease in length and branching of apical dendrites of hippocampal CA3 pyramidal neurons (Watanabe et al., 1992). But some studies suggest that repeated application of the same stressor can lead to habituation of the stress response (Melia et al., 1994); for this reason, we have chosen an adapted, more severe, protocol to induce stress based on an imposed physical immobility (Vyas et al., 2002; Meyer et al., 2013). This stressor is purely psychological. It does not involve pain, illness, or physical exhaustion. Psychological stressors are particularly potent for generating stress responses in humans (Hammen, 2005).

Experimental procedure

Animals were placed in Decapicone immobilization bags (Braintree Scientific) for 4 h each day (9 a.m. – 1 p.m.). Each bag was secured around the tail, and the animals were placed in clean, bedding-free cages for the duration of the stress. Unstressed animals were handled daily (20 s/day) and did not have

access to food and water during the same 4-hour period in which stressed animals were under immobilization.

3.3 Caffeine administration through the drinking water

Caffeine is widely used by humans as a psychoactive drug, and it can also cross the blood-brain barrier of rodents (McCall et al., 1982). Its effects on the central nervous system have been studied in different neurological conditions (Gomes et al., 2011).

For chronic treatment with caffeine, a new solution was prepared every 2 days (caffeine is stable in water at room temperature, see Alvi & Hammami, 2011), in a concentration of 0.3 g per liter of drinking water, the equivalent to about 2 cups of coffee for humans (Costenla et al., 2010). At this dose, caffeine antagonizes mostly A_1 and A_{2A} receptors (Fredholm et al., 1999).

3.4 Kainate-induced convulsion model

Kainate (KA) is a potent agonist of ionotropic glutamate receptors that is often used to induce excitotoxic cell death (Choi, 1988; Cognato et al., 2010). For that reason it is used as a model to study glutamate-induced excitotoxicity. In rodents, peripheral injections of KA induce recurrent seizures and consequent degeneration of neuronal populations in different brain regions, mainly in the hippocampus and the amygdala (Ben-Ari et al., 1980; Coyle, 1983, Sperk et al., 1985). In the hippocampus, KA administration induces cell death especially of the pyramidal cells of the CA1 and CA3 regions and interneurons in the dentate gyrus (Coyle, 1983; Sperk et al., 1985; Tauck & Nadler, 1985).

<u>Experimental procedure</u>

The experiments were carried out in CaMKII-α gene promoter-driven forebrain A_{2A}R KO mice (fb-A_{2A}R-KO) and the respective wild type. These animals were previously characterized as displaying a selective elimination of A_{2A}R from principal neurons and interneurons of the forebrain (Shen et al., 2008; Yu et al.,

2008). Animals were either injected subcutaneously (s.c.) with 0.9% saline or 35 mg/kg of kainate (Tocris, Cookson, Bristol, UK) in saline. After the injections, the animals were placed in individual cages kept at 30ºC and observed for 2 h to score the kainate-induced convulsions according to the Racine scale adapted to mice, as described by Schauwecker and Steward (1997), (Table 2). The animals were sacrificed 24 h later for histochemical analysis.

Table 3 – Convulsions scale for mice after kainate injection (from Schauwecker & Steward (1997), adapted from Racine (1972))

Level	Description
1	Immobile and crouching
2	Stretches body out, tail becomes straight and rigid, ears laid back, bulging eyes
3	Repetitive head bobbing, rears into a sitting position with forepaws resting on belly
4	Rearing and falling, tonic clonic seizures broken by periods of total stillness, jumping clonus, running clonus
5	Continuous level 4 seizures
6	Body in clonus, no longer using limbs to maintain posture, usually precursor of death

3.5 Behavior tests

All behavioral tests were performed between 9 a.m. and 5 p.m. in a sound-attenuating room, under dim red light (5-10 lux). Bright lights are anxiogenic, while the red color is not visible by rodents, adding to the darkness effect perceived by the animals. Some tests were analyzed by manual scoring while later an automated tracking system based on video feed was used (ANYmaze v.4 software, Stoelting, Ireland, paired with a Logitech C270 webcam). The experimenter stayed in an adjacent room. After each trial, the apparatus were cleaned with 10% ethanol solution and dried with paper wipes in order to remove and disperse olfactory, tactile and visual clues.

3.5.1 Open field

The open field (OF) test was originally created to assess the locomotor activity of animals. Since rats and mice tend to avoid lit areas and open spaces, the relative exploration of the center of the maze is used as a measurement to estimate anxiety-like behavior (Belzung & Girebel, 2001; Walsh & Cummins, 1976).

The test consists in placing the animals in the center of an open field arena which they are allowed to explore for the duration of the test. For mice, the arena was 30 cm each side, divided in 9 squares, 10 by 10 cm each. For rats, for manual score, we used a black wooden arena, lined with plastic, measuring 1 m by 0.8 m, and divided in 20 squares, 20 by 20 cm each. For automated tracking, the arena was 1 m by 1 m, made of dark grey PVC, and the center of the body of the animal was used to determine the distance travelled. All tests lasted for 10 min.

Locomotion was assessed by counting the number of total crossings (i.e. the animal's four paws cross the lines between squares) or the total distance travelled (as assessed using the ANY-maze software).

Anxiety-like behavior was assessed by calculating the ratio between the number of central crossings and the total number of line crossings (or similarly, with the automated tracking, the distance travelled in the center and the total distance).

3.5.2 Object recognition

Rats and mice have a tendency to interact more with a novel object than with a familiar object. Pharmacologists and neuroscientists have used this tendency to study learning and memory. When placing an object in an open field, the animal will explore the novel object as it provides novel stimuli. When placing a new object and an object already familiar to the rodent in the same apparatus, the rat or mouse directs its interest towards the new object, exploring the familiar object for a shorter period. This test probes "recognition memory", a type of non-aversive, non-spatial memory. It avoids the use of non-specific motivational,

emotional and motor influences like electrical shocks and food deprivation, which makes it comparable to memory tests made in humans (Bevins & Besheer, 2006; Dellu et al., 1992; Ennaceur & Delacour, 1988; Pires et al., 2009). The test consists in 3 phases: habituation, acquisition trial, and test trial as described below:

1) Habituation corresponds to open field test described above.

2) In the acquisition trial, 24 h later, the animals were exposed to two identical objects (O1 and O2) in the same open field apparatus in which they were habituated and the animals were allowed to explore them for 5 min. After that, the mice were removed from the open field and placed in their home cages.

3) In the test trial, carried out 90 min after acquisition trial, mice were placed in the open field arena in the same conditions as in acquisition trial, except that this time one of the objects was novel (N) and the other object was identical to the ones the rat was familiarized with (O3). The locations of the objects were counterbalanced in each section in order to avoid any basal preference for any side of the maze that might have existed.

Exploration of an object is defined as directing the nose to the object at a distance equal to or less than 2 cm from the object and/or touching it with the nose. Data can be presented as the total amount of time novel and familiar objects are explored or as a discrimination index, defined as (N-O3)/(N+O3), being N the time exploring the novel object and O3 the time exploring the familiar object.

3.5.3 Object displacement

Also known as ‘object location’ test, the object displacement test (OD) was developed in rats as a test for spatial memory with a similar protocol to the object recognition test (OR) (Ennaceur et al., 1997). In the OD, animals are introduced to two identical objects in an experimental apparatus placed in a room with cues to allow the geographical localization of the objects. After a delay, the animals are exposed again to the same two objects, one of which has been displaced to a new location. The test was then adapted for mice and satisfactory protocols have been achieved, thus, making it also possible to

study spatial memory with this test in mice (Murai et al., 2007). The protocol followed was identical to the one used in the object recognition test, except that both objects remained the same in both acquisition and test trials. One of the objects was moved to a novel position, and memory performance was assessed comparing the time exploring the object in the novel location with the object in the familiar location.

3.5.4 Two-trial Y-maze

The Y-maze (YM) test was originally developed as a one-trial test to evaluate working memory in rodents (Dember & Fowler, 1958; Hughes, 2004). It was later modified by Dellu and colleagues (1992) as a two-trial test that is used to evaluate spatial memory in both rats and mice (Akwa et al., 2001; Dellu et al., 1992, Silva 2013). Similarly to the object recognition and the object displacement tests, the modified two-trial Y-maze (2TYM) test does not require non-specific motivation or emotional stimuli.

The test was carried out in a Plexiglas apparatus with 3 arms (mice: w: 5 cm, l: 30 cm, h: 20 cm; rats: w: 10 cm, l: 35 cm, h: 25 cm) in a Y-shape, separated by equal angles, which is placed in a room with defined visual cues on the walls to allow the geographical orientation of the animals. The test consists in subjecting animals to two sessions separated by a 2-hour inter-trial interval (each session lasts 8 min for mice and 5 min for rats). During the first session, a guillotine wall separated one arm from the rest making it inaccessible to the animals so they were allowed to explore only two arms. During the second session the guillotine was removed so all arms could be explored. Memory performance was evaluated measuring the percentage of time spent exploring the ‘novel’ arm compared to the exploration of the other two arms (‘start’ arm and ‘other’ arm).

3.5.5 Cued Morris water maze

Some animals were tested in the Morris water maze (1984), with adaptations from Packard and McGaugh (1992) to evaluate habit learning. The test was

originally developed to assess spatial learning in rodents by Richard Morris, and has been subsequently altered and changed many times. The test has a lack of pre-training, is reliable across different tank configurations, protocols and species (Vorhees & Williams, 2006). The cued version was described in the original paper by Morris (1984) to be used as a control procedure, but has then been used as a test *per se* to evaluate habit learning, a task dependent on striatal function (Dias-Ferreira et al., 2009; White & McDonald, 2002).

The water maze is a circular tank (1.80 m diameter), walls are 60 cm high, and filled with water (25ºC) until 10 cm from the border. Non-toxic black paint was added to the water for better color contrast. An acrylic transparent platform was placed 2 cm below the water surface with a ball above it that was visible to the animals and was used as the cue.

The test consisted in 4 trials per day for 2 days. In each trial, the animal is placed in the water and allowed to find the escape platform for a maximum of 60 s. Both the start position of the animal and the position of the platform changed every trial, and the intertrial interval was 30 s. After the animal reached the platform, it was allowed to stay for 10 s. When it could not reach the platform in the 60-second cut off time, the experimenter guided it and let the animal remain there for 10 s. The time to reach the platform was recorded for each trial.

3.5.6 Elevated plus maze

The elevated plus maze is the most widely used test to evaluate anxiety-like behavior in rodents (Carobrez & Bertoglio, 2005). The test takes advantage of the natural tendency of animals to explore novel environments but at the same time to avoid open, well-lit spaces. Given the choice of exploring an unprotected open arm of the maze or stay in the enclosed protected arms, an approach-avoidance conflict is created, which is modulated by anxiogenic and anxiolytic drugs (Handley & Mithani, 1984; Walf & Frye, 2007).

The elevated plus-maze apparatus consisted of four arms of the same size (40 cm × 5 cm) arranged in the form of a cross and raised 50 cm above the floor. Two opposed arms were surrounded 30 cm high opaque black Plexiglas

walls, except for the entrance (closed arms) while the other two had no walls (open arms). Each animal was placed on the central square of the maze facing an enclosed arm and was allowed to explore the maze for 5 min (mice) or 10 min (rats). The number of entries and the time spent in both open and closed arms were recorded. The exploratory behavior upon the open arms was expressed as the mean percentage of time spent inside the open arms over the total time spent in all arms (the time in the central square was excluded from the analysis).

3.5.7 Forced swimming test

The forced swimming test (FST) is one of the most regularly used models to test the antidepressant effect of drugs. It was originally described by Porsolt and colleagues (1977) and is based on the fact that when animals are submitted to an inescapable situation, they become immobile after a period of vigorous activity. This immobility can be reduced by a wide range of clinically active antidepressants (Borsini & Meli, 1988).

The forced swimming test apparatus consisted of:

- Rat: a dark blue tank, 27 cm diameter base, 32.5 cm diameter surface, 43 cm high, filled with tap water (25 ± 2ºC) to a depth of 32 cm.
- Mouse: a transparent Plexiglas tank, 10 cm diameter base and surface, 24 cm high, filled with tap water (25 ± 2ºC) to a depth of 19 cm.

The animals' tail did not touch the cylinder floor. Water was replaced after each session. Each animal was placed inside the cylinder for a single 15-minute (rat) or 6-minute (mouse) forced swimming session. Immediately after each session, rats were dried with a cloth towel for 10-20 sec and placed back in their home-cage. The time of immobility was recorded for each animal. Additionally, for rats, the following behavioral measures were scored in blocks of 1 min by a trained observer during the first 8 min session: (1) swimming: smooth coordinated movements with the four paws immersed; the water surface was not broken by the paws; (2) climbing/struggling: strong movements with the four paws; the water surface was vigorously broken by the anterior paws; and (3)

passive/floating (immobility): the rat stays still or makes only movements necessary not to submerge. Observers were blind to experimental groups.

3.5.8 Tail suspension test

The development of the tail suspension test (TST) by Steru et al. (1985) was influenced by the previously developed FST. Similar to the FST, in the TST mice are placed in an inescapable but moderately stressful situation. Like the FST, the TST is a test best validated for the evaluation of antidepressant efficacy of drugs, but also used to evaluate the effects of environmental, neurobiological, and genetic manipulations (Can et al., 2012).
In this protocol, the mice's tail was wrapped in adhesive tape that was attached to a surface allowing the animals to be suspended by the tail. After initially trying to escape by engaging in vigorous movements, mice rapidly become immobile. The duration of immobility is reduced by a wide variety of antidepressants (Strekalova & Steinbusch, 2010). Thus, we recorded the time the animals were immobile, i.e. not struggling to escape, during a 6-minute period.

3.6 Synaptosomal, glial and total membrane preparations

One of the aims of the study presented in this thesis was the investigation of the modulatory role of the adenosinergic system in the re-organization of neural networks of the hippocampus and the amygdala in situations of emotional dysfunction. Adenosine receptors, especially A_1 and A_{2A}, in neurons outside the striatum, are located most abundantly in synapses (Rebola et al, 2003, Rebola et al, 2005) and play an important role modulating synaptic efficiency (Sebastião & Ribeiro, 2009). Moreover, synaptic dysfunction is considered one of the main factors in the etiology of depression (Duman & Aghajanian, 2011), therefore, we chose to isolate and study synaptosomal membranes, i.e. synaptosomes (Cunha, 1998). This preparation is constituted essentially by nerve terminals and post-synaptic densities, free of their integration in the neuronal networks (Gray & Whittaker, 1962). Synaptosomes are prepared by

applying shear forces to nervous tissues (Gray & Whittaker, 1962; Dunkley et al., 1988), causing the separation of the presynaptic buttons linked to the post-synaptic densities, whose membranes re-seal, forming morphologically isolated (Gray & Whittaker, 1962) and biochemically autonomous vesicles (Marchbanks, 1967).

We also wanted to evaluate if changes in adenosine receptors occurring in the synapse were accompanied by changes in other cerebral compartments like glial cells or non-synaptic regions of neurons. For that reason we also purified gliosomes and total membranes from the hippocampus. Gliosomes are resealed membranes of glial origin able to take up and release glutamate and aspartate in a Ca^{2+}-dependent manner (Dunkley et al, 2008; Stigliani et al, 2006). The total membranes fraction includes membranous material from cytosolic, mitochondrial and vesicular origin from both neurons and glia as well as non-purified synaptic membranes.

Hippocampal or amygdalar nerve terminals were prepared using a combined sucrose/Percoll centrifugation protocol as previously described (Lopes et al., 2002). Briefly, hippocampal tissue was homogenized in a sucrose-HEPES medium containing 0.32 M sucrose, 1 mM EDTA, 0.1% bovine serum albumin (BSA) and 10 mM HEPES (pH 7.4). The homogenate was centrifuged at 3,000 *g* for 10 min at 4ºC and the supernatant centrifuged again at 14,000 *g* for 12 min. The pellet was re-suspended in 1 mL of Percoll 45% (v/v) in Krebs-HEPES-Ringer (KHR) medium (NaCl 140 mM, EDTA 1 mM, KCl 5 mM, glucose 5 mM and HEPES 10 mM, pH 7.4) and centrifuged at 14,000 *g* for 2 min. Synaptosomes were then removed from the top layer, washed once with KHR medium and centrifuged for 14,000 *g* for 2 min.

Glial membranes were prepared as described in Matos et al (2012). Briefly, after tissue homogenization and a brief centrifugation to remove debris, the purification of gliosomes was achieved through a discontinuous Percoll gradient (2, 6, 15, and 23% v/v of Percoll in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4) and a centrifugation at 31,000 *g* for 5 min. Gliosomes were collected between the 2% and 6% layers of Percoll, and further centrifuged at 22,000 *g* for 15 min at 4ºC to remove myelin contaminants.

For the isolation of total membranes, the tissue was homogenized in a 0.32 M sucrose solution containing 1 mM EDTA, 10 mM HEPES and 1 mg/mL BSA, pH

7.4 at 4ºC. Then, the homogenates were centrifuged at 3,000 *g* for 10 min at 4ºC. The pellet was discarded and the supernatant was further centrifuged at 25,000 *g* for 60 min at 4ºC. The pellet corresponds to total cytoplasmic membranes.

For Western blot analysis, samples (synaptosomes, gliosomes or total membranes) were re-suspended in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris, pH 8.0) with a protease inhibitor cocktail (CLAPS, Sigma). For binding experiments, samples (synaptosomes, gliosomes or total membranes) were re-suspended in of an incubation solution (25 mM Tris and 10 mM $MgCl_2$, pH 7.4). All samples were frozen at -80ºC until use.

The protein concentration was determined using the BCA (bicinchoninic acid) protein assay reagent (Pierce Biotechnology, Rockford, USA).

3.7 Western blot analysis

After determining the protein concentration, all samples were normalized to the same concentration with reducing 6X Laemmli buffer [final concentrations: 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 0.063 M Tris-HCl, pH 6.8]. Samples were heated at 95ºC for 5 min, and then applied to the gel. 2-Mercaptoethanol reduces disulfide bonds and allows proteins to be denaturated and linearized by SDS, thus making epitopes accessible to antibodies and normalizing the charge density of proteins. Western blot gels were made of a polyacrylamide matrix (30% acrylamide/bis stock solution, BioRad, Portugal) and were 1.5 mm thick. The stacking gel [4% acrylamide, 0.125 M Tris-HCl, 0.1% SDS, 0.5% ammonium persulfate (APS), 0.1% tetramethylethylenediamine (TEMED)] was prepared at pH 6.8 (same pH as the sample buffer) and the resolving gel (7.5% acrylamide, 0.5 M Tris-HCl, 0.1% SDS, 0.1% APS, 0.7% TEMED) was prepared at pH 8.8 (optimum for the separation of negatively-charged proteins). Western blot analysis was performed using a Bio-Rad system (PowerPac power source coupled with a Mini-Protean Tetra electrophoresis chamber and a Trans-Blot Cell for the transfer). The samples (10-50 µL) and the pre-stained molecular weight

markers (5 µL; dual-color standards from BioRad, Portugal) were loaded and separated by SDS-PAGE electrophoresis (in 7.5% polyacrylamide resolving gels with 4% polyacrylamide stacking gels) under denaturating conditions by using a bicine buffered solution (20 mM Tris, 192 mM bicine and 0.1% SDS, pH 8.3). The samples were separated under 80-100 V, and transferred to activated polyvinylidene difluoride (PVDF) membranes (1 A, 2 h, in CAPS [3-(cyclohexylamino)-1-propane-sulfonic acid] buffered solution with methanol [10 mM CAPS, 10% (v/v) methanol, pH 11.0], at 4ºC under agitation). Membranes were then blocked for 1 h at room temperature (RT) with 5% low fat milk in Tris-buffered saline (20 mM Tris, 140 mM NaCl, pH 7.6) with 0.1% Tween 20 (TBS-T). Afterwards, membranes were incubated with the primary antibodies diluted in TBS-T with 5% milk, overnight, at 4ºC. After being washed three times, 15 min each, in TBS-T, the membranes were incubated with the phosphatase-linked secondary antibodies, also diluted in TBS-T with 5% milk for 1 h at RT. Again, membranes were washed three times, 15 min each, in TBS-T, then incubated with Enhanced Chemi-Fluorescence substrate (ECF, GE Healthcare, UK). Proteins were detected and analyzed with Molecular Imager VersaDoc 3000 and Quantity One software (BioRad, Portugal).

Table 4 – antibodies (Ab) used in Western blot protocol

Target (Primary Ab)	Supplier	Host	Dilution
A_1R	Affinity Bioreagents	rabbit	1:500
$A_{2A}R$	Santa Cruz	goat	1:500
SNAP25	Sigma	mouse	1:2000
Syntaxin	Sigma	rabbit	1:5000
Synaptophysin	Sigma	mouse	1:5000
vGluT1	Millipore	guinea pig	1:5000
Gephyrin	Abcam	Rabbit	1:500
Target (Secondary Ab)	**Supplier**	**Host**	**Dilution**
Mouse-AP	Santa Cruz	Goat	1:10,000
Rabbit-AP	Amersham	Goat	1:10,000
Goat-AP	Santa Cruz	Rabbit	1:2500
Guinea pig-AP	Sigma	Goat	1:5000

3.8 Binding assays

Binding experiments allow assessing the density of proteins in a biological sample by measuring the specific binding of ligands, and thus infer the protein density in the membranes of the original samples. In this work, binding experiments were performed as described by Cunha et al (2006) and Duarte et al. (2012). Binding of the selective A_1R antagonist, ^{3}H-1,3-dipropyl-8-cyclopentylxanthine (^{3}H-DPCPX) (DuPont NEN, Alfagene, Portugal) was for 2 h at 37 °C, in a final volume of 300 µL in the incubation solution containing 2 U/mL adenosine deaminase (ADA, Roche, Amadora, Portugal), to remove endogenous adenosine. To evaluate the binding density of $A_{2A}R$, we used ^{3}H-SCH58261. Binding of ^{3}H-SCH58261 (prepared by Amersham, Buckinghamshire, UK and generously offered by Dr. Ennio Ongini, Shering-

Plough, Milan, Italy) was for 1 h at RT (23–25 °C) in a final volume of 300 μL in the incubation solution containing 4 U/mL ADA. A single supra-maximal and selective concentration of ^{3}H-DPCPX and ^{3}H-SCH58261 was used to evaluate the density of A_1 and A_{2A} receptors. Specific binding was determined by subtraction of the non-specific binding, which was measured in the presence of 1 μM 8-{4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl} xanthine (XAC) (Research Biochemical Inc., Sigma-Aldrich), a mixed A_1/A_2 receptor antagonist. The binding reactions were stopped by vacuum filtration through glass fiber filters (GF/C filters) using a 12 well Millipore harvester. The filters were then placed in scintillation vials and 4 mL of scintillation liquid (Ready Safe, Pharmacia-Portugal) was added. Radioactivity was determined after at least 12 h with a counting efficiency of 55–60%. To ensure an error lower than 5% of counts, the samples were counted for 10 min. The protein concentration was determined using the BCA protein assay reagent (Pierce Biotechnology, Rockford, USA).

3.9 Stereotaxic injection into the BLA of lentiviral vector encoding an siRNA to downregulate A_{2A}R

Various classes of retroviruses, adenoviruses and adeno-associated viruses have been successfully adapted for the development of recombinant vectors with the aim of long-term gene delivery to different cell types in different tissues (Kootstra & Verma, 2003). Lentiviruses belong to a class of retroviruses that efficiently infect both dividing and non-dividing (postmitotic) cells, making the recombinant lentiviral vectors applicable for stable, long term gene delivery to neurons (Déglon & Aebischer, 2002).

Lentiviral vectors provide a unique tool to integrate small interference RNA (siRNA) expression constructs with the aim to locally knockdown the expression of a specific gene, enabling to assess the function of a gene in a defined neuronal pathway. Although the delivery of siRNA *in vivo* is inefficient, lentiviral vector-mediated delivery of one type of siRNA, the short hairpin RNA (shRNA) results in persistent knockdown of gene expression in the brain (Scherr et al, 2003). Therefore, the use of lentiviruses for stable expression of siRNA in brain

is a powerful aid to probe gene functions *in vivo* and for gene therapy of diseases of the central nervous system (Dreyer, 2011).

In this work we chose this approach to interfere with the translation of $A_{2A}R$ in the BLA, thus, 'silencing' $A_{2A}R$ in this region. Viral vectors were produced in house by Nélio Gonçalves according to Gonçalves et al. (2013). Concentrated viral stocks were thawed on ice. After anaesthesia of rats with 14 mg/kg xylazine and 128 mg/kg ketamine administered intraperitoneally (i.p.), lentiviral vectors encoding short hairpin $A_{2A}R$ (shA2AR) or control sequence (shCTR) were stereotaxically injected into the basolateral amygdala (BLA) in the following coordinates: antero-posterior (AP): -2.8 mm; medial-lateral (ML): ±4.8 mm; dorso-ventral (DV): -9.0 mm. Rats received 1.5 µL solution of lentivirus (500'000 ng of p24/mL) into the BLA, in both hemispheres. Animals were allowed to recover for one week before stress procedure started.

3.10 Histological and immunohistological analyses

3.10.1 Preparation of fixed brain slices

Rats were anesthetized with an overdose (150 mg/kg) of thiopental-sodium (Sigma-Aldrich, Portugal) and deep anesthesia was verified by tail and paw pinch. The heart of the anesthetized animal was exposed, the descending aorta clamped, a catheter was inserted in the ascending aorta through the left ventricle, and the right atrium was opened to allow the outflow of perfusate. The animal was then perfused with 200 mL cold saline solution (0.9% NaCl) with 4% sucrose, followed by 200 mL of 4% paraformaldehyde in saline solution (0.9% NaCl, 4% sucrose). Brains were removed and kept 24 h in the same paraformaldehyde solution at 4ºC. Subsequently, the brains were transferred to 30% sucrose in phosphate buffer saline (PBS) at 4ºC. Once at the bottom of the solution (at least 48 h later), the brains were embedded in Tissue-Tek (Sakura-Americas, USA), frozen at -21ºC and cut in coronal sections using a cryostat (CM3050 S from Leica Microsystems, Portugal). The sections were stored in PBS with 0.01% sodium azide and mounted in 2% gelatin-coated slides before use (all reagents from Sigma-Aldrich, Portugal, unless stated otherwise).

3.10.2 Acetylcholinesterase (AChE) staining

Within the amygdala and in neighboring regions, labeling sections with cholinergic markers yields labeling patterns that allow the distinction of different amygdala nuclei (Ichikawa et al., 1997; Schäfer et al., 1998). In particular, acetylcholinesterase (AChE) is highly abundant and yields a strong staining of the basolateral nucleus that contrasts with the pale labeling of the neighboring nuclei, like the lateral nucleus and basomedial nucleus (Ben-Ari et al., 1977). Thus, this pattern offers the ideal reference to confirm if the EGFP labeling provided by the viral vectors is selectively present in the BLA.

The protocol was based on that described by Stenman (2003). Brain sections (30 μm) were mounted on slides and dried at 37°C for 1 h before placing in incubation medium (3 mM cupric sulfate, 10 mM glycine, 15 mM acetic acid, 35 mM sodium acetate, 0.08 mM tetraisopropyl pyrophosophoramide, 1.5 mM acetylthiocholine iodide, adjusted to pH 5.0) at 37°C for 4 h. They were then developed in a 40 mM sodium sulfide solution (pH 7.5). Sections were visualized under transmission on a Zeiss Axiovert 200 microscope.

3.10.3 Fluoro-Jade C

Fluoro-Jade C staining was used to evaluate general neurodegeneration in brain sections, regardless of the specific insult or mechanism of cell death (Schmued et al., 2005). Brain sections on slides were defrost, dried and immersed for 5 min in 0.01% sodium hydroxide (NaOH pellets from Sigma-Aldrich, Portugal) prepared in an 80% ethanol solution. After rinsing for 2 min in 70% ethanol and for 2 min in distilled water, slides were transferred to a 0.06% potassium permanganate solution during 10 min, under agitation and protected from light. Sections were again rinsed in distilled water, for 2 min, and immersed in 0.0001% Fluoro-Jade C (Histo-Chem Inc., Jefferson, AR, USA; in 0.1% acetic acid vehicle) for 10 min, protected from light and under agitation, before being rinsed three times, 1 min each, in distilled water. Slices were dried on a slide warmer, dehydrated by passing through an ethanol gradient (50%, 70%, 100%) and cleared in xylene. Finally, slices were coversliped with DPX (distyrene,

plasticizer, xylene) non-aqueous mounting medium (Merck, Portugal). Images were acquired with Zeiss Axiovert 200 microscope and mean fluorescence intensity was quantified using ImageJ software (NIH, Bethesda, USA).

3.10.4 GFAP and CD11b imunohistochemistry

Microglia are immunocompetent cells in the brain, and their presence and activation was assessed through CD11b (cluster of differentiation molecule 11B) immunostaining. Astrocytes, which support neuronal function, were stained for GFAP (glial fibrillary acidic protein).

Sections were washed 3 times in PBS at RT and then blocked with 5% horse serum in PBS for 45 min. Sections were subsequently incubated with the primary antibody anti-CD11b or anti-GFAP (CD11b: rat, 1:200, Serotec; GFAP: rabbit, 1:500, Millipore) in PBS containing 5% horse serum and 0.25% Triton-X100 for 48 h at 4ºC, washed 3 times with PBS containing 0.25% Triton-X100, and incubated with secondary antibodies (donkey anti-rat, 1:500; conjugated with Alexa-Fluor 488; goat anti-rabbit, 1:500, conjugated with Alexa-Fluor 647) for 2 h at RT in PBS containing 5% horse serum and 0.25% Triton-X100. After washing 3 times, the sections were washed 3 times in PBS and mounted in gelatin-coated slides with Vectashield antifade mounting medium with DAPI, for nuclear staining (Vectorlabs, USA). Sections were observed and images acquired in a Zeiss Axiovert 200 microscope. Mean intensity fluorescence was calculated using ImageJ software (NIH, Bethesda, USA).

3.11 Statistical analysis

All data are presented as mean ± S.E.M. (standard error of the mean), unless otherwise stated. Student's *t* test was used to compare two independent groups and an analysis of variance, two-way ANOVA, was used to compare four independent groups with independent variables, followed by a Bonferroni *post hoc* test to assess caffeine/A_{2A}R-related genotype/A_{2A}R silencing effect. In the two-trial Y-maze, intra-group one-way ANOVA followed by a Bonferroni *post*

hoc test was used to compare arms against ‘novel arm’, and one tail one sample *t* test was used to assess ‘learning’ *vs*. chance (33.3%) in the ‘novel arm’. $p<0.05$ was considered to represent a significant difference.

4. RESULTS AND DISCUSSION

4.1 CAFFEINE SELECTIVELY REVERTS MEMORY BUT NOT MOOD DEFICITS IN A GENETIC MOUSE MODEL OF DEPRESSION

4.1.1 Introduction

Depression is the leading cause of disease in Europe but has an unknown etiology and far from optimal therapeutic solutions (Andlin-Sobocki et al, 2005). Besides the mood changes, one characteristic of depressed individuals is a decreased cognitive function with a strong impact on their quality of life (Clark et al, 2009; Kim and Diamond, 2002). Accordingly, animal models with mood-related alterations, display an impaired memory performance, which underlying mechanism is still incompletely understood.

In recent years, epidemiological and animal studies have concurred to conclude that the chronic consumption of caffeine prevents memory impairments associated with diverse conditions (Cunha & Agostinho, 2010; Rivera-Oliver & Díaz-Ríos, 2014). The only known molecular mechanisms of caffeine action in non-toxic doses is the antagonism of adenosine receptors, namely of adenosine A_1 (A_1R) and A_{2A} receptors (A_{2A}R) (Fredholm et al, 2005). Accordingly, both caffeine and A_{2A}R antagonists prevent memory dysfunction (reviewed in Cunha & Agostinho, 2010) and A_{2A}R activation is sufficient to trigger memory dysfunction (Li et al., 2015; Pagnussat et al., 2015). However, it is unknown if caffeine can revert memory impairment associated with a depressive state.

To explore this question, we took advantage of a bred-based model of depression, ‘helpless’ mice (HM), obtained by selective breeding of mice with poor responses in the tail suspension test (TST) (El Yacoubi et al., 2003), a paradigm predictive of antidepressants (Steru et al., 1985); HM show several features associated with major depressive disorder like helplessness, sleep-wakefulness alterations, higher basal serum corticosterone levels and decreased brain serotoninergic tone (El Yacoubi et al, 2003) and display face value predictability of clinically used antidepressants (Bougarel et al, 2011), allowing us to test if memory impairments are present in this model and if both memory and mood changes might be reverted by caffeine consumption.

4.1.2 Experimental design

Adult helpless (HM) and non-helpless (NHM) mice were first behaviorally tested during 3 days, in a hereafter called “Block 1”. Following the last day of testing, each group of NHM and HM was sub-divided in two groups: one drinking regular water (“water”) and a treated group drinking water containing 0.3 g/L caffeine (Sigma, Sintra, Portugal) (“Caff”) during 4 weeks; this eliminates the acute effects of caffeine while preserving its long-term neuroprotective effects (Cognato et al, 2010; Duarte et al, 2012).

After the last day of caffeine treatment, we carried out another series of behavior experiments, “Block 2”. Both blocks of behavioral testing were identical and took place as follows:

Day 1, morning: open field

Day 1, afternoon: object recognition

Day 2, morning: two-trial Y-maze

Day 2, afternoon: tail suspension test

Day 3: forced swimming test

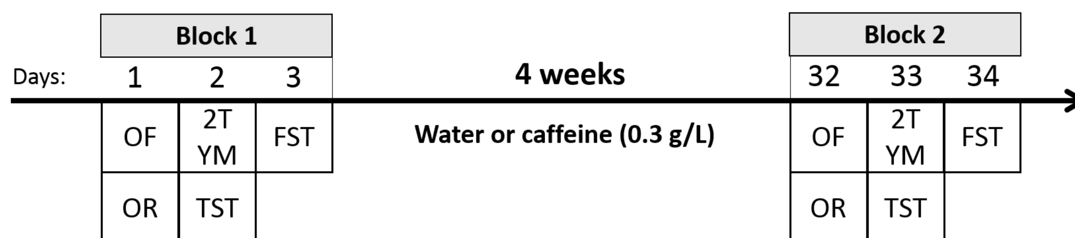

Figure 4: Representation of the experimental design for assessing the behavioral effects of caffeine in a genetic model of depression.
Legend: 2TYM – two-trial Y-maze; FST – forced swimming test; OF – open field; OR – object recognition; TST – tail suspension test.

The experiments in Block 2 occurred in a different behavior room in order to avoid habituation, especially, in the OF and modified Y-maze tests. Also, all objects used in the object recognition task were changed for the same reason.

After behavior analysis, mice were sacrificed by deep anesthesia with halothane atmosphere before decapitation, for biochemical analysis.
In the scope of this work, we mostly focused on the analysis of the density of adenosine receptors A_1 and A_{2A} using a binding protocol, to provide a first insight into the involvement of the adenosinergic system in depression. Furthermore, we also investigated alterations of synaptic markers to consolidate our hypothesis that the onset of memory impairment is associated with synaptic changes.

4.1.3 'Helpless' mice display memory impairment and depressive-like behavior

As described by Yacoubi and colleagues (2003), the 'helpless' mice used in this study were selected based on the behavioral profile of their previous generations in the tail suspension test (TST). This was confirmed in the TST (Fig. 5A): thus, HM animals struggled to escape from the downward position during a much shorter time than NHM during the 6-minute duration of the test (immobility time; NHM: 4.0 ± 3.6 s, n=17; HM: 227.4 ± 9.5 s, n=16; $p<0.0001$). To test whether these animals possess indeed a depression-related phenotype or, instead, this difference is non-translational and inherited through the breeding selection, the forced swimming test (FST), another widely used paradigm to assess anti-depressant effects (Porsolt, 1977, reviewed in Petit-Demouliere et al, 2004), was used. The results in the FST were very similar to the TST (immobility time; NHM: 22.0 ± 7.2 s, n=17; HM: 258.8 ± 6.8 s, n=16; $p<0.0001$) (Fig. 5B), thus, HM were immobile for the vast majority of the time in the FST thus displaying robust "behavioral despair" (see Porsolt et al., 1977). Basal locomotor activity was also tested in the open field arena (Fig. 5C): although the changes in the FST are not related to changes in locomotion (Hilakivi & Lister, 1990), we have observed that HM displayed a clear and consistent decrease in the number of line crossings to about half of NHM in the OF (crossings; NHM: 104 ± 8.1 s, n=17; HM: 50.8 ± 2.8 s, n=16; $p<0.0001$). Yacoubi et al (2013) have also observed the same effect, which is consistent with symptoms of depression that include a reduction of general activity or

fatigue. It should be noted that reduced locomotor activity might interfere with the interpretation of the 'despair' behavior (longer immobility) in TST and FST. However, during the creation of the H/Rouen line (HM), the decrease of locomotor activity in the OF and the increase in immobility time in the TST and FST were not correlated, meaning these are independent effects (Yacoubi et al., 2003).

To assess memory performance, we used the object recognition task (Fig. 5C). In this particular test we did not observe differences in the recognition index (discrimination index; NHM: 0.10 ± 0.05, n=15; HM: 0.12 ± 0.06, n=15; Student's *t* test) but that is due to the fact that the discrimination index of NHM was too low, thus creating a ceiling effect ($p>0.05$, NHM *vs*. zero, one-sample *t* test). The discrimination index, expected to be close to 0.5, depends on several factors including species, size of arena, the objects used and the experimental room (Bevins & Besheer, 2006). The objects were changed for the object recognition task in experimental Block 2 (section 4.1.4): color, shape and material were different between the familiar and novel objects in order to increase the discrimination index of 'non-helpless' mice.

Another task used to assess memory performance was the two-trial Y-maze (Dellu et al., 1992; Dellu et al., 2000). This task probes spatial memory, dependent on hippocampal processing. One primary measurement in this task is the total number of arm entries (Fig. 5F). In this regard, HM entered the arms less times than NHM (arm entries; NHM: 36.1 ± 2.3, n=17; HM: 28.1 ± 1.2, n=14; $p<0.01$), consistent with the locomotion quantified in the OF arena. But the memory-related results (permanence in the novel arm; Fig. 5E) are displayed as a quotient of time, thus the lower locomotion of HM is not expected to hamper the analysis of memory performance. In this regard 'helpless' mice displayed a performance that was lower than 'non-helpless' animals: thus, compared to NHM, HM entered less times (entries in novel arm; NHM: 45.5 ± 1.7%, n=17; HM: 40.6 ± 1.6%, n=14; $p<0.05$) and remained for shorter periods (time in novel arm; NHM: 48.2 ± 2.3%, n=17; HM: 39.6 ± 2.1%, n=14; $p<0.05$) in the novel arm.

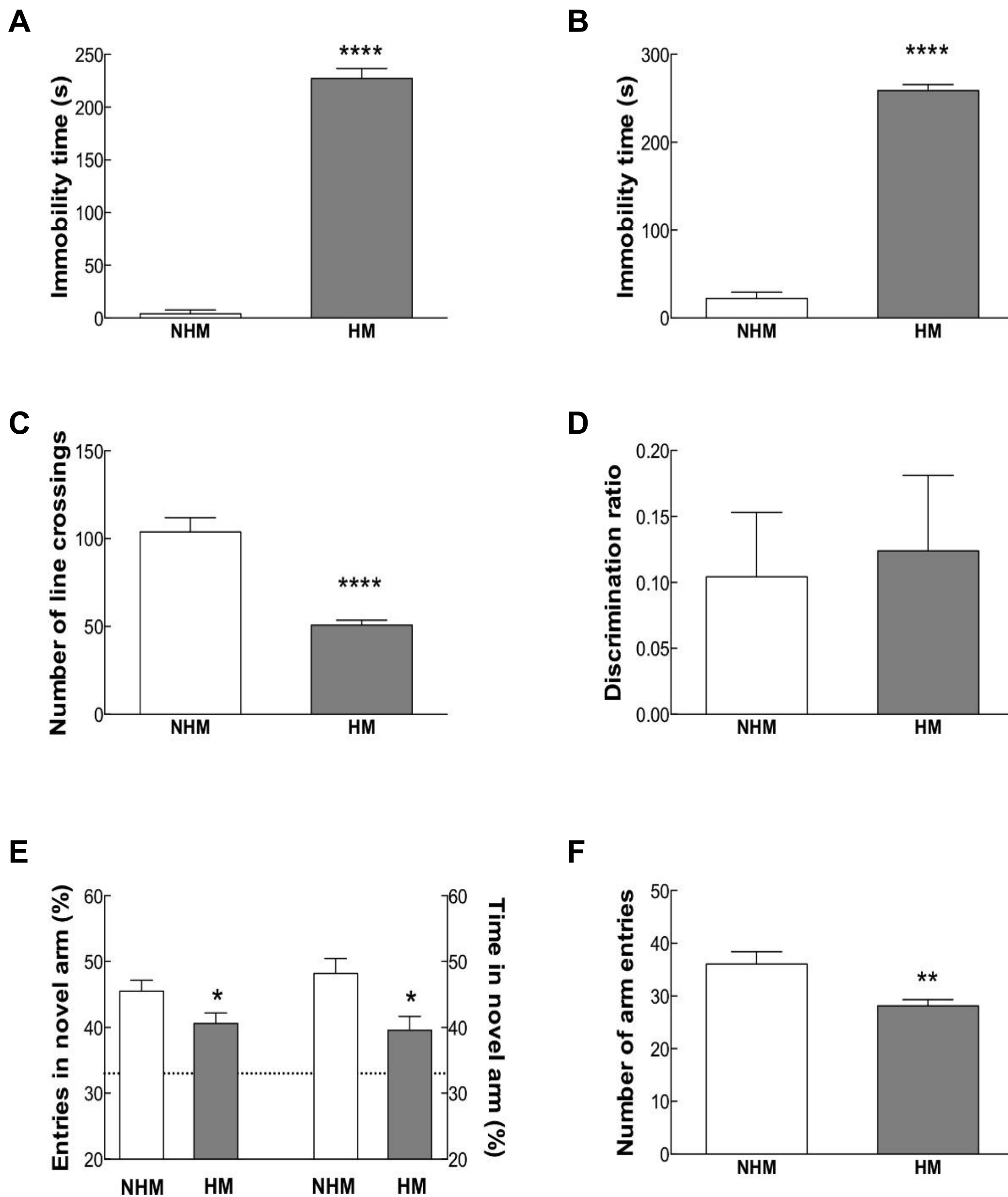

Figure 5: **Bred-based 'Helpless' mice (HM) display depressive-like behavior, decreased locomotor activity and memory impairment compared to non-helpless mice (NHM)**. Mice were tested prior to caffeine treatment. Depressive-like behavior was assessed using the tail suspension (A) and the forced swim (B) paradigms; locomotor activity was tested in the open field arena (C); memory was tested in the object recognition task (D); spatial hippocampal dependent memory was tested in the two-trial Y-maze task (E, dotted line represents chance at 33.3%) as well as the locomotor activity in the same apparatus (F). Bar graphs represent mean + SEM. Group means were compared using Student's *t* test. *p<0.05, **p<0.01, ****p<0.0001 *vs*. NHM group, n=14-17. NHM, non-helpless mice; HM, helpless mice.

The results in the Y-maze were in line with our hypothesis of the occurrence of learning and memory deficits in this model of depression. These data are suggestive of a hippocampal impairment that can be detrimental to the performance in these test (Silva et al., 2013).
Overall, HM displayed depressive-like behavior, lower locomotion and impaired memory that are among the main features of a depressive state.

4.1.4 Caffeine recovers memory performance of 'helpless' mice

After the first set of behavioral experiments, mice drank either caffeine (0.3 g/L; Caff) or regular drinking water for 4 weeks, thus sub-dividing each group into two. The resulting groups are: HM/water (n=10), HM/Caff (n=10), NHM/water (n=9), NHM/Caff (n=10). All animals were tested in a second set of experiments (Block 2) as before, albeit in a different behavioral room. The striking difference between the performance of HM and NHM in the depression-related tests (TST and FST, Figs. 6A and 6B) was observed once again (immobility in TST and FST: $p<0.0001$, NHM *vs*. HM effect, 2-way ANOVA, n=9-10/group). Remarkably, caffeine intake did not revert or attenuate the difference between groups (immobility in TST: HM/water: 307.2 ± 8.9 s, n=10; HM/Caff: 323.3 ± 5.6 s, n=10; adjusted $p=0.33$; immobility in FST: HM/water: 307.1 ± 14.2 s, n=10; HM/Caff: 335.2 ± 3.2 s, n=10; adjusted $p=0.50$; mean ± SEM, Bonferroni *post hoc* test) nor did it significantly change the behavior of NHM mice (immobility in TST: NHM/water: 8.2 ± 6.8 s, n=9; NHM/Caff: 21.3 ± 10.1 s, n=10; adjusted $p=0.54$; immobility in FST: NHM/water: 85.1 ± 27.5 s, n=10; NHM/Caff: 70.5 ± 17.2 s, n=10; adjusted $p>0.99$; mean ± SEM, Bonferroni *post hoc* test).
When we measured the locomotor activity of NHM in the OF (Fig. 6C), we observed that the chronic caffeine intake decreased horizontal locomotion (number of crossings: NHM/water: 191.9 ± 15.2, n=9; NHM/Caff: 128.8 ± 15.5, n=10; adjusted $p<0.01$; mean ± SEM, Bonferroni *post hoc* test). This has not been reported before, but it should also be noted that the NHM line was produced in parallel with the HM, and both present an extreme behavior in the TST and FST, although in the opposite extremes. This means that NHM are not the 'normal' control group, corresponding to 'wild-type'. NHM are also

selectively bred and that might be related to this observed effect of caffeine on their pattern of locomotion. Caffeine intake had no significant effect on 'helpless' mice (number of crossings: HM/water: 58.3 ± 6.4, n=10; HM/Caff: 75.5 ± 9.8, n=10; adjusted p=0.64; mean ± SEM, Bonferroni *post hoc* test) and, as in experiments in Block 1, NHM presented a marked decreased of locomotor activity (p<0.0001, NHM *vs*. HM effect, 2-way ANOVA, n=9-10/group).

Regarding memory performance, in the object recognition task (Fig. 6D), NHM displayed what is expected to be the discrimination ratio in normal controls (NHM/water: 0.42 ± 0.09, n=6) and caffeine did not affect their performance (NHM/Caff: 0.41 ± 0.07, n=8; adjusted p>0.99, *vs*. NHM/water, Bonferroni's *post hoc* test). HM drinking water showed a clear memory impairment (NHM/water: 0.14 ± 0.10, n=8) that was reverted by the four-week caffeine intake (HM/Caff: 0.44 ± 0.05; adjusted p<0.05, *vs*. HM/water, Bonferroni's *post hoc* test). Moreover, HM/water animals where the only ones that did not statistically differentiate the two objects (NHM/water: p<0.01; NHM/Caff: p<0.001; HM/water: p=0.20; HM/Caff: p<0.0001; *vs*. zero, one-sample *t* test). These results show a full recovery from memory impairment in 'helpless' mice and, thus, confirming the initial hypothesis.

Lastly, we tested the animals in the two-trial Y-maze task (Figs. 6E,F). Although HM tended to spend less time in the novel arm, the difference was not significant and caffeine had no effect on either group (time in novel arm: NHM/water: 37.7 ± 2.3%; NHM/Caff: 35.4 ± 2.3%; HM/water: 32.0 ± 2.4%; HM/Caff: 32.7 ± 3.3%). It is worth noting that the results obtained for NHM were lower than expected in this task, and not significantly different from chance of entering the novel arm (NHM/water: p=0.09, *vs*. 33.3%, one-sample *t* test). This is likely due to the spatial arrangement of the new behavior room used in Block 2 and thus rendering the new Y-maze test inconclusive.

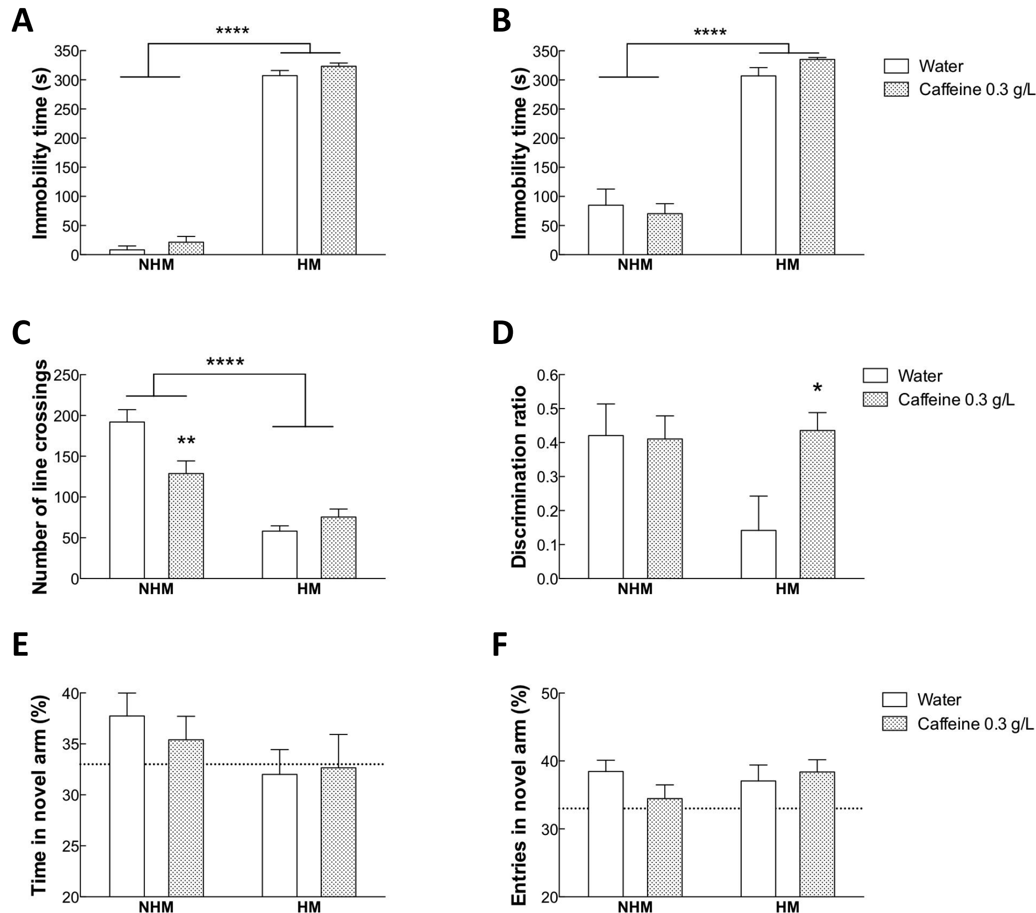

Figure 6: Caffeine reverts memory deficits of 'helpless' mice, a genetic model of depression. Mice were tested after either 4 weeks of normal drinking water or caffeine (0.3 g/L). Depressive-like behavior was assessed using the tail suspension (A) and the forced swim (B) paradigms; locomotor activity was tested in the open field arena (C); memory was tested in the object recognition task (D); spatial hippocampal dependent memory was tested in the two-trial Y-maze task by measuring the time (E) and the entries in the novel arm (F). Bar graphs represent mean + SEM. Group means were compared using 2-way ANOVA followed by Bonferroni's *post hoc* test for caffeine *vs*. water comparison. *$p<0.05$, **$p<0.01$, ****$p<0.0001$, n=9-10. NHM, non-helpless mice; HM, helpless mice.

4.1.5 The density of adenosine A_{2A} receptors, but not of A_1 receptors, is increased in hippocampal nerve terminals of 'helpless' mice

The memory deficits observed in 'helpless' mice suggest an impairment of hippocampal function in these animals. These data are consistent with the

expected symptoms of depression. It is also known that the adenosinergic system is involved in memory dysfunction (Cunha & Agostinho, 2010) and that the molecular targets of caffeine at the dose used are mostly of A_1 and A_{2A} receptors (Fredholm et al., 2005). The predominant effect of adenosine receptors is a modulation of synaptic plasticity (Cunha, 2008) impacting on memory formation (Cunha & Agostinho, 2010) and for that reason we tested if there were any alterations of the density of A_1R and $A_{2A}R$ in nerve terminals of the hippocampi of 'helpless' mice.

The day after behavioral testing, mice were sacrificed, the hippocampi dissected and frozen (-80ºC). The synaptosomal membranes were later prepared to run the binding experiment to assess A_1R and $A_{2A}R$ receptors density (Duarte et al., 2012).

Previous works have shown that $A_{2A}R$ density is increased in the hippocampus of animals with memory deficits, such as upon aging, Alzheimer's disease or diabetes (Cunha et al., 1995; Duarte et al., 2012; Matos et al., 2012). Accordingly, binding of the $A_{2A}R$ antagonist ^{3}H-SCH58261 showed that $A_{2A}R$ density was 71.9 ± 6.1% larger in HM compared to NHM (n=5, $p<0.05$; Fig. 7A), whereas the binding of the A_1R antagonist ^{3}H-DPCPX showed that A_1R density was not significantly modified (n=5, $p>0.05$; Fig. 7B).

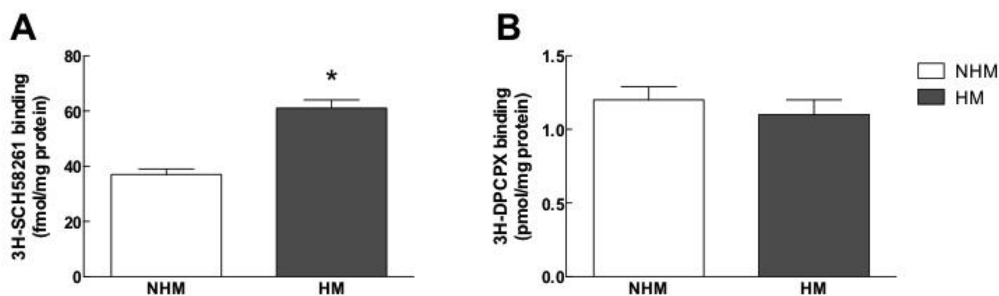

Figure 7: 'Helpless' mice have an increased density of adenosine A_{2A} receptors but a preserved density of adenosine A_1 receptors. The density of adenosine A_{2A} (A) and A_1 receptors (B) was measured as the specific binding of a supra-maximal concentration (6 nM) of the selective antagonists of A_{2A} receptors (^{3}H-SCH58261) or of A_1 receptors (^{3}H-DPCPX) in membranes from hippocampal nerve terminals of 'helpless' (HM) and 'non-helpless' mice (NHM). Bar graphs represent mean + SEM. Group means were compared using Student's *t* test. *$p<0.05$, *vs*. NHM group, n=5. NHM, non-helpless mice; HM, helpless mice.

4.1.6 Helpless mice display a reduction of synaptic markers that recovers upon caffeine intake

Since synaptic dysfunction is increasingly recognized as being tightly associated with memory impairment (Wishart et al., 2006; Cunha and Agostinho, 2010), we tested if HM displayed a modified density of synaptic markers. Western blot analysis (Fig. 8) revealed a reduction of the immunoreactivity of the synaptic markers, synaptophysin (-26.7 ± 6.9%, n=4, $p<0.05$; Fig. 8A) and SNAP25 (-18.5 ± 2.5%, n=5, $p<0.05$; Fig. 7B) in synaptosomal membranes of HM compared to NHM. To investigate if this corresponded to a modification of pre- or post-synaptic components of either glutamatergic or GABAergic synapses, we evaluated the density of presynaptic markers of glutamatergic (vesicular glutamate transporters type 1, vGluT1) or GABAergic terminals (vesicular GABA transporters, vGAT) and of markers of postsynaptic excitatory (postsynaptic density protein 95, PSD95) and inhibitory synapses (gephyrin). Figure 8 shows that HM had reduced vGluT1 (-21.9 ± 7.0%, n=5, $p<0.05$; Fig. 8C) and vGAT densities (-23.4 ± 8.4%, n=5, $P<0.05$; Fig. 8D) compared to NHM. In contrast, there was no significant difference in the density of either PSD95 (6.1 ± 3.7%, n=4, $p<0.05$; Fig. 8E) or gephyrin (8.9 ± 6.4%, n=4, $p>0.05$; Fig. 8F) between HM and NHM.

Caffeine consumption reverted the decrease of synaptophysin (Fig. 9A) and of SNAP25 density (Fig. 9B) in HM, whereas it was devoid of effects in NHM mice (Figs. 9A,B). Thus, caffeine prevented the synaptic deterioration that parallels memory impairment in HM.

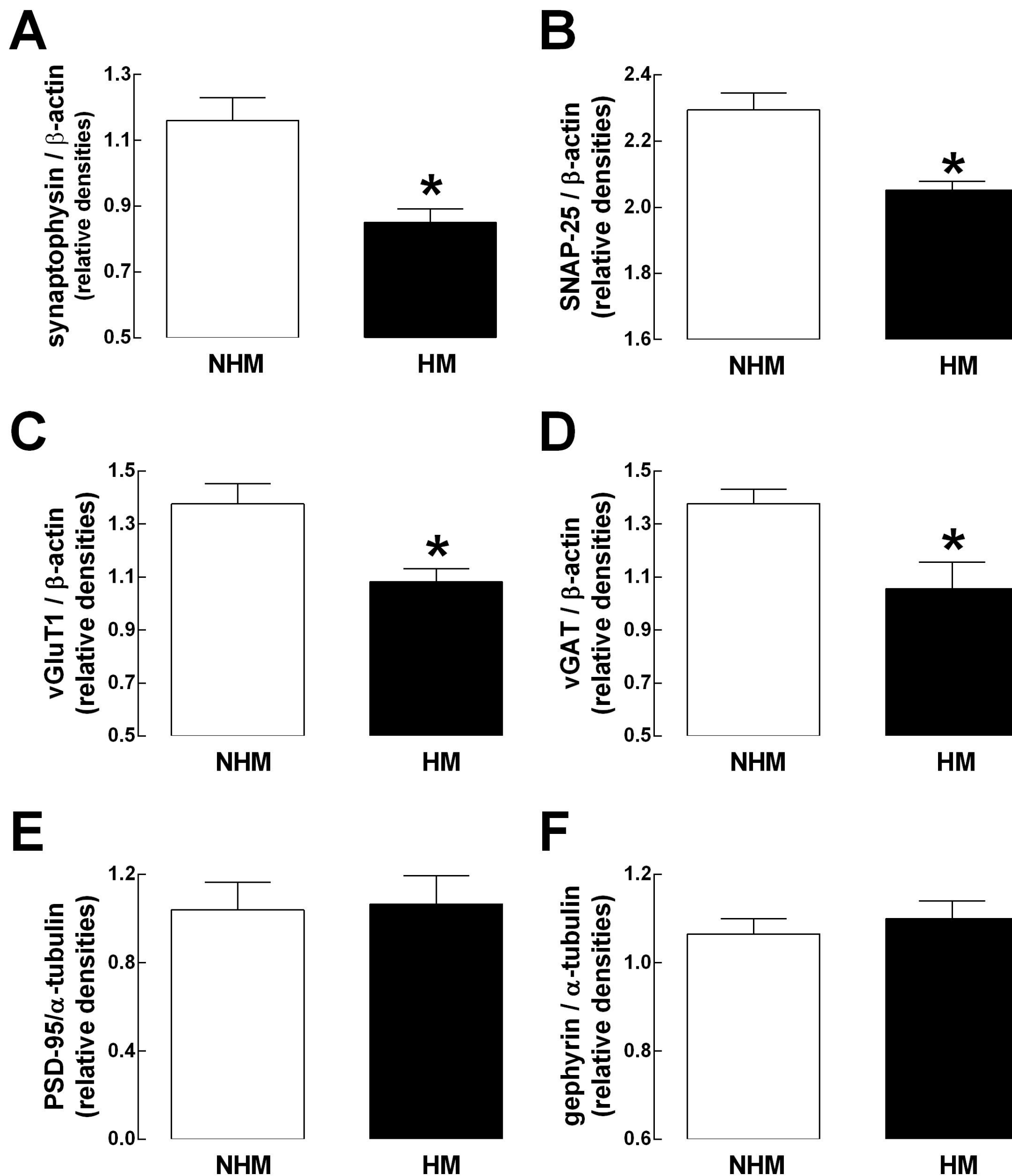

Figure 8: 'Helpless' mice display a selective loss of presynaptic markers, both of glutamatergic and GABAergic synapses. 'Helpless' mice (HM) displayed a lower immunoreactivity for general markers of nerve terminals, namely synaptophysin (A) and SNAP25 (B), as well as for markers of glutamatergic nerve terminals, namely vesicular glutamate transporter type 1 (vGluT1, C), and of GABAergic nerve terminals, namely vesicular GABA transporter (vGAT, D), but not of post-synaptic markers of excitatory (post-synaptic density 95 kDa protein, PSD95, E) or inhibitory (gephyrin, F) synapses, when compared with 'non-helpless' mice (NHM). Data are mean ± SEM of the Western blot immunoreactivity of each marker normalized with a re-probing for either β-actin or α-tubulin using membranes from hippocampal nerve terminals from 4-5 mice per group. * $p<0.05$ between indicated bars using an unpaired Student's *t* test. A representative Western blot is displayed below each bar graph.

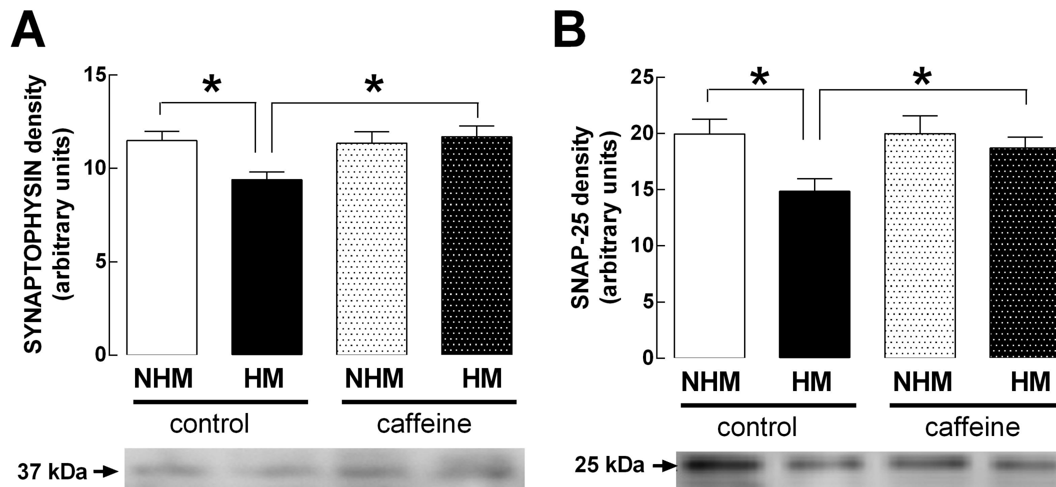

Figure 9: Chronic consumption of caffeine (0.3 g/L in the drinking water during 4 weeks) reverts the loss of presynaptic markers displayed by 'helpless' mice. After 4 weeks of consumption of water, Western blot analysis of membranes from hippocampal nerve terminals revealed that 'helpless' mice (HM) had a reduced immunoreactivity of both synaptophysin (A) and SNAP25 (B) when compared to 'non-helpless' mice (NHM). Notably, the consumption of caffeine (0.3 g/L) through the drinking water during 4 weeks reverted the loss of presynaptic markers in HM, while it was devoid of effects in NHM. Data are mean ± SEM of 4-5 mice per group in (A) and (B).* $p<0.05$ between genotypes and ** $p<0.05$ between caffeine treated and non-treated mice, using a two-way ANOVA followed by Newman-Keuls *pos-hoc* test.

4.1.7 Conclusion

The present study shows that genetically bred mice selected for spontaneous helpless behavior (helpless mice, HM) display impaired learning and memory abilities, which together with their anhedonia, anxiety, sleep disorders and responsiveness to antidepressant therapies (Bougarel et al., 2011; El Yacoubi et al., 2003), consolidate this animal model as a relevant model of depression. Additionally, we show that this memory impairment is associated with an increase in A_{2A} receptors density in the hippocampus. Finally, we found that caffeine consumption reverts these memory deficits.

The impairment of memory function is a core feature associated with major depression, which is also present in animal models pertinent to mood dysfunction (Clark et al., 2009; Kim & Diamond, 2002). Albeit the mechanism

underlying this memory impairment is still unresolved, synaptic dysfunction may play a key role: indeed, several studies have related synaptic dysfunction to the impairment of learning and memory associated with diverse neuropsychiatric conditions (reviewed in Cunha & Agostinho, 2010; Wishart et al., 2006)

The other main finding of this study was the ability of caffeine to revert the impairment of memory performance. Indeed, in recent years, several studies have concurred to conclude that the chronic consumption of caffeine has the ability to prevent memory impairments associated with diverse conditions (reviewed in Cunha & Agostinho, 2010). Epidemiological studies have shown an inverse association between the consumption of caffeine and the incidence of depression (Lucas et al., 2011) as well as with cognitive deficits in elderly individuals (e.g., Ritchie et al., 2007) or in demented individuals (e.g., Eskelinen & Kivipelto, 2010). Animal studies have confirmed that the chronic consumption of caffeine indeed prevents memory dysfunction in aging (e.g., Prediger et al., 2005) or Alzheimer’s disease (Arendash et al., 2006; Dall’Igna et al., 2007; Espinosa et al., 2013). Notably, caffeine displayed a parallel ability to prevent memory impairment and the loss of synaptic markers in several animal models of diseases where memory deterioration is observed (Cognato et al., 2010; Duarte et al., 2012; Vila-Luna et al., 2012). Overall, this suggests that the consumption of moderate doses of caffeine (0.3 g/L), which roughly corresponds to circa 3-4 cups of coffee daily (Fredholm et al., 1999), can revert the memory deficits associated with depression.

The mechanism operated by caffeine to revert memory impairment is likely to be the antagonism of adenosine $A_{2A}R$, as previously suggested (Cunha & Agostinho, 2010). Accordingly, $A_{2A}R$ antagonists mimic the beneficial effects of caffeine to prevent memory dysfunction caused by early life stress (Batalha et al., 2013; Cognato et al., 2010) as well as in animal models of aging (Prediger et al., 2005) or Alzheimer’s disease (Canas et al., 2009; Laurent et al., 2015). This seems associated with the increased density of hippocampal $A_{2A}R$ in these different conditions (e.g., Cunha et al., 2006; Espinosa et al., 2013; Rebola et al., 2003), which we now also observed in ‘helpless’ mice. These $A_{2A}R$ are mostly located in glutamatergic synapses in the hippocampus (Rebola et al., 2005) and play a crucial role in the control of synaptic plasticity and in the definition of information salience in hippocampal synapses (reviewed in Cunha,

2008). Notably, the blockade of $A_{2A}R$ prevents the abnormal synaptic plasticity and loss of synaptic markers associated with stressful brain insults and conditions (Batalha et al., 2013; Cognato et al., 2010; Costenla et al., 2010; Cunha et al., 2006).

In conclusion, the present study suggests that the ability of caffeine to revert memory impairment in the H/Rouen model of depression might be associated with the antagonism of an aberrant up-regulation of hippocampal adenosine $A_{2A}R$.

4.2 DELETION OF NEURONAL ADENOSINE A_{2A} RECEPORS PREVENTS DEPRESSIVE-LIKE ALTERATIONS IN A MOUSE MODEL OF CHRONIC UNPREDICTABLE STRESS

4.2.1 Introduction

As seen in Chapter 4.1, caffeine was able to revert cognitive changes in the genetic model of stress Rouen/H, the 'helpless' mice. But mood-related changes were not significantly altered by caffeine intake. As previously reported in animal studies, caffeine seems to yield its beneficial effects on pathological conditions in a preventive manner, for example in diabetes (Duarte et al., 2012), Alzheimer's disease (Espinosa et al., 2013; Dall'Igna et al., 2007) and Parkinson's disease (Miyoshi et al., 2002). For that reason it was necessary to understand the action of caffeine and adenosine receptors before the onset of the events causing the depressive state. With that aim, we used a behavior-based model emotional impairment, the chronic unpredictable stress (CUS) (Bondi et al., 2008). Repeated stress elicits neurochemical and morphological changes that negatively impact on brain functioning (de Kloet et al., 2005; McEwen, 2007) being a trigger or a risk factor for neuropsychiatric disorders, namely depression, in both humans and animal models (Kim & Diamond, 2002; McEwen, 2007).

Although the molecular target operated by caffeine to afford beneficial effects has not been defined, $A_{2A}R$ blockade affords a robust protection against noxious brain conditions (Cunha, 2005), an effect that might result from the ability of neuronal $A_{2A}R$ to control aberrant plasticity (Batalha et al., 2013; Costenla et al., 2011) and synaptotoxicity (Canas et al., 2009; Silva et al., 2007) or from the impact of $A_{2A}R$ on astrocytes (Matos et al., 2012) and microglia (Rebola et al., 2011). This prompts the hypothesis that $A_{2A}R$ antagonism may underlie the beneficial effects of caffeine upon chronic stress, in accordance with the role of synaptic (Magariños et al., 1997; Rebola et al., 2011) or glial dysfunction (Sanacora & Banasr, 2013) in mood disorders. This is suggested by previous observations that $A_{2A}R$ antagonists prolong escape behavior in two screening tests for antidepressant activity (El Yacoubi et al., 2003b; Yamada et

al., 2014) and prevent maternal separation-induced long-term cognitive impact (Batalha et al., 2013). We now used tissue-selective $A_{2A}R$ transgenic mice (Shen et al., 2008) to test if neuronal $A_{2A}R$ might control the modifications caused by chronic unpredictable stress (CUS).

4.2.2 Experimental design

Two genetic approaches were used. Global $A_{2A}R$ KO mice (g-$A_{2A}R$-KO) allowed us to observe if knocking down $A_{2A}R$ would replicate the ability of caffeine to prophylactically alleviate stress-induced alterations. CaMKII-α gene promoter-driven forebrain $A_{2A}R$ KO mice (fb-$A_{2A}R$-KO) do not express $A_{2A}R$ only in the neurons of the forebrain (including cortex, hippocampus and amygdala), as previously validated (Shen et al., 2008, 2013; Yu et al., 2008), and allow testing if it is neuronal $A_{2A}R$ that are selectively involved in the control of behavioral alterations and neurodegeneration resulting from chronic stress.

Mice of both knock out lines were individually housed and divided into 2 groups: the stress group was submitted to 3 weeks of chronic unpredictable stress protocol (Mineur et al., 2006; see Table 3), whereas control mice were handled daily. At the end of stress period, blood samples were collected at 9 a.m. and corticosterone was determined (radioimmunoassay, MP Biomedicals, Illkirch, France). Control (i.e., non-stressed) and stressed mice were individually housed during and after stress and were submitted to behavioral tests 24 h after the last stressor.

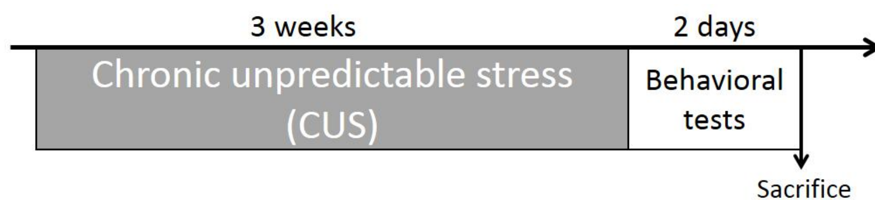

Figure 10: Schematic representation of the experimental design to evaluate the effects of chronic unpredictable stress in $A_{2A}R$ global knockout mice (g-$A_{2A}R$-KO) or in CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout mice (fb-$A_{2A}R$-KO).

4.2.3 Validation of the chronic unpredictable stress model

Chronic stress is expected to decrease gain of weight, increase corticosterone levels, trigger helpless-like and anxious-like behaviors, and decreased performance in memory tests. Accordingly, compared to controls (i.e., non-stressed), mice exposed to the 3-week protocol of CUS displayed: i) reduced weight gain (Fig. 11); ii) increased corticosterone plasma levels (Fig. 11); iii) increased immobility time in the forced swimming test (Fig. 12) indicative of a helpless-like state; iv) reduced time spent in the open arms of an elevated plus-maze (Fig. 12), indicative of an anxious state; v) impaired spatial reference memory, gauged as reduced time spent in the novel arm of a modified Y-maze test and lower recognition index in an object displacement test (Fig. 12).CUS decreased the immunoreactivity of syntaxin and SNAP25, synaptic markers, in the hippocampus (Fig. 13), in accordance with the critical impact of synaptic modifications in stress-induced behavioral alterations. Overall, these behavioral, biochemical and morphological alterations validate our CUS protocol.

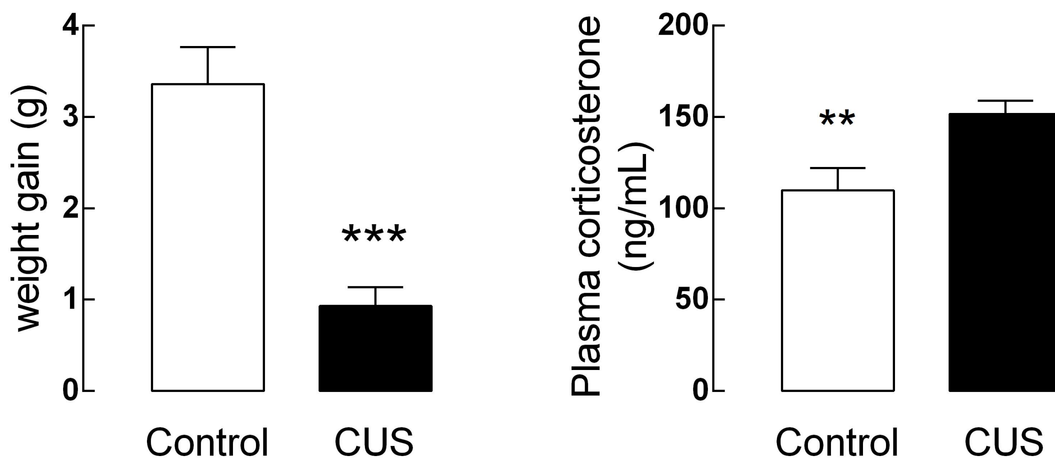

Figure 11: Chronic unpredictable stress (CUS) reduced gain of weight and increased plasma corticosterone levels in C57/Bl6 mice. Male mice (10 weeks old) were subjected to a 3-week period of CUS. Compared to control mice, CUS caused a decreased gain of weight in controls (**A**) and increased the plasma levels of corticosterone (**D**). Data are mean + SEM of n=7-10 mice/group. **p<0.01, ***p<0.001 using Student's *t* test.

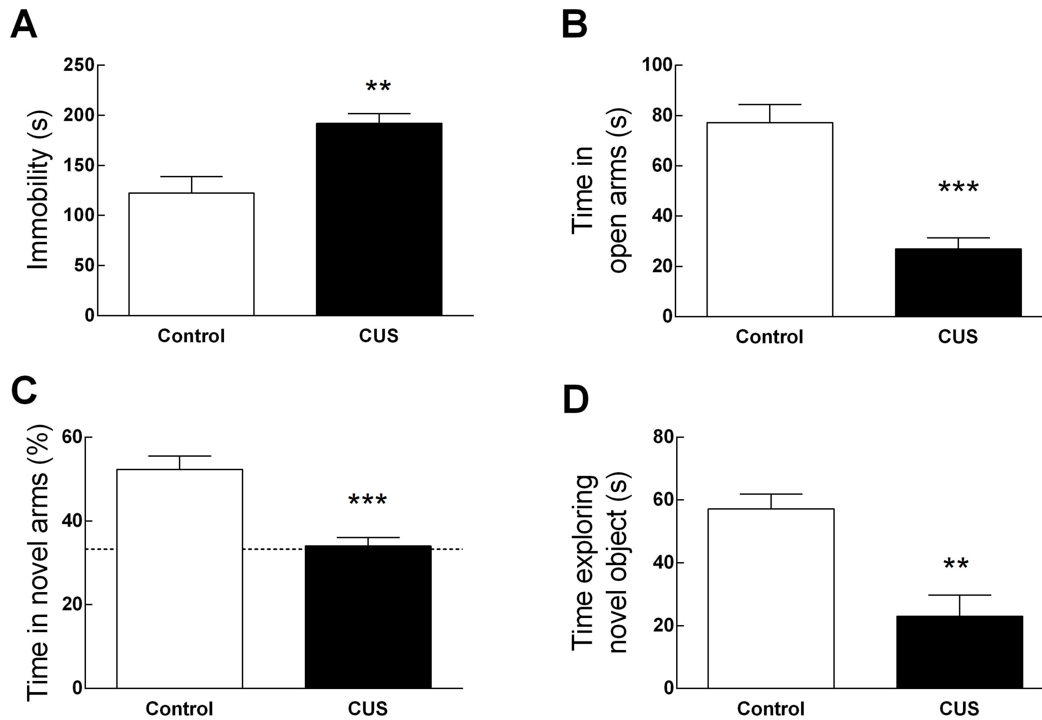

Figure 12: Chronic unpredictable stress (CUS) caused emotional and cognitive changes in mice. Male C57Bl/6 mice (10 weeks old) were subject to a 3-week period of CUS and were behaviorally evaluated after the last stressor. CUS caused an increase in despair-like behavior (**A**), an increase in anxiety-like behavior in the elevated plus maze task (**B**), and impaired memory performance in the two-trial Y maze (**C**) and in the object displacement paradigm (**D**). Data are mean + SEM of n=7-10 mice/group. **p<0.01, ***p<0.001 using Student's *t* test.

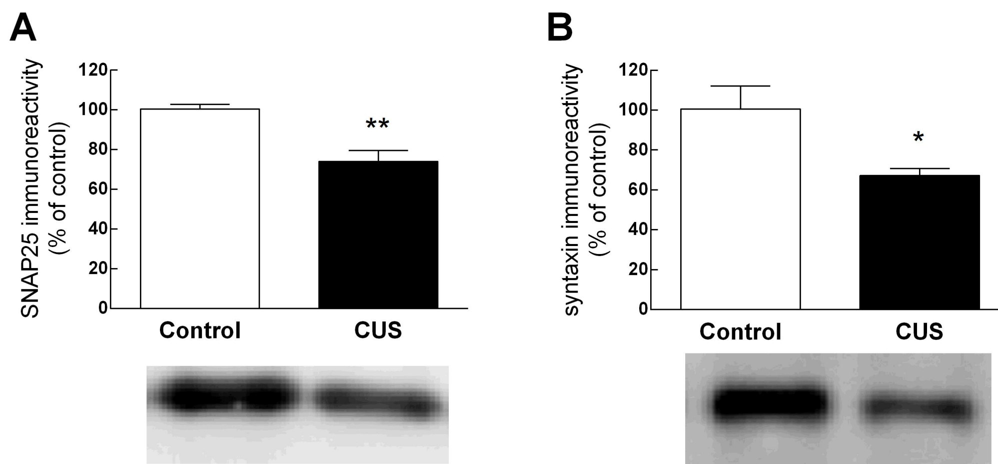

Figure 13: Chronic unpredictable stress (CUS) decreased synaptic markers' density in the hippocampi of mice. Male C57Bl/6 mice (10 weeks old) were subject to a 3-week period of CUS and behaviorally evaluated after the last stressor. CUS caused a decrease in synaptic proteins SNAP25 (**A**) and syntaxin (**B**). Data are mean + SEM of n=7-10 mice/group. *p<0.05, **p<0.01 using Student's *t* test.

4.2.4 Alterations of adenosine receptors upon chronic stress

We tested if CUS altered the density of A_1R and $A_{2A}R$ which could be the reason for the ability of caffeine to prevent CUS-induced changes while being largely devoid of effects in non-stressed mice (Kaster et al., 2015). CUS decreased the A_1R antagonist [^{3}H]DPCPX binding, both in total membranes and in synaptosomal membranes, but not in gliosomal (i.e. astrocytic, see Matos et al., 2012) membranes of the hippocampus (Fig.14A). By contrast, CUS enhanced the binding of $A_{2A}R$ antagonist [^{3}H]SCH58261 in synaptosomal (60.1 ± 8.7% larger in CUS compared to non-stressed mice; n=6, $p<0.05$), but not in total or in gliosomal membranes of the hippocampus (Fig.14B).

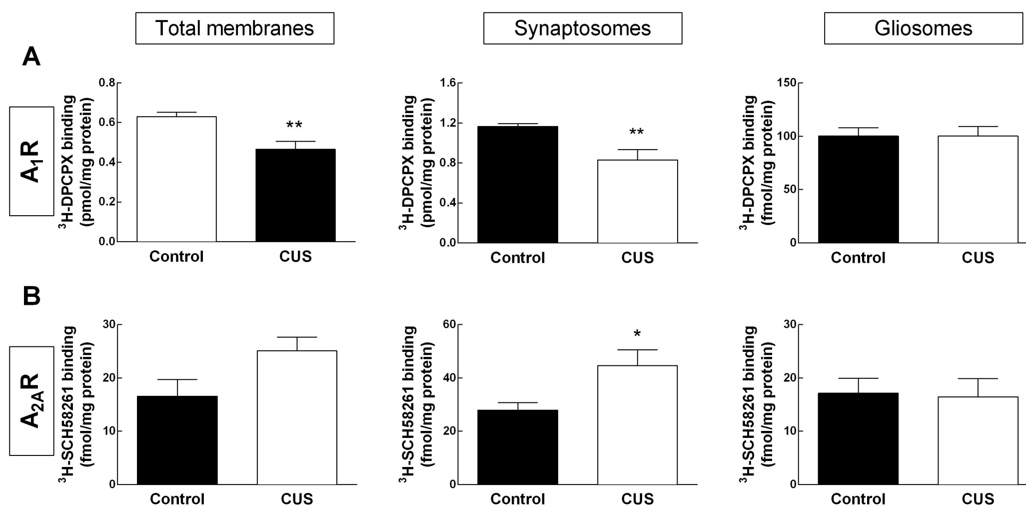

Figure 14: Chronic unpredictable stress alters the adenosine neuromodulation system in the hippocampus. Male mice (10 weeks old) were subjected to a 3-week period of CUS and sacrificed for the preparation of total, synaptosomal (i.e., from synapses) and gliosomal (i.e., from astrocytes) membranes from the hippocampus. (**A**) The binding density of adenosine A_1 receptors (A_1R), evaluated with the A_1R antagonist [^{3}H]DPCPX (10 nM), was decreased in total and synaptosomal membranes and unaltered in gliosomal membranes of CUS-subjected mice compared to non-stressed mice. By contrast, there was a selective increase of the binding density of the $A_{2A}R$ antagonist [^{3}H]SCH58261 in synaptosomal membranes from CUS-subjected mice, without changes in its binding density in total or gliosomal membranes (**B**). Data are mean + SEM of 5-6 mice per group; *$p<0.05$, **$p<0.01$ using an unpaired Student's *t* test.

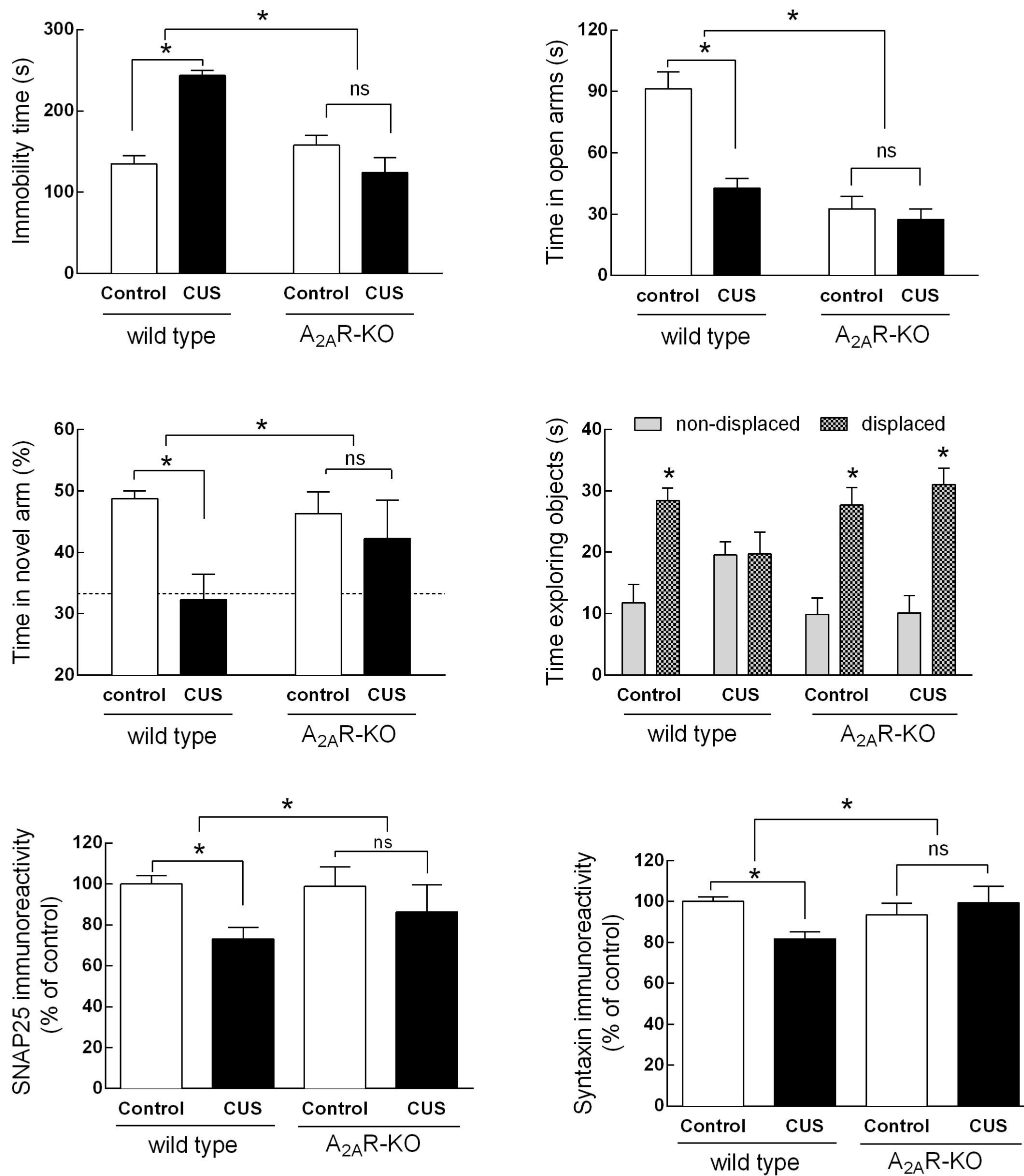

Figure 15: The genetic blockade of adenosine A_{2A} receptors prevents chronic unpredictable stress (CUS)-induced behavioral and neurochemical alterations in the hippocampus. Male mice (10 weeks old) were subjected to a 3-week period of CUS before behavioral evaluation 24 h after the last stressor. In CUS-subjected mice, as compared to wild type animals, the genetic elimination of A_{2A}R in global A_{2A}R knockout mice (**g-A_{2A}R-KO**) prevented the CUS-induced despair-like behavior evaluated in the forced swimming test (**A**), anxiety-like behavior evaluated in the elevated plus maze test (**B**), impaired memory performance evaluated in a two-trial Y-maze test (**C**) and object displacement task (**D**), and decrease of synaptic markers such as SNAP25 (**E**) and syntaxin (**F**) in hippocampal nerve terminals. Data are mean + SEM of n=8-10 mice per group in the behavioral assays (**A-D**) and n=5-6 in the neurochemical analysis (**E**, **F**); *$p<0.05$ using a two-way ANOVA followed by a Newman Keuls *post hoc* test, except the object displacement task in which a Student's *t* test was used; ns: non-significant.

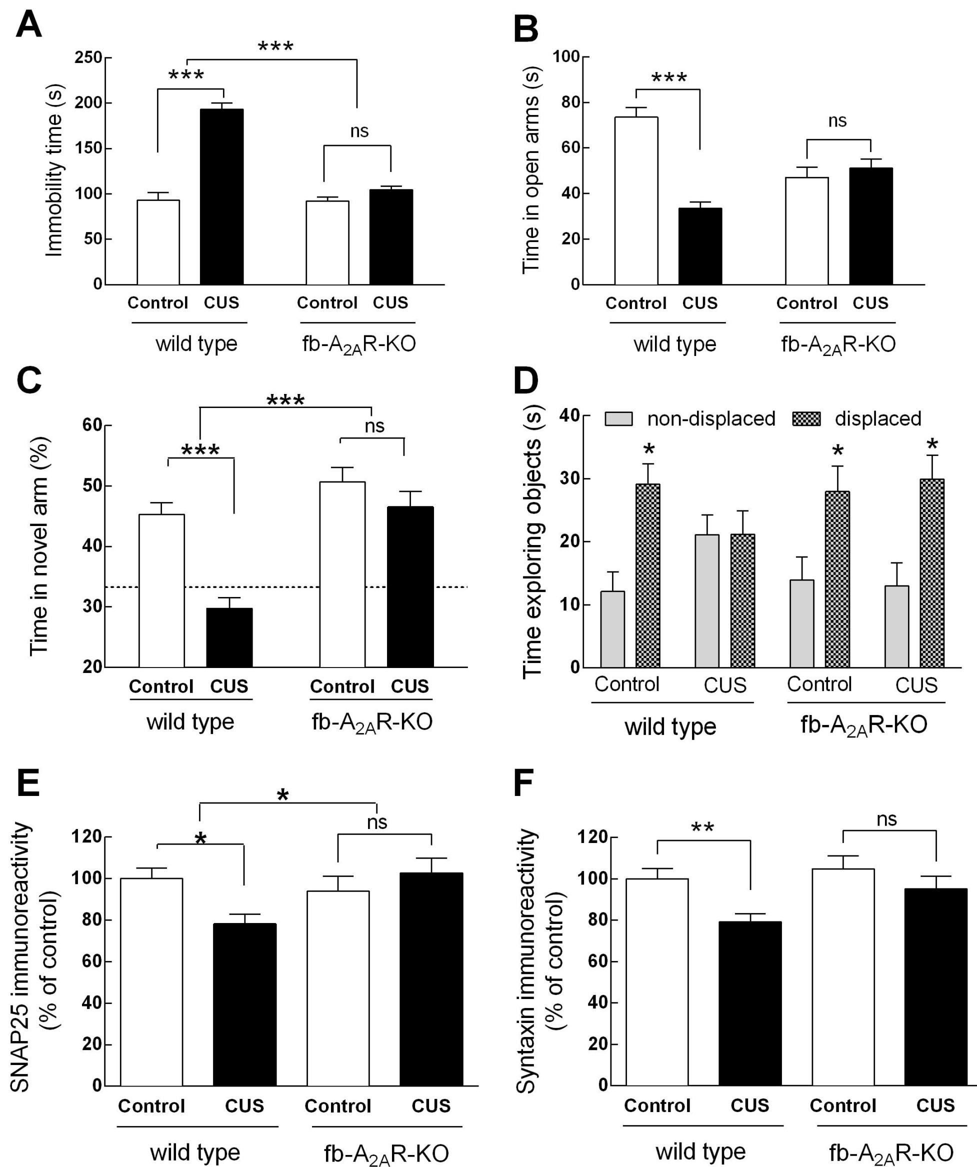

Figure 16: The selective deletion of neuronal A_{2A} receptors prevents chronic unpredictable stress (CUS)-induced behavioral and neurochemical alterations in the hippocampus. Male mice (10 weeks old) were subjected to a 3-week period of CUS before behavioral evaluation 24 h after the last stressor. By contrast with the impact of CUS in wild type mice, in CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout (**fb-A_{2A}R-KO**) mice, CUS failed to trigger despair-like behavior in the forced swimming test (**A**), anxiety-like behavior in the elevated plus maze test (**B**), impaired memory performance in a modified Y maze (**C**) and object displacement test (**D**). Also in fb-A_{2A}R-KO mice, CUS did not affect the density of synaptic markers such as SNAP25 (**E**) and syntaxin (**F**) in hippocampal nerve terminals. Data are mean + SEM of n=7-9 mice per group in the behavioral assay (**A**-**D**), and n=5-6 in the neurochemical analysis (**E**, **F**); *$p<0.05$ using a two-way ANOVA followed by a Newman Keuls *post hoc* test, except the object displacement task in which a paired Student's *t* test was used.

4.2.5 $A_{2A}R$ control the burden of chronic stress

In accordance with this CUS-induced enhancement of $A_{2A}R$, the global deletion of $A_{2A}R$ protected against CUS-induced alterations. CUS did not change the behavior of global $A_{2A}R$-KO mice in the forced swimming (Fig. 15A), EPM (Fig. 15B), modified Y-maze (Fig. 15C), and object displacement tests (Fig. 15D). The density of synaptic proteins such as syntaxin (Fig. 15F) or SNAP25 (Fig. 15E) in the hippocampus of global $A_{2A}R$-KO mice was also unchanged upon CUS by contrast to the alterations found in wild type littermates. Notably, in contrast to wild-type mice, stressed g-$A_{2A}R$-KO mice also failed to display alterations in the levels of vGluT1 (Fig. 17A), a selective marker of glutamatergic nerve terminals.

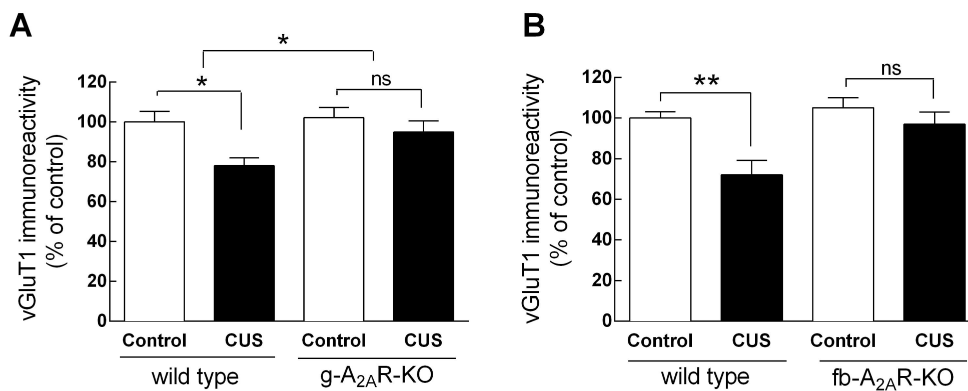

Figure 17: The deletion of A_{2A} receptors prevents chronic unpredictable stress (CUS)-induced decrease of vGluT1 density in the nerve terminals of the hippocampus. Male mice (10 weeks old) were subject to a 3-week period of CUS and sacrificed for the preparation of synaptosomal (i.e., from synapses) membranes from the hippocampus and the quantity of protein was probed through Western blot analysis. The density of the vesicular glutamate transporter type 1 (vGluT1) was decreased in synaptosomal membranes of CUS-subjected mice compared to non-stressed mice. The global genetic deletion (g-$A_{2A}R$-KO, **A**) and the neuronal deletion (fb-$A_{2A}R$-KO, **B**) of A_{2A} receptors prevented the CUS-induced decrease of vGluT1. Data are mean + SEM of n=5-6 mice per group. *$p<0.05$ using a two-way ANOVA followed by a Newman Keuls *post hoc* test, except the object displacement task in which a Student's *t* test was used; ns: non-significant.

4.2.6 Neuronal $A_{2A}R$ control the burden of chronic stress

Given the fact that $A_{2A}R$ density is changed by chronic stress in parallel to behavioral alterations and synaptic rearrangement, namely of glutamatergic terminals, we hypothesized that the neuronal $A_{2A}R$ would play a pivotal role in the emergence of CUS-induced changes. This was probed by testing the impact of CUS on CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout (fb-$A_{2A}R$-KO) mice (n=7-9 mice/group). These fb-$A_{2A}R$-KO mice selectively lack neuronal $A_{2A}R$ in the forebrain and display a blunted $A_{2A}R$-mediated control of glutamatergic synapses (Shen et al., 2008). Fb-$A_{2A}R$-KO mice had a behavior similar to wild type mice under control conditions (Figs. 16A-D); however, fb-$A_{2A}R$-KO mice did not display CUS-induced behavioral alterations neither of mood (Figs. 16A,B) nor memory (Figs. 16C,D). CUS also failed to modify the density of synaptic proteins such as syntaxin (Fig. 16F) and SNAP25 (Fig. 16E) as well as vGluT1 (Fig. 17) in fb-$A_{2A}R$-KO mice, by contrast to the effect of CUS in wild type mice.

4.2.7 Conclusion

This study shows that genetic blockade of adenosine $A_{2A}R$ mimics the beneficial effects of caffeine intake upon chronic stress (published in Kaster et al., 2015). We also show that it is the neuronal $A_{2A}R$ that play a critical role in controlling the burden of chronic stress in adult mice. The constellation of behavioral changes upon CUS are comparable to those observed in stressed or depressed individuals (Hill et al., 2012; Willner, 1997). Furthermore, the reported inverse relation between caffeine consumption and the incidence of depression (Lucas et al., 2011; Smith, 2009) or suicide (Kawachi et al., 1996; Lucas et al., 2014) prompts a pivotal role of $A_{2A}R$ controlling mood disorders. However, the role of $A_{2A}R$ on anxiety still remains unclear (Correa & Font, 2008) typified by the inconsistent effects of the different $A_{2A}R$ manipulations.

A major advance provided by this study is the identification that it is the neuronal $A_{2A}R$ that control behavioral dysfunction upon CUS, as concluded by

the elimination of CUS-induced changes in CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout (fb-$A_{2A}R$-KO) mice. This excludes a major participation of peripheral adenosine receptors (Minor et al., 2008) and does not support that glial $A_{2A}R$ might play a prominent role in controlling CUS-induced alterations, as occurs in animal models of Parkinson's disease (Yu et al., 2008) or upon treatment with lipopolysaccharide (Rebola et al., 2011). By contrast, the data are compatible with a key role of $A_{2A}R$ in glutamatergic terminals defining the synaptic dysfunction underlying the behavioral alterations associated with repeated stress (Duman & Aghajanian, 2012; Popoli et al., 2011). In fact, CUS leads to alterations of synaptic markers and synaptic function in the hippocampus, a brain region known to play a pivotal role in the maladaptive changes upon chronic stress. Furthermore, in the hippocampus, $A_{2A}R$ are most abundantly located in nerve endings and are selectively engaged to control synaptic plasticity (Costenla et al., 2011). Additionally, noxious brain conditions trigger an $A_{2A}R$ up-regulation, which is most evident in synapses (Cognato et al., 2010; Duarte et al., 2012), namely in glutamatergic synapses (Cunha, 2005). This $A_{2A}R$ up-regulation is accompanied by a gain of function of $A_{2A}R$ (reviewed by Cunha & Agostinho, 2010) leading to synaptic dysfunction, as testified by the ability of $A_{2A}R$ antagonists to prevent synaptic plasticity dysfunction upon aging (Prediger et al., 2005) and maternal separation (Batalha et al., 2013) as well as the loss of synaptic markers upon different noxious brain conditions, namely upon repeated restraint stress (Cunha et al., 2006). It still remains to be determined how an enhanced $A_{2A}R$ function triggers synaptic dysfunction, in view of our current ignorance of the transducing systems operated by these pleiotropic $A_{2A}R$ (Fredholm et al., 2007; Zezula & Freissmuth, 2008) and if this synaptic dysfunction is associated with the loss of excitatory synapses. Clarifying this aspect is of notorious importance given that it seems a mechanism common to the $A_{2A}R$-mediated control of memory and of mood alterations.

Overall, this robust ability of $A_{2A}R$ to control CUS-induced alterations provides a rationale to understand the ability of caffeine to attenuate the burden of stress. The prevention by regular (not acute) caffeine consumption of different alterations caused by repeated stress had previously been noted by others and is in tight agreement with the inverse correlation between caffeine intake and

the incidence of depression. Given that caffeine intake increases in stressed individuals (Harris et al., 2007), one can speculate that this may be a prophylactic anti-stress measure to normalize mood-related behavioral changes through a normalization of synaptic function resulting from $A_{2A}R$ blockade. The present results also show a therapeutic benefit resulting from the deletion of $A_{2A}R$, in a manner similar to the reversion of memory impairment in aged rodents (Prediger et al., 2005b).

In comparison with the genetic model of depression described in the previous chapter, H/Rouen, $A_{2A}R$ deletion was not only able to prevent the memory deficits (Y-maze and object displacement tests) associated with the CUS model but also behavioral changes related to depression (forced swimming test).

We then set to get more insight into two key questions:

1) Given the changes induced by $A_{2A}R$ in the glutamatergic nerve terminals, is the ablation of these receptors in neurons sufficient to prevent glutamate-specific noxious stimuli? – we approached that question by probing the neurodegeneration resulting from kainate-induced seizures in wild type compared to CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout mice (Chapter 4.3)
2) The synaptic alterations caused by $A_{2A}R$ in the hippocampus give us an insight on their role and how their blockade might bring benefits, but the prevention of all the stress-induced alterations by $A_{2A}R$ cannot be explained by the synaptic plasticity changes in the hippocampus alone. Thus, we investigated the impact of the neuronal $A_{2A}R$ down-regulation in the amygdala, a brain region pivotal in the control of the stress response and development of depression (Chapters 4.4 and 4.5).

4.3 DELETION OF NEURONAL ADENOSINE A_{2A} RECEPORS PREVENTS THE KAINATE-INDUCED SYNAPTIC AND NEURONAL DAMAGE

4.3.1 Introduction

Kainate is a natural marine acid present in some seaweed. It is a cyclic analog of L-glutamate, and is a potent agonist to its selective receptors, kainate receptors, and a partial agonist of AMPA receptors (Bettler & Mulle, 1995). Kainate receptors are ligand-gated channels permeable to cations, including Ca^{2+}, and are activated by low concentrations of kainate (Pinheiro & Mulle, 2006). Kainate receptors are present both pre- and postsynaptically with apparently opposite effects (Malva et al., 2003). Presynaptically, they inhibit glutamate release (Chittajallu et al., 1996), although the underlying mechanism has not been clarified. Postsynaptically, they increase excitability through the facilitation of cation transport (Vignes & Collingridge, 1997). The outcome seems to depend on the availability and concentration of agonists. In our experimental conditions, kainate induces postsynaptic hyperpolarization, resulting in epileptic seizures (Ben-Ari et al., 1980; Sperk, 1994) and neuronal degeneration (Coyle, 1983; Sperk et al., 1985). Moreover, kainate induces astrogliosis and microgliosis, causing the production of nitric oxide and cytokines that expand the injury and the delayed neuronal loss (Chen et al., 2005).

Adenosine is a neuromodulator that mainly acts in the brain through the activation of A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) (Fredholm et al., 2005). The predominant effect of adenosine is an inhibition operated by the more abundant A_1R (de Mendonça et al., 2000; Fredholm et al., 2005). However, in spite of the massive (During and Spencer, 1992) and sustained (Berman et al., 2000) release of adenosine upon seizures, this inhibitory A_1R system appears to have a limited time-window of effectiveness in epilepsy (reviewed in Dunwiddie, 1999). In fact, there is a decreased density of A_1R after the induction of seizures and a reduced efficiency of the A_1R system with the progression of this condition (Ochiishi et al., 1999; Rebola et al., 2003; Young and Dragunow, 1994;

but see Gouder et al., 2003). This is in accordance with the general idea that A_1R act as 'gate-keepers' of brain tissue viability, increasing the threshold chronic brain conditions (Cunha, 2005).

Chronic noxious brain conditions not only lead to a decreased density and function of A_1R but also increase the density of $A_{2A}R$ (reviewed in Cunha, 2005). In particular, the density of $A_{2A}R$ increases in limbic regions in different experimental models of epilepsy (Rebola et al., 2005a), and the pharmacological blockade or the genetic inactivation of $A_{2A}R$ confers a robust neuroprotection in animal models of epilepsy (El Yacoubi et al., 2008, 2009). However, it is unclear if $A_{2A}R$ control the emergence of seizures or if it is instead selectively controlling the development of seizure-induced neurodegeneration, which is a key component of the pathophysiological process of epilepsy (Meldrum, 2002; Pitkänen & Sutula, 2002). Furthermore, it is also unclear if it is neuronal $A_{2A}R$ that are selectively involved in the control of seizure-induced damage, as we reported for memory and mood dysfunction. Therefore, we decided to take advantage of forebrain-driven $A_{2A}R$ knockout (fb-$A_{2A}R$-KO) mice to define the role of neuronal $A_{2A}R$ in the control of convulsions and consequent brain damage caused by kainate administration, an animal model of temporal lobe epilepsy (Ben-Ari, 1985).

Since $A_{2A}R$ facilitate glutamatergic transmission, their blockade is potentially a beneficial strategy to prevent the neuronal damage caused by excessive activation of glutamate receptors, in particular kainate-induced toxicity. Indeed, the intra-hippocampal administration of a selective $A_{2A}R$ antagonist with kainate prevents hippocampal damage (Jones et al., 1998), and peripheral administration of caffeine or a selective $A_{2A}R$ antagonist prevent delayed, long-term memory impairment in rats as well as synaptic toxicity (Cognato et al., 2010). The effects of blocking $A_{2A}R$ probably counteract the action of increased release of adenosine induced by kainate (Carswell et al., 1997).

However, we do not know if the blockade of neuronal-only $A_{2A}R$ is able to prevent the behavioral and/or morphological changes cause by the kainate-induced excitotoxicity. That being confirmed, it would provide a plausible hypothesis for the mechanism underlying the protection provided by the deletion of $A_{2A}R$ upon chronic stress.

4.3.2 Experimental design

We used fb-A_{2A}R-KO mice and their wild type (WT). As described in the methods, we induced convulsions by subcutaneous injection of kainate, observed the animals for 2 h and scored the convulsive behavior according to the Racine scale. There were four groups in total: WT/control, WT/kainate, fb-A_{2A}R-KO/control, fb-A_{2A}R-KO/kainate (control being injected with saline 0.9%). Twenty-four hours after injection, the animals were killed, the brain hemispheres separated and either submersed in 4% paraformaldehyde solution (in 0.9% saline with 4% sucrose) for 24 h for histo- and immunohistochemical analyses or the hippocampus dissected and frozen at -80ºC for posterior synaptosomal preparation and Western blot analysis.

4.3.3 Deletion of neuronal A_{2A}R does not change the seizures severity caused by kainate injection

The administration of kainate (35 mg/kg, sc) to C57Bl6 mice triggered a convulsive period that started within 10 min up to 15 min and reached a maximal intensity of 5-6 in the modified scale of Racine (Schauwecker & Steward, 1997) after 65-80 min. Figure 18 presents the average maximal intensity of seizures as well as the rate of survival of the mice injected with kainate. Most animals presented a status epilepticus close to the maximum in the Racine scale, excluding score 6, which causes death (WT: 4.8 ± 0.2; fb-A_{2A}R-KO: 4.7 ± 0.2; n=10), and the genetic neuronal deletion of A_{2A}R did not decrease the severity of seizures (two-way ANOVA followed by Tukey *post hoc* test).

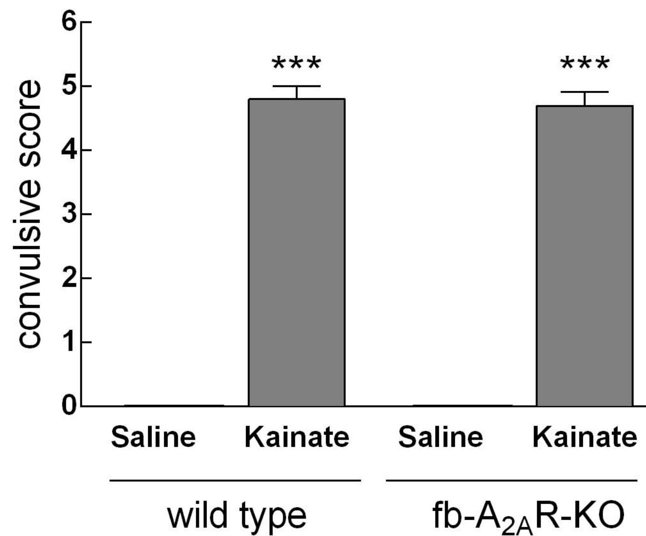

Figure 18: Kainate injection induces epileptic seizures that are not prevented by the neuronal deletion of $A_{2A}R$. Kainate (35 mg/kg) was injected into male adult CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout (fb-$A_{2A}R$-KO) or wild-type (WT) mice subcutaneously (s.c.) and animals were observed for 2 h. Seizure-related behavior was scored according to the Racine scale. Data are mean + SEM of n=10-11 mice per group. ***$p<0.001$ *vs.* control, using a two-way ANOVA followed by Tukey *post hoc* test. ns: non-significant

4.3.4 Elimination of neuronal $A_{2A}R$ abrogates kainate-induced toxicity in the hippocampus

Kainate administration also caused pyramidal cell damage in the *cornu Ammonis* of the hippocampus, although it was considerably more discreet than the pyramidal cell damage observed in rats, as previously reported (Benkovic et al., 2004; Schauwecker and Steward, 1997). However, at 24 hours post-treatment with kainate, there were pyramidal cell-like profiles stained with FluoroJade-C in the pyramidal cell layer of both CA1 and CA3 regions (Figs. 19A,20). Fluoro-Jade C fluorescence was increased over controls in both CA1 and CA3 region (CA1: 3.59 ± 0.34; CA3: 2.40 ± 0.60; n=5; fluorescence over background). Kainate-injected fb-$A_{2A}R$-KO mice, on the contrary, did not present significant fluorescence over background (CA1: 1.03 ± 0.02; CA3: 1.05

± 0.2; n=4). Both control groups did not present altered fluorescence (CA1: WT, 1.04 ± 0.01, fb-A_{2A}R-KO, 1.02 ± 0.02; CA3: WT, 1.03 ± 0.03, fb-A_{2A}R-KO, 1.04 ± 0.02; n=4-5).

In parallel with this putative damage of pyramidal neurons, the treatment with kainate triggered astrogliosis and microgliosis in both the CA1 and CA3 regions (Figs. 19B,C, respectively). In fact, there was an increased number of GFAP-stained profiles (2.5-fold increase in the number of GFAP-stained profiles, n=5) and each labeled astrocyte also displayed a more intense GFAP staining compared to saline controls (inserts in Fig. 20). There was also an increased number of profiles labeled with CD11b and, again, each labeled profile displayed a more intense CD11b staining compared to saline controls (inserts in Fig. 20), in accordance with the expected behavior of activated microglia (Jensen et al., 1997).

Kainate induced an increase of 62.6 ± 4.7% and 84.5 ± 5.3% of CD11b immunoreactivity over controls in CA1 and CA3 regions, respectively (n=5). Both fb-A_{2A}R-KO groups showed microglial activation similar to WT/control (CA1: control, +0.7 ± 1.9%, kainate, +7.6 ± 6.0%; CA3: control, -4.6 ± 2.7%, kainate, +6.2 ± 1.8%; n=5).

Astrocytic activation mimics astrogliosis: only WT animals injected with kainate displayed increased immunoreactivity to GFAP over WT controls (CA1: +72.4 ± 7.6%; CA3: +74.9 ± 12.4%; n=5). Neither fb-A_{2A}R-KO groups displayed increased astrogliosis (CA1: control, -0.8 ± 4.0%, kainate, +7.9 ± 1.7%; CA3: control, +1.2 ± 3.6%, kainate, +6.8 ± 3.7%; n=5).

4.3.5 Deletion of neuronal A_{2A}R prevents the loss of synaptic markers in the hippocampus induced by kainate

Kainate causes excitotoxicity that leads to synaptic loss, neuronal death and glial activation. The previous two sections have shown that the deletion of A_{2A}R from neurons prevents both neuronal death and glial activation. In this section the results related to synaptic loss are shown.

All synaptic markers analyzed through Western blot were reduced from synaptic membranes from the hippocampus after kainate injection. The glutamate

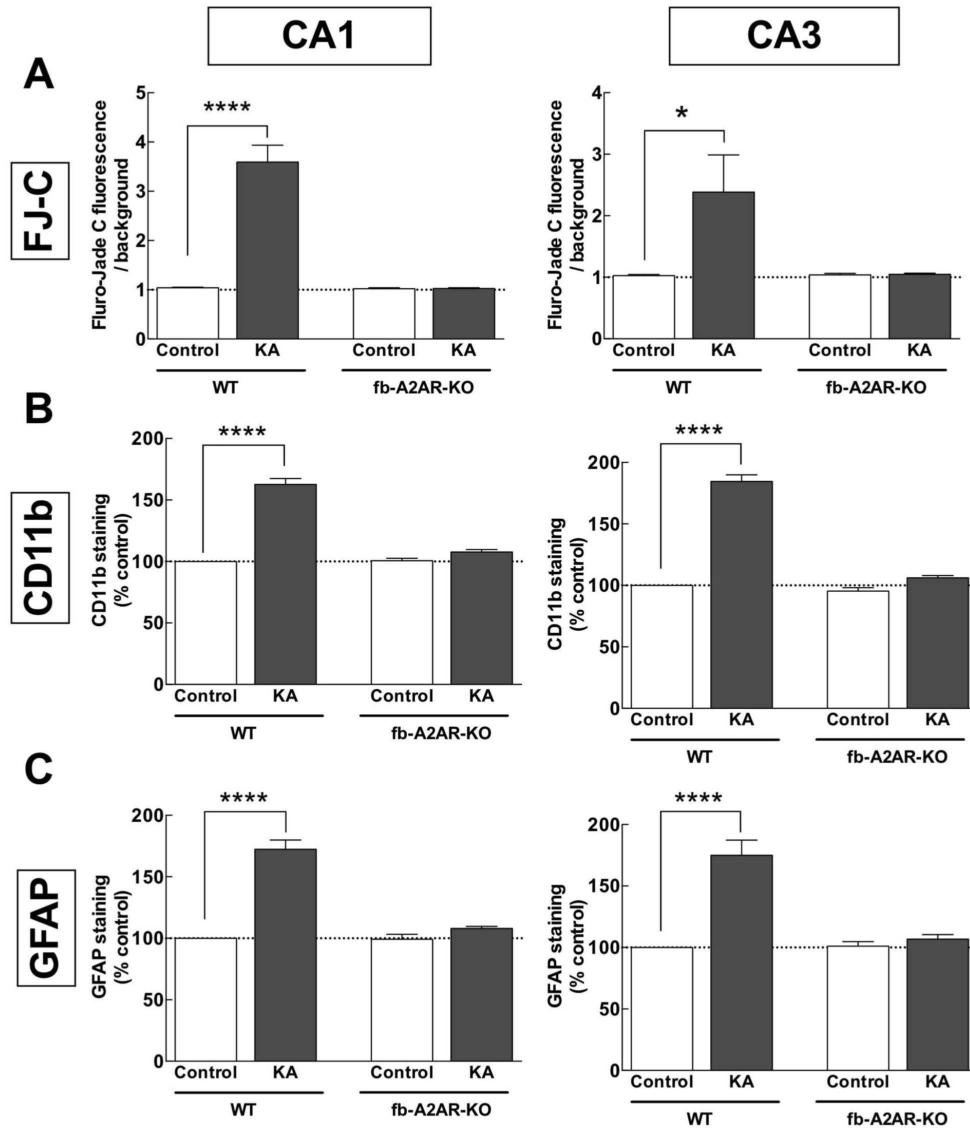

Figure 19: Selective deletion of neuronal $A_{2A}R$ from neurons prevents neuronal death, microglial and astrocytic activation in both CA1 and CA3 regions of the hippocampus caused by kainate injection. Kainate (35 mg/kg) was injected into male adult CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout (fb-$A_{2A}R$-KO) or wild type (WT) mice subcutaneously (s.c.) and animals were observed for 2 h. Twenty-four hours later, one hemisphere was submersed in 4% paraformaldehyde, 24 h later, in 30% sucrose, and subsequently sectioned (20 μm thick) and mounted. Kainate induced neuronal death as observed by increased Fluoro-Jade C staining, while fb-$A_{2A}R$-KO mice injected with kainate presented values similar to vehicle-injected animals (control) (**A**). Similarly, kainate induced microglial activation (**B**) and activation of astrocytes (**C**), and fb-$A_{2A}R$-KO mice presented values close to controls. Data are mean + SEM of n=4-5 mice per group. ****$p<0.0001$ using a two-way ANOVA followed by Tukey *post hoc* test. Legend: CA1, *cornu ammonis* region 1; CA3, *cornu ammonis* region 3; CD11-b, cluster of differentiation molecule 11B; FJ-C, Fluoro-Jade C; GFAP, glial fibrillary acidic protein; KA, kainate.

transporter vGluT1 was reduced by 18.6 ± 1.8% ($p<0.001$, n=6, one-sample *t* test) whereas fb-A_{2A}R-KO displayed values similar to control (-0.6 ± 1.3%, n=6). fb-A_{2A}R-KO animals do not display changes when compared to wild types (+0.9 ± 1.5%, n=6). Similarly, kainate reduced SNAP25 (Synaptosomal-Associated Protein, 25kDa) and syntaxin-I density (-16.5 ± 1.9% and -13.0 ± 2.0%, $p<0.01$, n=6, one-sample *t* test). fb-A_{2A}R-KO mice injected with kainate presented values similar to controls (SNAP25: -2.9 ± 1.6%; syntaxin-I: -0.3 ± 0.6%; n=6). Knocking out A_{2A}R in neurons did not change either SNAP25 (+1.2 ± 2.0%, n=5) nor syntaxyn-I density (+2.7 ± 0.8%, n=5).

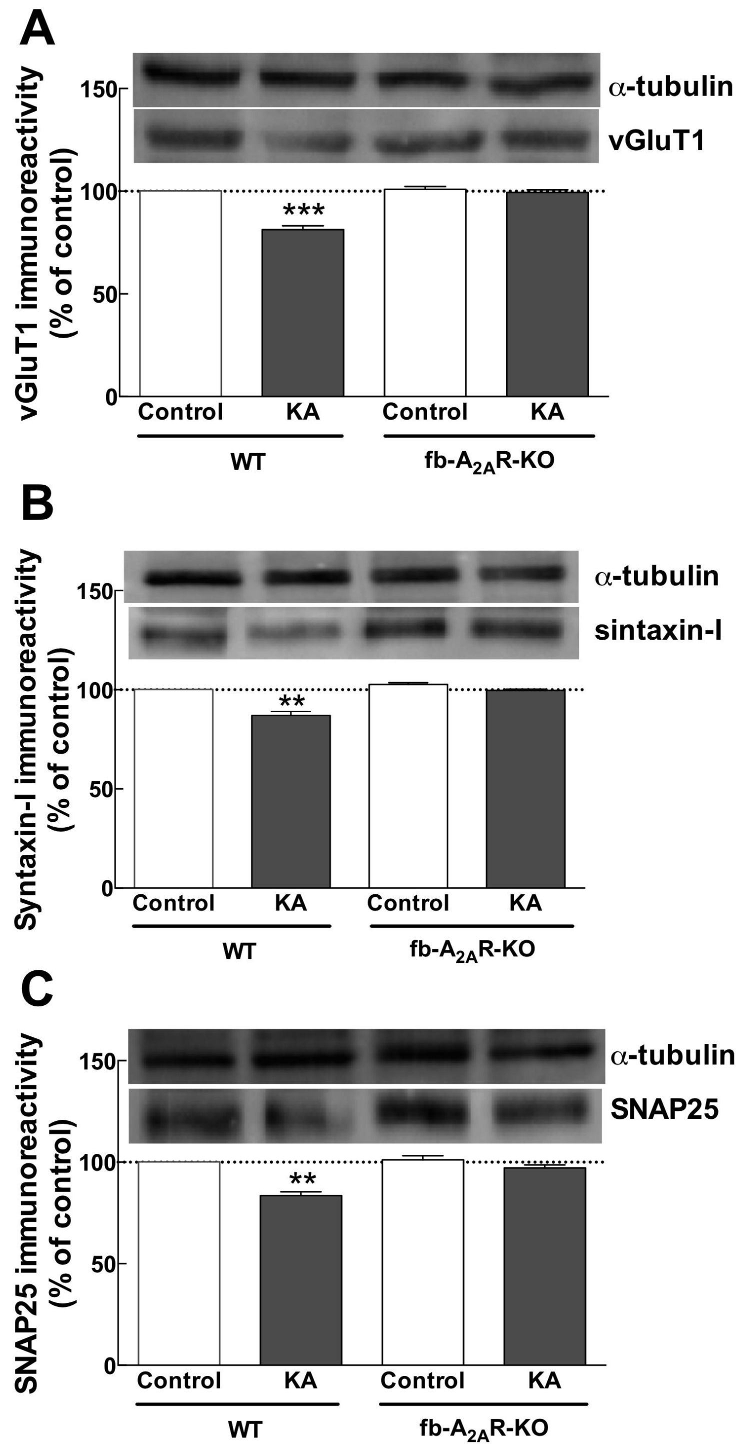

Figure 20: Selective deletion of neuronal $A_{2A}R$ from neurons prevents synaptic loss in the hippocampus caused by kainate injection. Kainate (35 mg/kg) or saline (control) was injected into male adult CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout (fb-$A_{2A}R$-KO) or wild type (WT) mice subcutaneously (s.c.). Twenty-four hours later, mice were killed and the hippocampus dissected for synaptosomal preparation. Subsequently, Western blot analysis was carried out for synaptic markers. Kainate induced loss of glutamate transporter vGluT1 (**A**), syntaxin-I (**B**) and SNAP25 (**C**). Deletion of $A_{2A}R$ (fb-$A_{2A}R$-KO) from neurons was able to prevent the decrease of all synaptic markers analyzed. **$p<0.01$, ***$p<0.001$, one-sample *t* test *vs.* 100% (WT/control), n=5-6.

4.3.6 Conclusion

In previous chapters, it was shown that the antagonism of $A_{2A}R$ was able to prevent memory impairment and mood-related changes in two animal models of mood disorders, one genetic and one behavioral. These behavioral changes were accompanied by alterations in the synaptic content in the hippocampus, which were also rescued by the blockade of $A_{2A}R$. Importantly, the use of the CaMKII-α gene promoter-driven forebrain $A_{2A}R$ knockout (fb-$A_{2A}R$-KO) mice allowed isolating the effects of neuronal $A_{2A}R$ upon stress. Neuronal $A_{2A}R$ ablation proved to be able to prevent both behavioral and neurochemical changes at the same extent as the global ablation or pharmacological blockade, thus these data position them as pivotal in controlling the effects of noxious conditions.

As previously described in this thesis, we hypothesized that the noxious effects of stress on the hippocampus occur through excitotoxic effects leading to impaired hippocampal function and ultimately neurodegeneration. Thus, we wanted to study the effects of the neuronal $A_{2A}R$ in a model of excitotoxicity.

One of the most widely used models is the peripheral injection of kainate. In our experiments, kainate injection caused convulsions, which were not prevented by the deletion of $A_{2A}R$. In contrast, mice lacking neuronal $A_{2A}R$ did not present morphological changes following kainate-induced convulsions: neuronal death (FJ-C), microglial and astrocytic activation (CD11b and GFAP). These results suggest that neuronal $A_{2A}R$ deletion is not sufficient to prevent the immediate excessive depolarization caused by kainate but rather the delayed consequences of that depolarization.

Our data suggest that astrogliosis and microgliosis occur as a response to neuronal death, in agreement with the role of neuroinflammation in the brain and upon damage (Zhang & Zhu, 2011). Since fb-$A_{2A}R$-KO mice still express $A_{2A}R$ in astrocytes and microglia, the primary protection occurs by the ablation of neuronal $A_{2A}R$. Nevertheless, the role of glia in aggravating the neuronal damage should not be disregarded, as excessive release of cytokines promotes neuronal dysfunction and the pharmacological blockade of $A_{2A}R$ confers protection through the control of neuroinflammation (Rebola et al., 2011).

Kainate causes excitotoxicity by acting as an agonist of glutamate receptors (kainate and AMPA) and, in the hippocampus, $A_{2A}R$ are mainly localized in synapses (Rebola et al., 2005), thus, it is likely that $A_{2A}R$ facilitate kainate-induced neurodegeneration at the synaptic level. Accordingly, synaptic deregulation was not observed in fb-$A_{2A}R$-KO mice. SNAP25 and syntaxin-I are part of the SNARE (Soluble NSF Attachment Protein REceptor) complex, which mediates the fusion of vesicles with the plasmatic membrane, primarily for the release of neurotransmitters. Thus, the reduction of these proteins from synaptic membranes (synaptosomes) indicates a loss of overall synaptic function. Kainate injection caused a decrease of both SNAP25 and syntaxin-I densities in hippocampal synaptosomes; deletion of neuronal $A_{2A}R$ prevented this outcome. vGluT1, the most abundant vesicular glutamate transporter in the hippocampus, is located presynaptically in glutamate-releasing, i.e. glutamateric, neurons. After glutamate is released into the synaptic cleft it must be degraded or recycled: it is up taken by the presynaptic neuron (or astrocytes) and subsequently transported to vesicles for novel release (Liguz-Lecznar & Skangiel-Kramska, 2007). As the other synaptic proteins, vGluT1 density was reduced by kainate injection. This result suggests that the SNAP25 and syntaxin-I reduction in synapse might be associated with a reduction of glutamatergic transmission.

With this work we did not directly demonstrate that the mechanism through which the deletion of $A_{2A}R$ prevents noxious changes upon chronic stress is by preventing the excitotoxicity-mediated synaptic loss. But excitotoxicity has been argued to play a pivotal role in the etiology of mood disorders (Popoli et al., 2011) and $A_{2A}R$ have been shown to play an important role in other excitotoxic-related diseases, e.g. Alzheimer’s disease (Canas et al., 2009). Thus, we have here strengthened the hypothesis that the antagonism of neuronal $A_{2A}R$, and not glial, is sufficient to prevent excitotoxic processes, which can explain its neuroprotective effects upon chronic stress.

4.4 BEHAVIORAL MODELS OF DEPRESSION

4.4.1 Introduction

The heterogeneity of depressive symptoms suggests the existence of different subtypes of depression. Thus, animal models used to study this disease might be valid for one or a few of these subtypes, and one must take into account the individual differences in the response to the stimuli leading to depression, as happens in humans. This means that it is unlikely to achieve the goal of establishing a single animal model that is able to reflect the various subtypes of depression. Therefore, it is of particular importance to probe if any given modification associated with an altered emotional behavior is actually present in different manipulations aimed at triggering the emergence of the array of behavioral modifications that tentatively allow building a model of repeated or chronic stress.

Given that environmental stress is one of the pivotal factors associated with the development of depression, animal models are often built upon the exposure to different types of stressors, presented repeatedly, that represent stressors that patients with depression might have faced. In chapter 4.2, we have described one of these models in mice (chronic unpredictable stress, CUS), but other more etiologically pure models exist, some of which will be described below (physical stress, social stress and immobilization).

When choosing the stressor to create a depression model, one ought to consider the type and the characteristics of the stressor (reviewed by Anisman & Matheson, 2005). We used two types of stressors: neurogenic (physical stress) and psychogenic (social defeat and immobilization). The characteristics are similar in all models: low controllability, high predictability, low uncertainty, chronicity and intermittence. This combination of characteristics led us to consider the 3 manipulations to be “chronic mild stressors”. Aversive stimuli should have a low degree of control to elicit a proper stress response, as defined by Koolhas and colleagues (2005): “chronic stress is the state of an organism that occurs when relevant environmental aspects have a low predictability and are not, or not very well, controllable over a long period of

time". Being predictable and certain allows lowering the intensity of the stressor (Anisman & Matheson, 2005; Willner, 1997) triggering neurochemical changes that are transient. However, the chronic daily exposure creates sensitization that re-elicits and reinforces those neurochemical (Gray et al., 2014) and behavioral changes (Vyas et al., 2006).

There is an increased awareness that some fundamental neurochemical modifications associated with the maladaptive behavioral response to chronic and/or repeated stress, are related to synaptic alterations (Duman & Aghajanian, 2012). Apart from changes of synaptic plasticity and alterations of the density of synaptic markers in different brain regions associated with emotional processing, we have observed that the up-regulation of adenosine A_{2A} receptors ($A_{2A}R$) seems to be an neurochemical hallmark of the neurodegeneration caused by noxious brain insults (Cunha and Agostinho, 2010). Taking these aspects into account, we behaviorally characterized 3 models of repeated stress to evaluate if all 3 models were indeed associated with an up-regulation of $A_{2A}R$ together with alterations of synaptic markers.

We have so far focused on the impact of $A_{2A}R$ in the hippocampus. However, the hippocampus alone does not control the constellation of changes caused by stress. Different brain structures react to stressors and control the behavioral and physiological responses to chronic stress (e.g., Dias-Ferreira et al., 2006; Vyas et al., 2002). Among them, the amygdala plays a pivotal role: it is one of the few brain regions displaying enhanced activity, and its lateral and basolateral nuclei (BLA) display increased dendritic branching and spine density upon chronic stress (Vyas et al., 2002; Vyas et al., 2006), which are correlated with increased anxiety (Vyas et al., 2004). After assessing the impact of adenosine receptors in the hippocampus, a role that has been widely described by our group and others (Gomes et al., 2011), we intended to understand the role of the adenosinergic system in the amygdala and its impact in controlling amygdalar responses upon stressful events. As a first step, we evaluated if any of the chronic stress models changed the adenosine A_1 and A_{2A} receptors density in the synaptic membranes of BLA.

4.4.2 Experimental design

Regarding behavioral testing, among the symptoms of depression, in this chapter we describe cognitive dysfunction (memory), motor activity and anxiety.
Memory impairment allowed us to bridge the data obtained from the previous models (H/Rouen and CUS). In both models, consistent memory impairment and protection through $A_{2A}R$ blockade were observed both behaviorally and neurochemically in the hippocampus.
Psychomotor activity decreases in some depressive patients (Nestler & Hyman, 2010), thus evaluating the total locomotor activity will provide not only a control measurement for the other behavioral tests but also the evaluation of a depression related symptom.
Anxiety and depression have a comorbidity of more than 50% (Hirschfeld, 2001), thus evaluating an increased anxiety-like behavior in rats is a valuable tool to assess the efficacy of the stress model.
All 3 models consisted in stressing male adult (8-10 weeks) Wistar rats for 2 weeks and evaluate their behavior in the 2 following days (Fig.21).

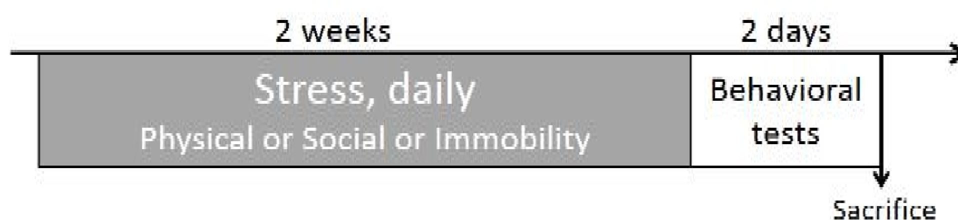

Figure 21: Schematic representation of the experimental design to evaluate the behavioral and neurochemical changes caused by three different stress paradigms: physical stress, social stress and immobility, to which adult male Wistar rats were exposed, once daily, for 2 weeks and followed by behavioral analysis in the following 2 days.

Briefly, in physical stress, animals had to run for one hour every day at rates that would vary daily. In social stress, tested animals were placed in a cage with an older and heavier male Wistar rat; rats were left in that cage to interact and fight for dominance for 10 min and then placed back in the ‘winner’ rat’s cage, physically separated by a metal grid (the tested animal is invariably the ‘defeated’ one). This allowed for a constant visual contact and odor exposure

during the next 24 h. In the immobility model, animals were placed in a DecapiCone plastic bag for 4 h a day, every day of the protocol.

The day after the last stressor, behavioral analysis took place. In the first day, we carried out the open field test. Total locomotion (total number of crossings) and anxiety (relative central crossings) were assessed for 10 min. On the same day, memory performance was tested using the two-trial Y-maze, similar to the protocol used with CUS-exposed mice. On the second day, anxiety was assessed in the EPM (5 min trial) as the relative exploration of the open, anxiogenic arms.

The day after the last behavioral test, all animals were sacrificed, the BLA dissected and kept at -80ºC until processed for binding assay of adenosine A_1R and $A_{2A}R$, as well as Western blot analysis of general markers of synapses (synaptophysin, SNAP-25 and syntaxin I), both glutamatergic and GABAergic synapses, namely vesicular transport type I (vGluT1) and vesicular GABA transporter (vGAT), and post-synaptic proteins associated with excitatory (PSD95) and inhibitory (gephyrin) transmission.

It is important to emphasize that this group of results is the first step of the study intended to assess the similarities and differences between the 3 models of repeated stress. In this optimization step, as long as there were enough samples for the biochemical analyses, no unnecessary animals were used and

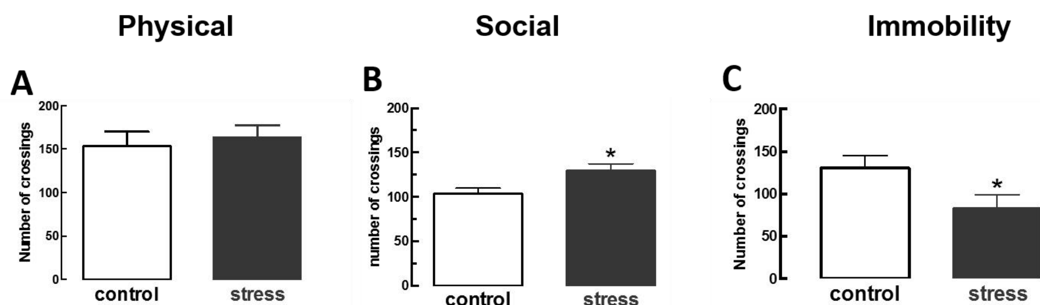

Figure 22: Locomotor activity in the open field arena. Adult (8-10 weeks old) male Wistar rats were subjected to physical stress (running on a treadmill for 1 h), social defeat stress (being dominated for 15 min and then placed in the same cage as the dominant rat for the rest of the time) and immobility stress (placed in a DecapiCone bag for 4 h with restrained movements) daily, for 2 weeks, until they were behaviorally tested. Locomotor activity was unchanged in animals submitted to physical stress (**A**), slightly increased in animals submitted to social stress (**B**) and decreased in animals submitted to immobilization (**C**). Data are mean + SEM of n=3-10 rats/group. *p<0.05, using Student's *t* test.

killed exclusively for the behavior tests (based on the 3R's policies, originally from Russell and colleagues (1959)). Consequently, the results obtained in the behavioral and neurochemical analysis often only indicate clear tendencies since they were derived from a low number of animals.

4.4.3 Different stressors induce varied locomotor changes, impair memory performance and increase anxiety

Regarding the OF test, the animals submitted to the physical stress do not seem to behave as expected in a model of depression. Locomotion (Fig. 22A) was similar between control and physically stressed animals, social defeat caused animals to crawl longer distances (Fig. 22B) and immobilization led to a decrease in locomotion (Fig. 22C).

While the OF test can be used to assess anxiety-like behavior in rodents, its predictive validity is not fulfilled, i.e., most anxiolytic drugs do not change the

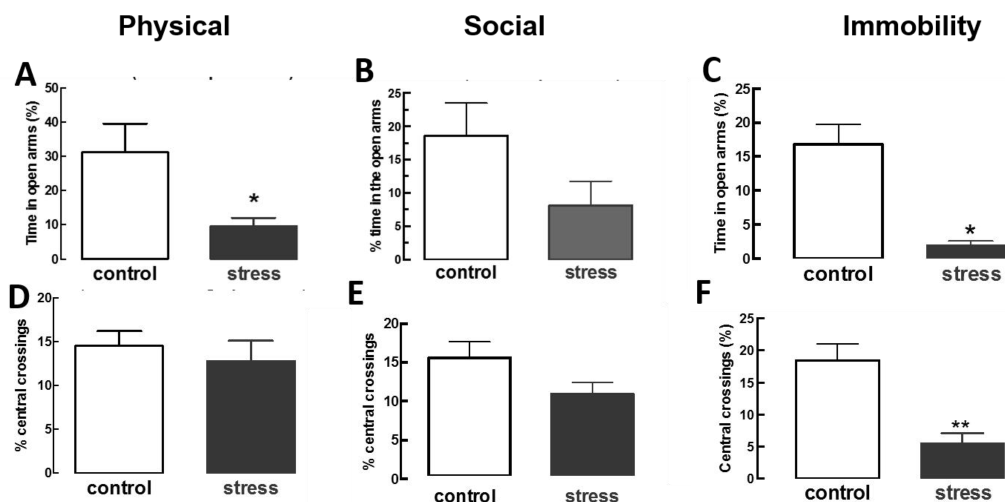

Figure 23: All stressors induced anxiety-like behavior. Adult (8-10 weeks old) male Wistar rats were subjected to physical stress (running on a treadmill for 1 h), social defeat stress (being dominated for 15 min and then placed in the same cage as the dominant rat for the rest of the time) and immobility stress (placed in a DecapiCone bag for 4 h with restrained movements) daily, for 2 weeks, until they were behaviorally tested. Physical stress, social stress and immobility induced anxiety-like behavior, measured as a lower exploration of the open arms in elevated plus maze (**A-C**). Physical and social stress protocols did not change significantly the exploration of the central anxiogenic region of the open field (**D**, **E**) while immobility stress (**F**) confirmed the results from elevated plus maze. Data are mean + SEM of n=5-9 rats/group. *p<0.05, **p<0.01, using Student's *t* test.

profile of central exploration (Prut & Belzung, 2003). For that reason we chose to complement the information provided by the OF test with another task, the EPM test, that fulfills predictive, construct and face validity (Walf & Frye, 2007). The downside of this test is its high variability (Hogg, 1996).

Physical stress increased anxiety-like behavior in rats, as assessed by the decrease in the time exploring the open arms of the EPM (Fig. 23A). Similarly, in the social stress cohort (Fig. 23B), stressed animals explored the open arms for shorter periods, albeit not significantly so. Animals submitted to immobilization (Fig.23C) also presented decreased exploration of the open arms when compared to their controls. These data was confirmed by the decrease in exploration of the central area of the OF by the immobilization model (Fig. 23F), however physical and social stress (Figs. 23D,E) did not induce the decrease in central exploration that would be consistent with increased anxiety.

Memory performance was impaired by all 3 stressors (Fig. 24). While control groups explored the novel arm above 40% on average, stress groups performed worse than their respective controls with the exploration of the novel arm close to chance (33%).

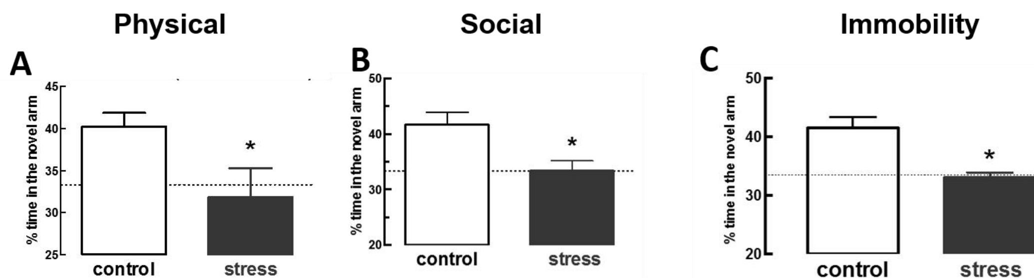

Figure 24: Chronic stress tended to decrease memory performance in the two-trial Y maze. Adult (8-10 weeks old) male Wistar rats were subjected to physical stress (running on a treadmill for 1 h), social defeat stress (being dominated for 15 min and then placed in the same cage as the dominant rat for the rest of the time) and immobility stress (placed in a DecapiCone bag for 4 h with restrained movements) daily, for 2 weeks, until they were behaviorally tested. Memory performance was assessed by measuring the time exploring the novel arm in the two-trial Y-maze. (**A**), socially defeated animals tended to have a lower performance (**B**) and animals subjected to immobilization had a clear tendency to perform worse than controls (**C**). Data are mean + SEM of n=3-5 rats/group. *p<0.05, using Student's *t* test.

4.4.4 Immobilization stress changes A_{2A} receptors but not A_1 receptors density in synaptosomal membranes from the amygdala

In both chronic unpredictable stress (CUS) and H/Rouen models, we observed an increase in the density of $A_{2A}R$ in the hippocampus as well as a decrease of A_1R. We now assessed the density of these two adenosine receptors in a

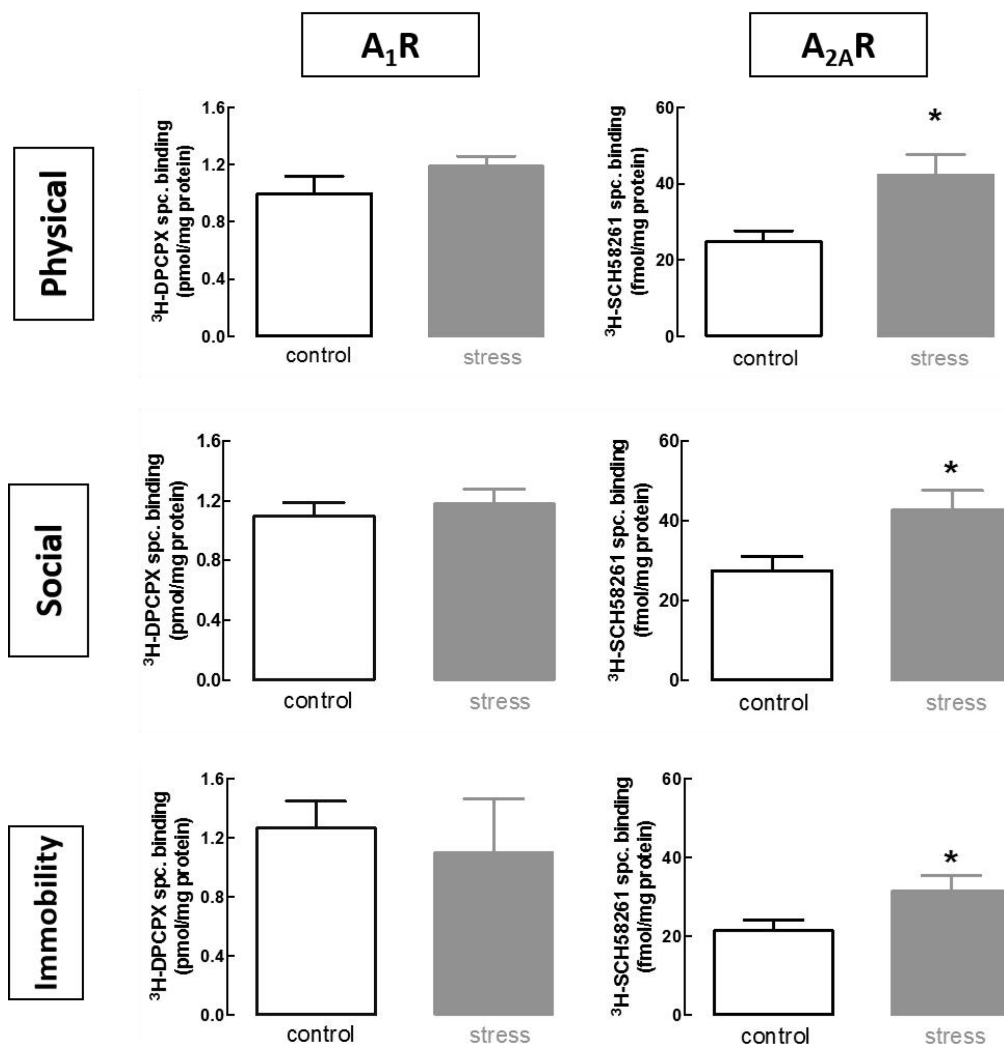

Figure 25: Chronic stress altered A_1R and $A_{2A}R$ density in the amygdala. Adult (8-10 weeks old) male Wistar rats were subjected to physical stress (running on a treadmill for 1 h), social defeat stress (being dominated for 15 min and then placed in the same cage as the dominant rat for the rest of the time) and immobility stress (placed in a DecapiCone bag for 4 h with restrained movements) daily, for 2 weeks, until they were behaviorally tested. The animals were subsequently sacrificed, decapitated, the basolateral amygdala (BLA) was dissected and synaptosomes were prepared for binding assays. No significant changes were observed in A_1R density but all stressors caused an increase in the density of $A_{2A}R$ in synaptosomes of the amygdala. Data are mean + SEM of n=3-5/group. * $p<0.05$, using the Student's *t* test.

different brain region pivotal in the control of stress responses, the amygdala. After dissecting the basolateral nuclei of the amygdala (BLA), proteins extracted from synaptosomal and total membranes were prepared for binding assays.
Regarding A_1R density, no changes were observed in the 3 stress models, with stressed animals consistently presenting A_1R density close to that of controls (Fig. 25). On the contrary, $A_{2A}R$ density was consistently increased, as observed in the afore mentioned models, and in accordance with the facilitatory role of these receptors and the increased amygdalar activity upon stress.

4.4.5 Immobilization stress increases the immunoreactivity of synaptic markers in the amygdala

As previously described, the chronic unpredictable stress (CUS) model has induced changes in behavior, in adenosine receptors density and synaptic arrangement (Chapter 4.2). In comparison, physical stress, social defeat and immobilization models induced behavioral changes that resemble those in depressive individuals, albeit immobilization induced more robust differences in anxiety-like behavior, in both the OF arena (central crossings, Fig. 23C) and the EPM (open arms exploration, Fig. 23F). Moreover, the daily exposure to immobilization increased the density of $A_{2A}R$, but not A_1R, in the BLA (Fig. 22C), in contrary to the decrease observed in the hippocampus after CUS.
These behavioral changes and adenosine receptors' alterations in the amygdala strongly suggest that synaptic changes might also occur; thus we investigated some neurochemical changes associated with synaptic plasticity in the amygdala.
Synaptophysin, SNAP25 and syntaxin-I are involved in vesicle fusion in the presynaptic terminal of neurons, allowing the release of neurotransmitters Kwon (e.g., Kwon & Chapman, 2011; Rizo & Rosenmund, 2008). This means that these 3 proteins are widely spread and are often used as markers for synaptic density in tissues. vGAT is used as a marker of GABAergic synapses and is localized in the presynaptic fraction, while gephyrin is a post-synaptic protein present in inhibitory terminal, albeit not exclusively in GABAergic ones (Craig et

al., 1996). vGluT1 and PSD95 are present in excitatory synapses: the former in the presynaptic terminal, the latter in the post-synaptic neuron.

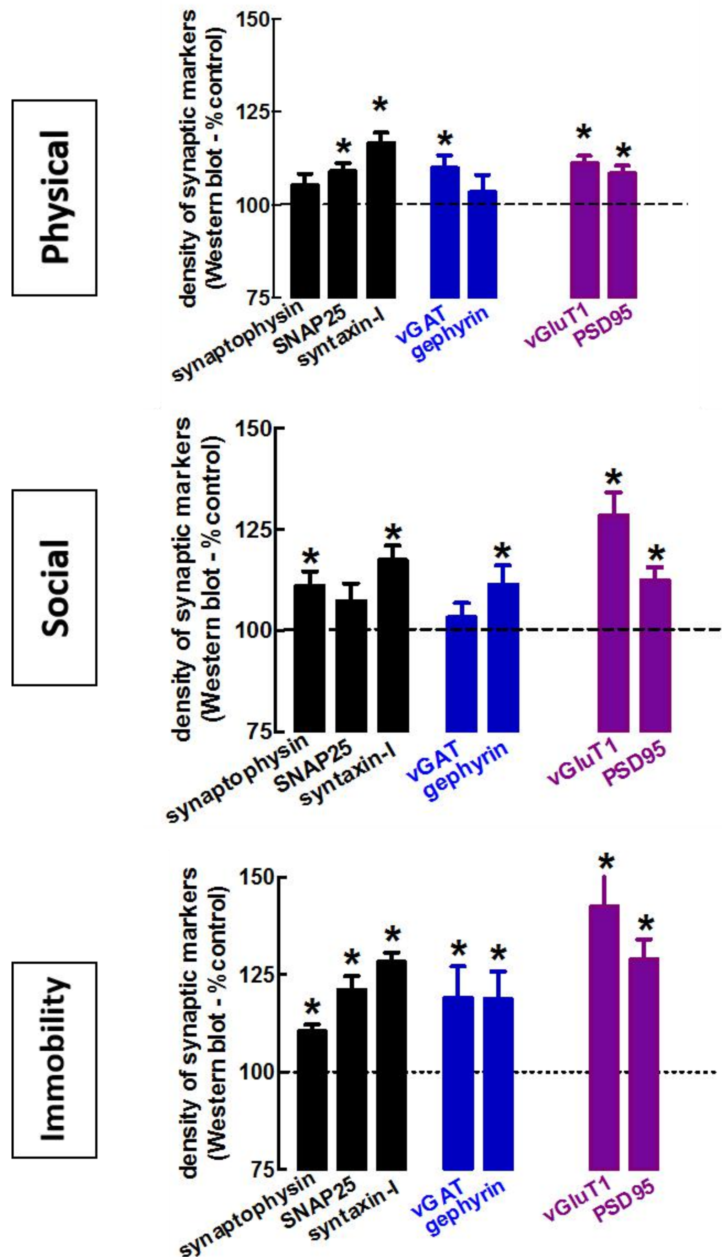

Figure 26: Repeated stress increases the density of synaptosomal markers in the amygdala. Adult (8-10 weeks old) male Wistar rats were subjected to physical stress (running on a treadmill for 1 h), social defeat stress (being dominated for 15 min and then placed in the same cage as the dominant rat for the rest of the time) and immobility stress (placed in a DecapiCone bag for 4 h with restrained movements) daily, for 2 weeks, until they were behaviorally tested. The animals were subsequently sacrificed, decapitated, the basolateral amygdala (BLA) was dissected and synaptosomes were prepared for binding assays. An overall increase of synaptic proteins occurs after repeated stress. Immobility stress causes an increase in proteins of both excitatory synapses (vGluT1, PSD95) and inhibitory synapses (vGAT, gephyrin) and, accordingly, non-specific presynaptic markers (synaptophysin, SNAP25, syntaxin-I) are also increased. Physical stress induced less pronounced changes and synaptophysin and post-synaptic protein gephyrin are not significantly different from control, while all other markers are changed. Social stress did not significantly change SNAP25 neither vGAT. Data are mean + SEM of n=4/group. *$p<0.05$ using the one sample *t* test. Legend: PSD95 – post-synaptic density protein 95; SNAP25 – synaptosomal-associated protein 25; vGAT – vesicular GABA transporter; vGLUT1 – vesicular glutamate transporter type I.

synaptic markers (Fig.26). Indeed this increase was not specific to excitatory or inhibitory synapses since VGAT, gephyrin, vGluT1 and PSD95 were all increased comparing to controls. This increase was unsurprisingly accompanied by an increase of the ubiquitous presynaptic markers analyzed (synaptophysin, SNAP25 and syntaxin-I).

Physical and social stress induced changes that were more modest, albeit also pointing to an increase in synaptic transmission. Although all proteins analyzed had increased density, synaptophysin and gephyrin were significantly changed by physical stress when compared to controls. In animals submitted to stress, SNAP25 and vGAT were not significantly changed.

4.4.6 Discussion

The study of the three different stress models described in this chapter (physical, social, immobility stress) provided an insight on the role of adenosine receptors in the amygdala.

As previously mentioned, different stressors can have different behavioral outcomes. In general, repeated stress increased anxiety-like behavior (EPM and OF) and impaired memory performance (two-trial Y maze), but induced less consistent changes in locomotor activity (OF).

Physical stress contributed to these conclusions with the exception that the exploration of the central area of the open field arena did not decrease when compared to controls. Conversely, the exploration of the open arms of the EPM was lower indicating higher anxiety.

Social stress tended to increase the anxiety-like behavior in both tests but these changes were not significant. One reason for the lack of significance might be the low number of animals used in this pilot study. Nevertheless, social stress seems to have a lower impact on anxiety when compared to the other 2 models.

The immobility model was the most consistent throughout: exploration of open, novel spaces was significantly decreased in both the EPM and the OF. Repeated immobility also impaired memory performance in the two-trial Y maze, a change that was consistent in all 3 models.

Regarding locomotion, social defeat increased it while immobilization caused the opposite effect. Increased locomotion has been reported in some studies after chronic stress models inducing depressive-like alterations, which seems to be especially related to experimental conditions (moderate lighting) and the stress model used (Strekalova et al., 2011). On the other hand, most reports show that after social defeat, animals decrease their locomotor activity (Patki et al., 2014), in accordance to the reduced psychomotor activity reported in depressed patients. Repeated immobilization stress followed this pattern in a significant manner.

As reported with the CUS model, synaptic markers in the hippocampus are down-regulated, which is in agreement with the fact that hippocampal activity is compromised and dendritic arborization is decreased after repeated stress and in models of depression (McEwen, 2001). For this reason, the stress protocols in rats described in this chapter were expected to mimic the memory deficits described previously in the CUS and H/Rouen models.

The hippocampus is pivotal in memory processing and other brain regions, importantly, the amygdala, modulate its activity. Emotional responses enhance memory formation and the basolateral nuclei (BLA), but not the central nuclei, of the amygdala facilitate long-term potentiation (LTP) in the hippocampus (Frey et al., 2001) and memory consolidation (Paré, 2003). This BLA influence on hippocampal LTP must be an indirect one and, interestingly, neuronal projections out of the BLA are glutamatergic (Smith & Paré, 1994). The adenosinergic system, through $A_{2A}R$, can facilitate glutamatergic excitation, which can lead to excitotoxicity in different brain regions, like the hippocampus after stress (Cunha et al., 2008). Thus blocking amygdalar $A_{2A}R$ during stress seems a compelling anti-depressant strategy. For that reason, we first analyzed the A_1 and A_{2A} receptors density in the BLA.

While we observed an increase of $A_{2A}R$ in synaptosomal membranes and no significant changes in A_1R density in the hippocampus after CUS and the amygdala and the hippocampus undergo opposite plastic changes after chronic stress, the BLA of rats exposed to repeated stress presented similar results to mice submitted to CUS. Concurrently, synaptic markers changed in opposite ways: in mice submitted to chronic stress (CUS model) the increase in $A_{2A}R$ in the synaptic membranes of the hippocampus was accompanied by a decrease

in synaptic proteins density; on the other hand, in the amygdala of stressed rats, the increase of $A_{2A}R$ was accompanied by an overall increase of synaptic markers. The changes in the density of synaptic proteins are in agreement with the decrease of hippocampal activity observed in stressed individuals as opposed to the increase in the amygdala (Pruessner et al., 2010). The loss of function in the hippocampus is hypothesized to be due to an excitotoxic process (Gomes et al., 2011), which explains the elevated levels of $A_{2A}R$, in a similar way to what has been described in neurodegenerative diseases (Cunha et al., 2005). In the BLA, the fact that $A_{2A}R$ are elevated means that there is a facilitation of neuronal plasticity, thus, increasing synaptic markers. The reasons behind the inability of the hippocampus to withstand prolonged synaptic facilitation in contrast to the amygdala's ability are largely unknown and pose a challenge and an opportunity to understand the development of mood disorders. Nevertheless, the data collected in this thesis supports these facts: elevated $A_{2A}R$ in both regions seems to promote increased synaptic plasticity that ultimately leads to neurodegeneration in the hippocampus but increased dendritic arborization in the amygdala, with compatible cognitive and mood consequences.

Regarding the synaptic markers, it must be noted that gephyrin, a post-synaptic protein present in inhibitory glycinergic and GABAergic neurons (Craig et al., 1996), is increased in the BLA, while an overall increase of amygdalar activity is suggested by the behavioral data. This data seems surprising at first since gephyrin density is decreased in the BLA after fear conditioning (Chhatwal et al., 2005), we have reported the impact of $A_{2A}R$ in glutamatergic synapses and there is no information reporting that $A_{2A}R$ in the amygdala control inhibitory plasticity. But it has also been reported that corticosterone peripheral injections, that increase the anxiety-like profile in rats, increase the density of α-2 subunits of $GABA_A$ receptors after chronic stress (Skórzewska et al., 2014).

Overall, while all models seem appropriate to induce mood changes compatible with mood disorders, immobilization stress has proved in our experiments to be the model yielding data more consistent with clinical depression features (decreased locomotor activity, increased anxiety-like behavior, lower memory performance), with changes in the adenosinergic system consistent with the two behavioral and genetic mice models of depression (CUS and Rouen/H) and

increased density of synaptic markers that are consistent with the behavioral changes observed.

These factors led us to select the immobilization stress model to be used in subsequent studies and assess the role of $A_{2A}R$ in the BLA.

4.5 THE DOWN-REGULATION OF ADENOSINE A_{2A} RECEPORS IN NEURONS OF THE BASOLATERAL AMYGDALA PREVENTS SELECTIVE IMMOBILIZATION STRESS-INDUCED BEHAVIORAL AND SYNAPTIC ALTERATIONS

4.5.1 Introduction

The amygdala, an important site for regulating emotional behavior, particularly during aversive experiences, is critically impacted by chronic stress. In fact, postmortem and imaging studies of depressed patients identified abnormalities in several brain areas, namely in the amygdala (reviewed in Lorenzetti et al., 2009; Diener et al., 2012; Price & Drevets, 2012). Accordingly, chronic stress is a potent risk factor for developing amygdala-based disorders of fear and anxiety. Indeed, individuals with post-traumatic stress disorder (PTSD) exhibit enhanced activity in the amygdala (Hull, 2002), and facilitated performance on amygdala-dependent tasks involving fear (Grillon, 2008). In several studies (e.g., Vyas et al, 2002), chronic stress was shown to produce enhanced dendritic branching in the lateral and basolateral nuclei of the amygdala (BLA), and also increased spine density in these regions (Vyas et al., 2006). These effects were correlated with increases in anxiety (Vyas et al., 2004). Thus, chronic stress seems to lead to a hyperfunction of neural circuits in the amygdala. Section 4.4 presents data supporting these contention based on the findings of increased anxiety and increased synaptic markers in the BLA that were associated with behavioral alterations caused by chronic stress.

Very few other brain regions have been found to display enhanced function following chronic stress. To date, one of the few to display neuronal growth following chronic stress is the dorsolateral striatum (Dias-Ferreira et al., 2009). This is a region within the basal ganglia that is essential for the expression of habitual behaviors, and is important for sensorimotor processing by cortico-basal ganglia circuits (Graybiel, 2008). These “habit circuits” of the basal ganglia are increasingly being recognized as being involved in the genesis of disorders in humans leading to unwanted, and often involuntary, repetitive

thoughts. Dysfunction of circuits regulating habit formation may be involved in anxiety disorders such as obsessive-compulsive disorder (OCD), in which behavioral flexibility is lost and motor habits are expressed repetitively. Major life stressors can precipitate episodes of compulsive habit-like behavior in apparently normal individuals (Rajarethinam et al., 2000). In this chapter, possible behavioral changes associated with dysfunction of the dorsolateral striatum were probed.

In sharp contrast to the enhancement of function observed in the BLA and dorsolateral striatum after chronic stress, many brain regions display a loss of function following chronic stress. For example, the hippocampus, a region of the brain that functions in many forms of learning and memory, displays atrophy, not expansion, of dendritic arbors (Vyas et al., 2002; Watanabe et al., 1992) as well as impairments in synaptic plasticity (Diamond and Rose, 1994; Foy et al., 1987) following chronic stress. These effects lead to the functional impairments on hippocampus-dependent memory tasks observed following chronic stress as observed by others (Garcia, 2001; Kim & Yoon, 1998) and described in this thesis in the chronic unpredictable and immobilization stress models and the H/Rouen model of depression. The decrease of synaptic markers in the hippocampus, in contrast to the amygdala, will also be described.

All these changes attest that no brain region acts in isolation. The high degree of interconnectivity within neural circuits means that changes in one brain region may lead to changes in another. Therefore, although the importance of the amygdala is undisputable, we wanted to test if lowering the density of $A_{2A}R$ in this region affects other regions in a way that can change behavior.

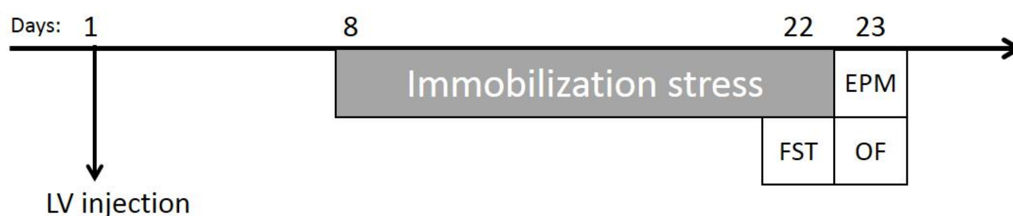

Figure 27: Schematic representation of the experimental design of animals selected to perform mood-related behavioral tests. After lentivirus injection into the basolateral amygdala (BLA) for silencing $A_{2A}R$, animals recovered for 1 week, were submitted to stress for 2 weeks, and were subsequently tested for despair-related behavior (forced swimming test, FST), anxiety-like behavior (elevated plus maze, EPM) and spontaneous locomotor activity (open field, OF).

4.5.2 Experimental design

We have thus far described how neuronal $A_{2A}R$ could prevent memory and mood changes in animal models of depression. Considering the relevance of the amygdala in emotional processing, and the fact that especially the BLA is over-active after chronic stress, we were lead to study the importance of the neuronal $A_{2A}R$ in the BLA in the immobilization stress model, selected in chapter 4.4.

First, adult (10 weeks) male Wistar rats were injected with a lentiviral vector encoding for either a short hairpin RNA sequence for A_{2A} receptors (**shA2AR**), thus rendering $A_{2A}R$ RNA untranslatable, or a scrambled control sequence (**shCTR**) into the BLA through stereotaxic surgery. After one week of recovery from surgery, half the animals were assigned to the stress group (4 h immobilization stress, daily, from 9 a.m.) or the control group (same procedure without immobilization, including no access to food and water). The stress was repeated for 15 days. At the end of stress all animals were evaluated either for mood-related tests (Fig. 27) or for memory tests (Fig. 28).

If assigned for mood-related tests (Fig. 27), at 4 p.m. of the last day of stress one session of forced swimming test (despair, FST) was carried out. In the morning of the next day, the elevated plus maze test (anxiety, EPM) was carried out and, in the afternoon, the animals explored the open field arena (locomotion and anxiety). The following day, the animals were sacrificed by decapitation after anesthesia with halothane, and the blood from the trunk was collected for corticosterone ELISA essay.

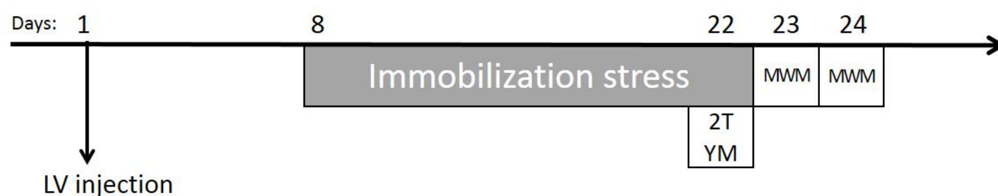

Figure 28: Schematic representation of the experimental design of animals selected to perform behavioral memory tests. After lentivirus injection into the basolateral amygdala (BLA) for silencing $A_{2A}R$, animals recovered for 1 week, were submitted to immobilization stress for 2 weeks, and were subsequently tested for hippocampal-dependent memory (two-trial Y-maze, 2TYM) and habit learning (Morris water maze, MWM) in the following two days.

If assigned to the memory tests (Fig. 28), at 3 p.m. of the last day of stress, the two-trial Y-maze (2TYM) test was performed. In the next 2 days, in the morning, animals were tested in a version of the Morris water maze (MWM), assessing habit learning, composed by four 60-second trials each day.

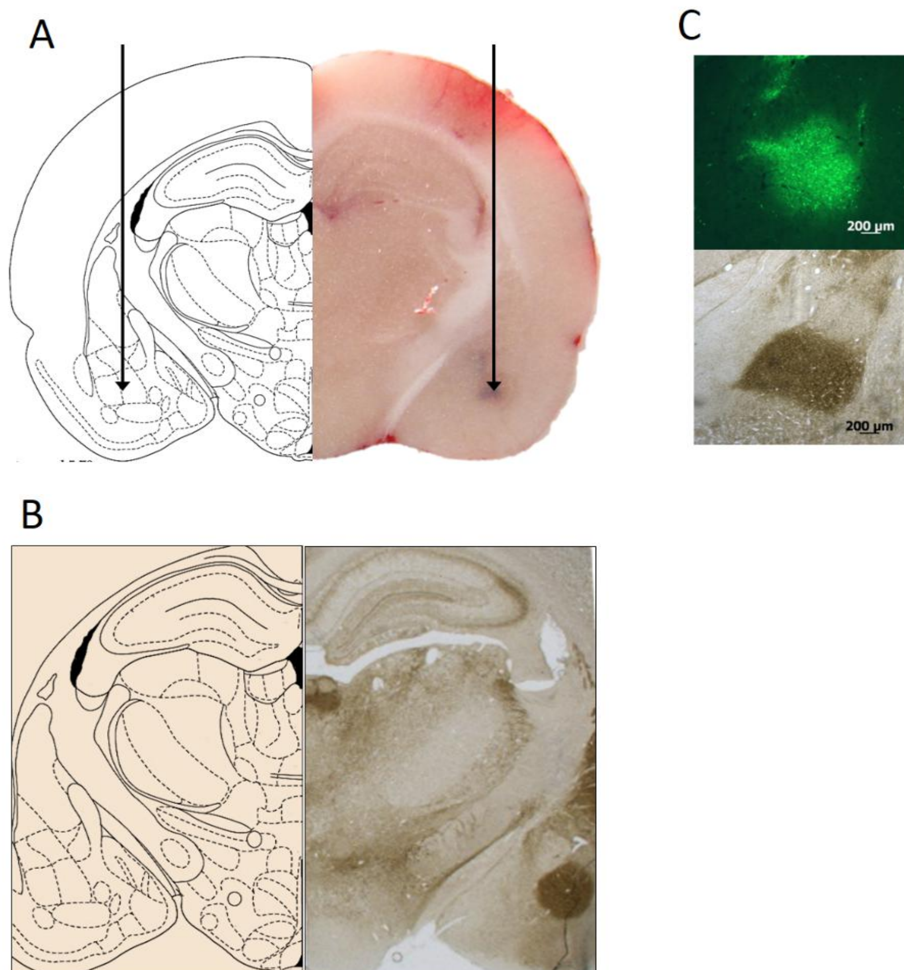

Figure 29: Intracraneal injection was optimized to target the basolateral amygdala and allowed to spread. Firstly, the injection coordinates were optimized to target the basolateral nuclei by performing stereotaxic surgery and injecting Brilliant Blue dye. The blue dye in the representative picture (A, right side) is in the BLA area, as compared to the representative coronal section (from Paxinos, 1997) at the same scale and approximate antero-posterior coordinate (A, left side, AP +3.3 mm). The arrows´ tips are at the same distance from the bottom of the pictures and the center (**A**). AChE was labeled through histochemistry to identify the basolateral nucleus of the amygdala (**B**) and with adjacent sections we were able to label both EGFP-positive cells (indicating the presence of the lentivirus, upper image) and AChE (lower image) in the same animal (**C**). Abbreviations: AChE – acetylcholinesterase; AP – antero-posterior; EGFP – enhanced green fluorescent protein.

4.5.3 Optimization of virus injection

Before the effects of silencing $A_{2A}R$ in the amygdala were studied, optimization for the location of the injection and the spread of the virus was assessed (Fig. 29). Three animals were submitted to stereotaxic surgery and Brilliant Blue dye was injected into the BLA at the following coordinates measured from bregma: antero-posterior (AP), -2.80 mm; lateral (L), 4.80 mm; dorso-ventral (DV), -9.00 mm. The dye was not harmful to the animals as they were sacrificed before the anesthesia effect subsided. We reached the target area successfully in all three animals, as shown in representative Figure 29A.

After the spatial determination of the injection, we determined the time interval needed before the beginning of stress in order to allow the virus to spread in the BLA and the viral machinery could effectively silence $A_{2A}R$ RNA. In previous studies (Gonçalves et al., 2013), the injection of lentivirus encoding a mutant ataxin-3 gene produced overt synaptic loss in only two weeks, meaning that the production of physiologically relevant ataxin-3 mRNA happened long before the end of that time window. Thus, we hypothesized that one week could be enough to spread sufficient viral vector in the BLA and for the expression of $A_{2A}R$-silencing shRNA. To do so, we used stock control lentivirus, encoding for EGFP, in order to identify the viral vectors position in the brain through fluorescent histochemical analysis. We observed that the fluorescent labeling coincided with and spread little around the position of acetylcholinesterase (AChE) labeling (Fig. 29C), which is observed mainly in the basolateral nucleus (BLA) of the amygdala (Stenman et al., 2003). The expression of lentiviral proteins, in this case EGFP, meant that shRNA for $A_{2A}R$ should also be expressed. Indeed, 3 weeks after injection, a decrease of two thirds of the $A_{2A}R$ mRNA content in the BLA was observed (data not shown).

4.5.4 Automation of behavioral analysis

While in previous studies all behavioral counting was performed manually, for most tests used hereafter automated data collected with a video feed will be presented (ANYmaze software v4, Stoelting) for increased accuracy and additional control data. Consistency with previous observations and determination of correct arm entries is essential for the quality of the data collected. The identification of the position of the animal can be divided into two categories: body center and whole body area.

The body center of the animal was used to track its position in the open field arena and during the Morris water maze test. In Figure 30A, a representative image of the temporal occupancy of one animal is shown for a 10-minute test in the 1-meter open field. As can easily be seen, animals spend most of their time in thigmotaxis, i.e., moving next to the wall. The separation chosen between the peripheric and central areas allows for a clear distinction of the animals 'venture' into the center of the maze (approximately 36% of the total arena area). The total body area, instead of the center of body, was used in tests in which determining when an animal enters or exits one arm is essential to the outcome of the test. Case in point, the two-trial Y-maze and the EPM. As the criterion for entries is stricter than for exits when collecting data manually, the minimum

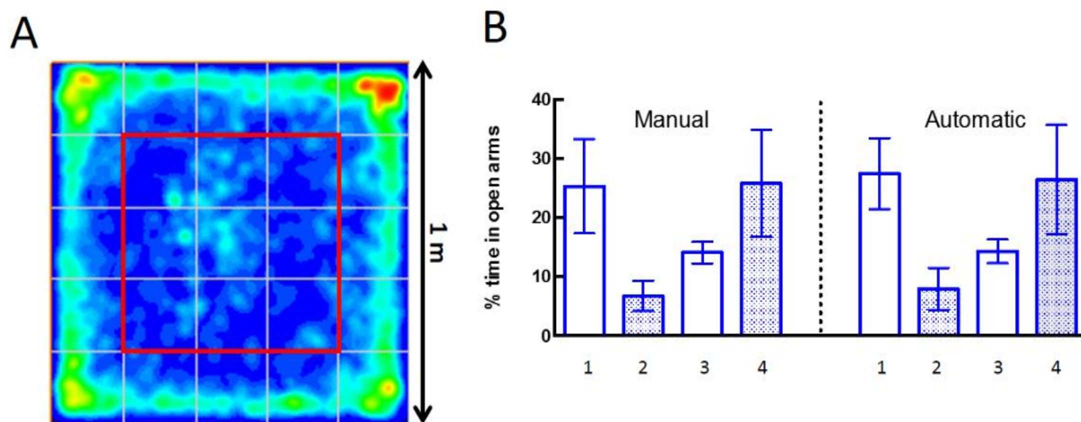

Figure 30: Automated data collection was controlled for reliability check. The center of the animal's body was the reference point for tracking the animal's movement and positioning in a square open field area (**A**). Colors represent permanence time in each position: increasing permanence time from dark blue to light blue to green to red. The red line represents the separation between periphery and center of the arena for anxiety-like measurement. In the elevated plus maze (**B**), using 60% of body area for entry and 40% for exit as criteria yielded identical data when compared to blind manual count. Groups 1-4 are arbitrarily chosen.

proportion of body area necessary inside the arm for an entry to be counted was 100% while for an exit it was 40%. These criteria yield data from the software analysis that perfectly matches data from manual counting (Fig. 28B, a sample of animals in the EPM).
The forced swimming test immobility/swimming/climbing times were counted manually.

4.5.5 Weight changes and corticosterone levels after stress and A_{2A} receptors 'silencing' in the BLA

Depression is often correlated with weight changes, either weight gain or loss (Nestler & Hyman, 2010). In animal models weight loss is often described, including after chronic immobility stress (Meyer et al., 2013). We weighed animals on the day of surgery, the day when the stress protocol started, halfway through stress period and in the last day of stress. All groups gained weight after surgery 2.9 – 3.4 g/day until the beginning of stress. After immobilization stress started both stressed groups stopped gaining weight in the first week (stress shCTR: +0.0 ± 0.3 g/day, n=26; stress shA2AR: +0.3 ± 0.4 g/day, n=24) while controls, though not having access to neither food nor water for 4 h per day, continued normal weight gain (control shCTR: 3.2 ± 0.4 g/day, n=22; control shA2AR: 3.1 ± 0.2 g/day, n=24). Silencing $A_{2A}R$ in the BLA had no effect on weight gain in neither group (Fig. 31A).
It is known that high levels of cortisol, a hormone associated to stress response, are often observed in depressed patients (Clark et al., 2009). In rodents, the equivalent hormone, corticosterone, has been reported to be elevated in models of depression (Belzung & Lemoine, 2011). In our model, we measured a three-fold increase in corticosterone ($p<0.01$, two-way ANOVA, stress effect; control shCTR: 132.2 ± 46.0 ng/mL, n=5; stress shCTR: 415.2 ± 72.2 ng/mL, n=6, $p<0.01$, Bonferroni *post hoc* test) with little effect upon $A_{2A}R$ silencing (control shA2AR: 206.5 ± 66.0 ng/mL, n=4; stress shA2AR: 335.3 ± 13.8 ng/mL, n=5, $p=0.06$ *vs*. control shCTR, Bonferroni *post hoc* test) (Fig.31B).

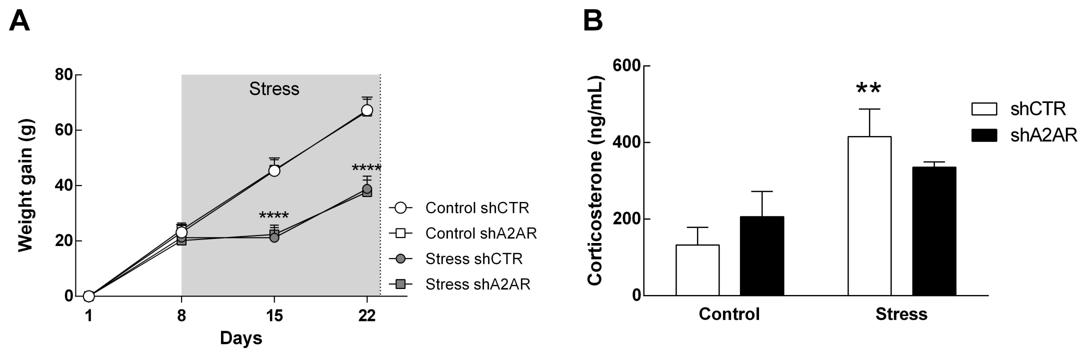

Figure 31: Immobility stress decreases weight gain and increases plasmatic corticosterone concentration. After lentivirus injection (day 1) into the basolateral amygdala (BLA) for silencing $A_{2A}R$, animals recovered for 1 week, were submitted to immobilization stress for 2 weeks (days 8 to 22), mood behavior tests were carried out (days 22 and 23) and blood was collected after decapitation (day 24). Both control (shCTR) and shA2AR virus-injected groups recovered weight from surgery normally, but immobility stress decreased weight gain irrespectively of viral serotype (**A**). ****$p<0.0001$ *vs*. Control shCTR within each day. Two weeks of stress increased corticosterone concentration in the plasma and silencing $A_{2A}R$ in the BLA had a mild (non-significant) effect on stress (**B**). **$p<0.01$ *vs*. Control shCTR. Two-way ANOVA followed by Bonferroni *post hoc* test.

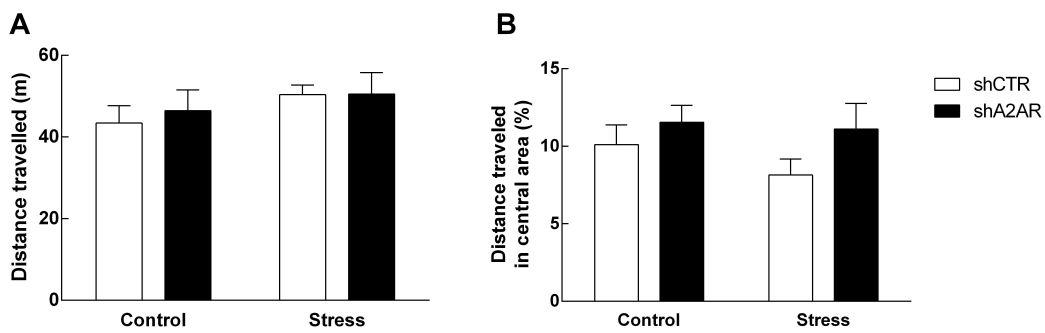

Figure 32: Stress did not change locomotor activity and only mildly affects anxiety-like behavior in the open field arena. After lentivirus injection into the basolateral amygdala (BLA) for silencing $A_{2A}R$, animals recovered for 1 week, were submitted to immobilization stress for 2 weeks and behaviorally tested. Locomotor activity was unchanged by stress and $A_{2A}R$ silencing (**A**). A mild (non-significant) decrease in central exploration (anxiety-like behavior) was caused by chronic stress but stressed animals injected with shA2AR lentivirus presented similar central exploration like control animals (**B**). Statistically tested with two-way ANOVA.

4.5.6 Stress did not alter locomotion in the open field arena

After the chronic immobilization stress protocol, animals were behaviorally tested. In the open field arena, the distance traveled (Fig. 32A) was unchanged by stress and silencing $A_{2A}R$ in the amygdala had no effect (control shCTR: 43.4 ± 4.3 m, n=10; control shA2AR: 46.4 ± 5.1 m, n=10; stress shCTR: 50.4 ± 2.4 m, n=10; stress shA2AR: 50.5 ± 5.2 m, n=9).
We assessed anxiety-like behavior by analyzing central exploration of the OF (Fig. 32B). Stress caused a mild (non-significant) reduction of the exploration of the central area while stressed animals with silenced $A_{2A}R$ presented a mean exploration closer to control (control shCTR 10.1 ± 1.3%, n=10; control shA2AR: 11.5 ± 1.1%, n=10; stress shCTR: 8.2 ± 1.0%, n=10; stress shA2AR: 11.1 ± 1.6%, n=9).

4.5.7 Stress-induced memory deficits dependent on hippocampal function but did not change habit learning performance

As referred above, two other brain regions involved in depression are the hippocampus and the striatum. As reported in other models described in this thesis, hippocampal function is impaired in the depressive-like state (see Chapters 4.1 and 4.2), which we propose being at least partially explained by a neurotoxic effect that could be prevented and/or reversed by deletion or antagonism of adenosine $A_{2A}R$. By down-regulating $A_{2A}R$ specifically in the BLA, we wanted to assess if memory performance dependent on the hippocampus was affected. In the two-trial Y-maze test, a spatial reference-based task, stress induced memory impairment (Fig. 33A) (exploration of novel arm; two-way ANOVA, $F_{(1,19)}=5.77$, $p<0.05$, stress effect). While controls explored the novel arm significantly longer than chance (control shCTR: 43.1 ± 3.7%, n=5; control shA2AR: 39.9 ± 3.1%, n=6; $p<0.05$, one-tailed one sample *t* test *vs*. 33.3% chance), both stress groups did not appear to be able to recognize the novel arm (stress shCTR: 35.7 ± 3.4%, n=6; stress shA2AR: 33.4 ± 2.4%, n=6).

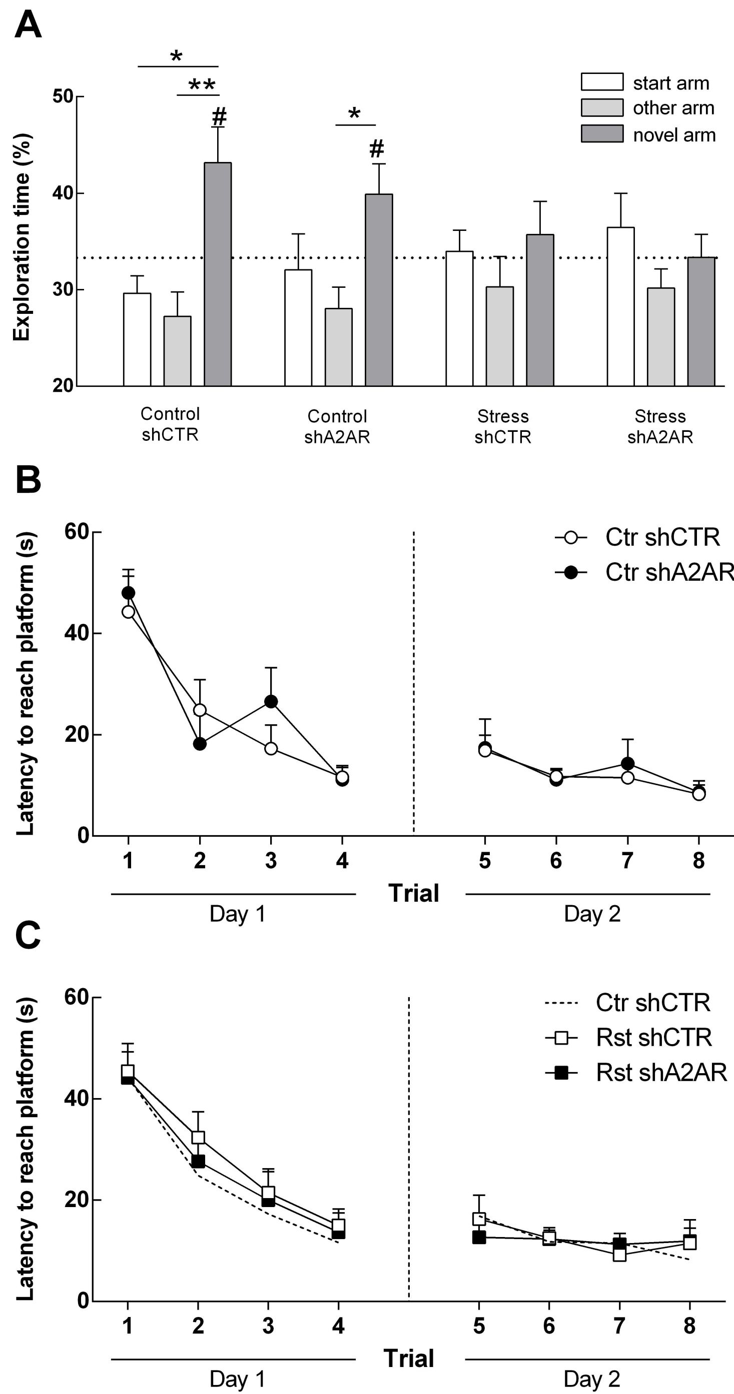

Figure 33: Immobilization stress induced hippocampus-dependent memory deficits but did not change habit learning. After lentivirus injection into the basolateral amygdala (BLA) for silencing $A_{2A}R$, animals recovered for 1 week, were submitted to immobilization stress for 2 weeks and behaviorally tested for memory and learning performance in the two-trial Y-maze and a habit learning version of the Morris water maze. Stress induced memory deficits as assessed by the Y-maze test that could not be prevented by silencing $A_{2A}R$ receptors in the amygdala (**A**). Down-regulating $A_{2A}R$ did not alter habit learning in the Morris water maze (**B**), which was also unaffected by stress (**C**). $^{*}p<0.05$, $^{**}p<0.01$, one-way ANOVA followed by Bonferroni *post hoc* test; $^{\#}p<0.10$, one-tailed one sample *t* test *vs*. 33.3%.

In contrast to the hippocampus, the dorsolateral striatum, which is involved in the regulation of habit learning, has its activity increased in depression (Dias-Ferreira et al., 2009). But in this model of stress we did not observe changes in habit learning (Fig. 33B,C) caused by either stress or $A_{2A}R$ silencing in the amygdala (average latency in day 2; control shCTR: 12.1 ± 1.0 s, n=8; control shA2AR: 12.9 ± 2.5 s, n=9; stress shCTR: 12.4 ± 2.1 s, n=11; stress shA2AR: 12.0 ± 1.8 s, n=12).

4.5.8 $A_{2A}R$ down-regulation in the BLA prevented stress-induced mood changes

In separate groups of animals, mood changes caused by chronic stress were tested and a possible preventive effect of silencing $A_{2A}R$ in neurons of the basolateral amygdala (BLA) was assessed. Stressed animals displayed a tendency (non-significant) for increased anxiety-like behavior, as measured by the permanence in the open arms of the EPM (Fig. 34A) (control shCTR: 17.1 ± 7.7%, n=8; stress shA2AR: 9.3 ± 3.1%, n=8), but values were closer to control in stressed animals injected with lentivirus silencing $A_{2A}R$ (stress shA2AR: 18.1 ± 9.1%, n=6). Interestingly, the silencing has an effect *per se* in non-stressed animals, increasing anxiety-like behavior (non-significant) (control shA2AR: 9.3 ± 3.1%, n=8).
Depressive-like behavior was assessed with the forced swimming test. Stressed animals displayed higher despair by stopping movement (except the strictly necessary to keep afloat) for longer periods (Fig. 34B) (control shCTR: 290.6 ± 26.2 s, n=10; stress shCTR: 327.0 ± 6.7 s, n=11; p=0.18, Student's *t* test) and silencing $A_{2A}R$ in amygdalar neurons was sufficient to lower immobility (stress shA2AR: 263.4 ± 14.6 s, n=9; p<0.05, *vs.* stress shCTR, two-way ANOVA followed by Bonferroni *post hoc* test). As in most tests, silencing $A_{2A}R$ in the BLA had no effect on non-stressed animals (control shA2AR: 297.5 ± 12.7 s, n=10). Moreover, the differences in immobility time are mostly due to the fact that stressed animals injected with control virus are immobile during the first 2 min of the test that is as high as all other groups 1 min after, which indicates that the struggle to escape the water stops earlier (Fig. 34D). However, when

injected with shA2AR virus, the immobility profile over time is similar to control animals. Accordingly, even while moving, stressed shCTR animals tended to try escaping by climbing next to the wall for a shorter period when compared to controls (climbing/swimming time ratio; control shCTR: 2.5 ± 0.5, n=10; stress shCTR: 1.5 ± 0.2, n=11; p=0.11, Student's *t* test) (Fig. 34C). Both shA2AR control and stress groups displayed similar behavior to controls (control shA2AR: 2.5 ± 0.2, n=10; stress shA2AR: 3.1 ± 1.1, n=9).

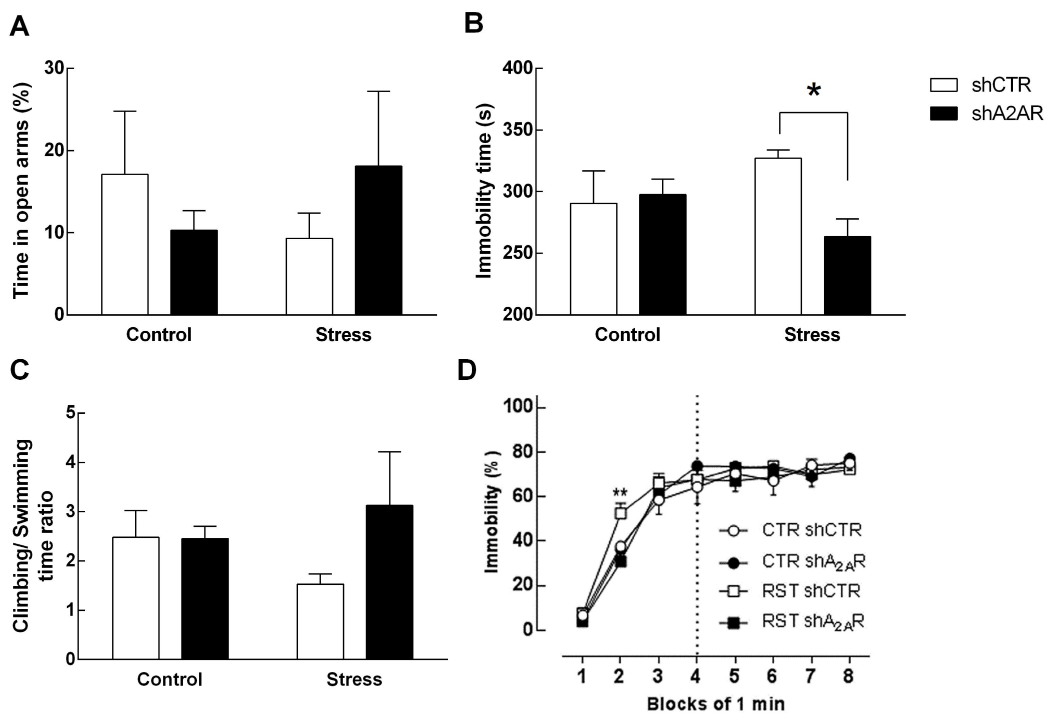

Figure 34: Immobilization stress induced anxiety-like and depressive-like behaviors that were prevented by silencing $A_{2A}R$ in the amygdala. After lentivirus injection into the basolateral amygdala (BLA) for silencing $A_{2A}R$, animals recovered for 1 week, were submitted to immobilization stress for 2 weeks and behaviorally tested for mood changes in the elevated plus maze (anxiety) and forced swimming test (despair). Stress induced (non-significant) increase in anxiety-like behavior (decreased open arm exploration in the elevated plus maze), while $A_{2A}R$ silencing in stressed animals yielded results similar to controls (**A**). In the forced swimming test, stress induced higher immobility time (**B**), especially in the first 2 min (**D**), and lower climbing to swimming ratio (**C**). Silencing $A_{2A}R$ in the amygdala prevented these changes. *$p<0.05$, two-way ANOVA followed by Bonferroni *post hoc* test.

4.5.9 Discussion

In this section, we aimed at assessing the role of $A_{2A}R$ in a pivotal brain region involved in the control of stress response and depression, the amygdala. However, we first performed two steps of experimental controls.

Importantly, it was necessary to optimize the injection coordinates, the time interval between injection of the virus and the beginning of the stress protocol and the spreading of viral diffusion. We consider that we were able to demonstrate that we injected the lentivirus into the basolateral nuclei of the amygdala (BLA) and one week was sufficient for lentiviral-mediated protein expression in the target region. In the following three weeks, an effective silencing of $A_{2A}R$ in the BLA occurred since a marked decrease in $A_{2A}R$ mRNA quantity was measured. This decrease is consistent with the long-term persistent expression of lentiviral proteins (Gascón et al., 2008; Scherr et al., 2003). Moreover, the fact that the lentiviral vector is not able to perform retrograde transfection (Osten et al., 2006) means that the $A_{2A}R$ knock down effects are confined to the BLA region and its efferents.

The BLA has neurons that project to varied brain regions to control the response to positive or negative-valence stimuli. Among these, there are projections to the ventral hippocampus (Felix-Ortiz et al., 2013) and the dorsolateral striatum (Lingawi & Balleine, 2012). These two regions are important in learning and memory of two different types: respectively, declarative/explicit memory and procedural/implicit memory.

This work showed that different stressors impair declarative-like memory inferred from the Y-maze and object displacement tests, and that these deficits could be prevented by the abrogation of neuronal $A_{2A}R$ function. However, the effect of BLA-only $A_{2A}R$ was unknown. In this chapter, chronic immobilization stress could induce memory deficits tested in the Y-maze, but silencing $A_{2A}R$ in the BLA did not afford beneficial effects. As for the amygdala, the hippocampus is part of a complex network of input and output connections. While the amygdala exerts some control over memory acquisition dependent of hippocampal function, the specific down-regulation of $A_{2A}R$, herein proposed to normalize neuronal function of the BLA, does not seem sufficient to normalize memory performance dependent on hippocampal function. It might be due to

the fact that the amygdala projects mainly to the ventral region of the hippocampus (Felix-Ortiz et al., 2013), which is involved in the control of emotional behavior (Henke, 1990), while spatial memory control occurs mostly in the dorsal region (Moser et al., 1995). One should not dismiss the indirect cross-talk between the dorsal and ventral regions of the hippocampus (reviewed by Fanselow & Dong, 2010), but the fact is that $A_{2A}R$ silencing in the BLA did not prevent hippocampal-dependent memory deficits caused by stress.

The connections from the amygdala to dorsal regions of striatum, especially the dorsolateral, are not as strong as to the hippocampus and ventral striatum (Cho et al., 2013). Thus, the direct amygdalar control over the dorsal striatum and its associated functions is likely limited. In the habit learning task, run in the Morris water maze, we did not find any effect of BLA's $A_{2A}R$ *per se*. Moreover, the immobility stress model did not alter habit learning, with all groups learning the task with equal efficiency; thus, possible modulatory effects of amygdalar $A_{2A}R$ on striatal function could not be assessed. A similar situation was observed for locomotor activity, in which the striatum also plays a pivotal role. There were no significant changes caused by stress and $A_{2A}R$ silencing did not alter the performance of either the control or stressed groups.

Another way in which the brain modulates stress responses is through the hypothalamic-pituitary-adrenal (HPA) axis, specifically, through the hypothalamus. This region is critically involved in the release of several hormones that maintain the homeostasis of essential body functions. Importantly, the hypothalamus releases corticotropin-releasing hormone (CRH) that acts on the anterior pituitary to release adrenocorticotropin hormone (ACTH), which in turn induces the adrenal glands to release corticosterone/cortisol (CORT) into the bloodstream. It has been widely described that CORT levels are elevated after chronic stress and in major depressive disorder (Belzung & Lemoine, 2011). Accordingly, immobilization stress caused a marked increase in CORT blood plasma concentration but the effect of silencing $A_{2A}R$ in the BLA was modest, at best. We proposed that $A_{2A}R$ in the BLA would be able to control the release of CORT by direct (stria terminalis and ventral amygdalofugal pathways) or indirect control of hypothalamic function. However, similarly to the hippocampus, the hypothalamus receives a multi-modal control from different brain regions, and

hampering $A_{2A}R$ function in BLA's neurons was not sufficient to prevent the changes of CORT levels caused by stress.

In addition to being part of the HPA axis, the hypothalamus exerts an important control over feeding behavior. This could be a pathway for $A_{2A}R$ silencing in the amygdala to prevent the reduced weight gain of stressed animals. However, like the increase of CORT, the decrease in weight gain after chronic immobilization was not affected by the silencing of $A_{2A}R$ in the BLA.

Regarding the classical role of the amygdala in controlling mood, the impact of $A_{2A}R$ in the BLA is more evident. Repeated immobilization stress reduced the exploration of the open arms in the elevated plus maze and of the central area of the OF, revealing an expected anxiogenic effect of stress. The BLA is particularly important in the control of anxiety responses, and, accordingly, animals with down-regulation of $A_{2A}R$ in this region displayed an anxiety-related behavior that closely resembled that of control animals. This preventive effect is probably due to a lower ability of the BLA to be activated upon chronic stress in a similar fashion to what has been described with models of neurodegenerative diseases and the role of $A_{2A}R$ in other brain regions, especially the hippocampus (Cunha et al., 2008).

Finally, in a test characterized by assessing depressive-like behavior, the forced swimming test, the effect of $A_{2A}R$ down-regulation was even clearer. Stressed animals stopped their escape bouts earlier than control animals, a behavior that indicates higher 'despair' in accordance to what has been described in depressive patients and that can be reversed by antidepressants in this behavioral test (Petit-Demouliere et al., 2004). However, -depleted stressed animals with $A_{2A}R$ down-regulated in the BLA showed a despair behavior profile similar to that of control animals. This preventive effect of the down-regulation of $A_{2A}R$ has been shown previously in global $A_{2A}R$ knock-out animals (El Yacoubi et al., 2001), but the fact that their confined abrogation in the BLA provides similar results is striking. This, once more, shows the pivotal role of the amygdala in controlling mood and shows for the first time the importance of the adenosinergic system within this brain structure. Considering the important role of $A_{2A}R$ in synaptic plasticity (Cunha, 2008) and that the BLA is a highly plastic region that has to constantly integrate and respond to different stimuli,

modulation of $A_{2A}R$ in the BLA has, accordingly, important consequences in the modulation of mood.

While the abrogation of $A_{2A}R$ normally has no effects *per se* and only plays a normalizing role in pathological-like conditions (Cunha, 2008), in the BLA it seems that $A_{2A}R$ might exert a tighter control of at least some neuronal networks. In the elevated plus maze, the down-regulation of $A_{2A}R$ in non-stressed animals caused an increase of the anxiety-profile, as measured by the decrease in the exploration of the open arms in the elevated plus maze test. The anxiogenic effect upon down-regulating $A_{2A}R$ has been noted in some studies using global $A_{2A}R$ KO mice but not with the use of selective antagonists (reviewed by Correa & Font, 2008). Indeed, we did not observe significant changes in anxiety of $A_{2A}R$ KO mice in this work, either with global deletion or selective deletion in forebrain neurons (including the amygdala). These apparently opposite data might be due to the varied roles of different structures in the control of mood. For example, the hypofunction of the ventral hippocampus results in increased anxiety and depressive-like behavior while amygdalar hyperfunction causes similar effects (Felix-Ortiz et al., 2013; Vyas et al., 2004). By abrogating $A_{2A}R$ in brain regions playing contrasting roles in the control of mood responses, no clear effects are expected to be observed in either direction. But when down-regulating $A_{2A}R$ selectively in the BLA, we can suggest that these receptors have a greater impact in the main efferent pathways from the BLA, including the stria terminalis, hippocampal projections and glutamatergic enervation to the central nuclei of the amygdala, all of which exert an anxiogenic effect (Janak & Tye, 2015). Moreover, the activation of the cell bodies of neurons in the BLA has been shown to be sufficient to induce an anxiety-like profile in the EPM test (Tye et al., 2011).

Overall, the down-regulation of $A_{2A}R$ in the BLA had a stronger effect on stress-induced behavioral changes that are most associated with amygdalar function and control, i.e., mood, as tested for anxiety and depression-like behaviors. Effects on behavioral changes that depend greatly on other brain regions (spatial memory and habit learning) were not observed. If this is due to limited control of BLA over these functions or limited effect of $A_{2A}R$ in the BLA to modulate changes in memory is not clear.

5. FINAL REMARKS

Throughout the work presented in this thesis, the deletion or blockade of A_{2A} receptors was continuously narrowed down, while the models of depression continuously became more specific.

We started studying the effects of the non-selective $A_{2A}R$ antagonist caffeine, given orally, then specifically knocking down $A_{2A}R$ globally and, in parallel, knocking down $A_{2A}R$ selectively in neurons of the forebrain. Finally, we reduced the amount of $A_{2A}R$ present in neurons of the basolateral amygdala.

Regarding the models of depression, we started using a bred-based genetic model (H/Rouen), then mimicking depressive-like state through chronic exposure to various stressors (CUS), and finally assessing the effects of a purely psychological repeated stressor (immobilization).

It seems clear that both genetic and pharmacological blockade of adenosine $A_{2A}R$ indicate that the antagonism of these receptors can be a potential therapeutic strategy in treating depression. This thesis describes improvements in memory impairment and mood changes that were accompanied by biochemical features, especially synaptic markers, in two important regions in the control of both memory and mood: the hippocampus and the amygdala.

Another important conclusion is that as $A_{2A}R$ blockade was narrowed down to only neurons and became region-specific, with the injection of the lentiviral vector encoding shRNA against $A_{2A}R$, the scope and influence of the potential therapeutic effects diminished. That might be, at least partly, explained by the limited influence of one brain region alone on the behavior of an animal.

This means that antagonizing $A_{2A}R$ in situations of depression and chronic stress can yield more robust and holistic benefits when done non-specifically. However, in this work, $A_{2A}R$ in the amygdala have been proven sufficient to control mood responses associated with depression and anxiety, which opens the possibility of testing the influence of these receptors in different brain regions.

We did not test if silencing $A_{2A}R$ in the hippocampus specifically could prevent memory deficits associated with stress and depression, but the extensive description of the role of $A_{2A}R$ in this brain region in neurodegenerative diseases, as referred throughout this text, leads us to consider it very likely. This hypothesis is further supported by the consistent memory rescuing effects of antagonizing $A_{2A}R$ in the chronic unpredictable stress and H/Rouen models.

Mechanistically, we propose that in situations of chronic stress and depression, neuronal $A_{2A}R$ are the ones facilitating the evolution of the disorder, as they are first increased in synapses but not in glial membranes. In the hippocampus it may result in neurodegeneration, while in the amygdala it results in the facilitation of its amygdalar function. Both processes probably act through the facilitation of glutamate neurotransmission: we have observed that knocking down $A_{2A}R$ hampers convulsions-induced neurodegeneration, others have described that the neurodegenerative process in the hippocampus is mediated by glutamate signaling, and the excitatory processes in and from the basolateral amygdala are mostly glutamatergic.

Clearly, there was throughout this work a correlation between synaptic changes and behavioral responses. Whenever hippocampal-dependent memory was impaired, and consequently hippocampal function was compromised, we have observed a decrease in the density of synaptic markers in the hippocampus (in CUS and H/Rouen models). In parallel, the increased response to negative valence stimuli, traditionally associated with increased amygdalar function, was accompanied by increased density of synaptic markers in the amygdala (physical load, social defeat and immobilization stress models). Synaptic plasticity is thus the driving force of memory performance and mood changes, and is also associated with changes in $A_{2A}R$ density. Thus, by changing the activity of $A_{2A}R$ we can control synaptic plasticity in different brain regions, namely the hippocampus and the amygdala, and ultimately control behavioral outcomes, a relevant strategy to treat mood disorders. This is what this work suggests: by blocking $A_{2A}R$ we are able to normalize and preserve normal synaptic function in brain regions affected by stress and depression-related disorders. Although we provide a strong argument for the role of $A_{2A}R$, the mechanisms through which their blockade might exert positive results, including the protection against synaptic damage and the normalization of synaptic function, are still unknown and further work in this field is required the understand the full picture.

Additionally, with the relevance of $A_{2A}R$ in neurons, their tight interaction with glutamate transmission, and the role of $A_{2A}R$ in specific brain regions impacting on the behavior of animals, it seems clear that these receptors might have an important role in other regions as well, which can be studied using models of

depression that cause a constellation of behavioral changes. Some candidate regions are the striatum that controls psychomotor activity, the nucleus accumbens that controls reward consumption and the prefrontal cortex that controls decision making.

Overall, this work shows that antagonizing $A_{2A}R$ is a promising novel strategy for the treatment of depression. Moreover, animal models of stress also provide a flexible and powerful tool to further understand the role of the adenosinergic system in different brain regions.

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