DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Adenosine A_{2A} Receptors and Stress-induced alterations in the rat ventral striatum

Inês Margarida Dias Cabaço Amaral

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Paula Margarida Gomes Canas (Centro de Neurociências e Biologia Celular) e do Professor Doutor Ângelo José Ribeiro Tomé (Departamento de Ciências da Vida, Universidade de Coimbra).

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ABSTRACT

Chronic stress is a major risk factor for the implementation of depression, a mental disorder that affects nearly 121 million people worldwide and is known to be associated with increased risk of suicide. The neurobiology underlying this pathology is not fully understood and available treatments for depression appear to be ineffective in a subset of patients, thus making this illness a public health burden. However, certain aspects of depression seem to result from maladaptive stress-induced modifications in specific brain regions and neural circuits. Synaptic dysfunction is a key aspect of depression and is a well-known consequence of chronic stress, as well as an imbalance between glutamatergic and GABAergic neurotransmission.

The ventral striatum is one of the regions affected in depression. Decreased functioning in this brain area has been evidenced in depression patients and also correlated with some symptoms of this disease.

To study mechanisms involved in depression is possible to use animal models such as social defeat stress and restraint stress. Distinct features observed in depressive syndrome, such as anhedonia, anxiety and social avoidance can be successfully replicated in laboratory animals.

Adenosine is a purine nucleoside present in all cells which in noxious brain conditions, like stress, is increased and activates adenosine A_{2A} receptors. Activation of these receptors allows controlling neuronal excitability and thus neurotransmitter release and the action of some receptors. Consumption of caffeine, a non-selective A_{2A} receptors antagonist, is inversely correlated with the risk of depression and suicide, and manipulation of A_{2A} receptors, either by blockade or depletion, has proven to modify several behavioral responses relevant for mood function in humans. Furthermore, the adenosinergic system is able to modulate dopaminergic, glutamatergic, serotoninergic and corticotrophin systems, known to be altered in depression.

Since there is not much information about A_{2A} receptors in the ventral striatum, we aimed to explore a possible role for these receptors and also stress-induced alterations in the ventral striatum.

Aims of this study were: (i) to validate the social defeat stress model; (ii) investigate the predominant localization of A_{2A} receptors in the ventral striatum; (iii) evaluate if A_{2A} receptors density is altered upon stress; (iv) explore if there are alterations in A_{2A} receptors upon stress in glutamatergic, GABAergic and dopaminergic nerve terminals, (v) as well as if restraint stress induced changes in synaptic markers such as synaptosomal-associated protein 25 (SNAP-25), syntaxin, synaptophysin and postsynaptic density protein 95 (PSD-95); and finally, (vi) evaluate if stress altered the balance between glutamatergic/GABAergic nerve terminals, assessed by their vesicular transporters.

Social defeat stress is a model that allows mimicking some of the symptoms of depression such as anhedonia, social avoidance, anxiety and depressive like-behaviors, in mice. In order for us to validate the use of this model, we performed a series of behavioral tests that informed us about the

model's reproducibility. Social defeat stress induced social avoidance in mice but failed to reproduce other key features of depression. Since we could not validate the model (i), this aim was left behind and alterations in the ventral striatum were then explored using restraint stress animal samples.

In the present work we used synaptosomes, structures that comprise both pre- and post-synaptic nerve terminal components, isolated from our region of interest - the ventral striatum, of animals subjected to restraint stress and also nonstressed animals, in order to assess alterations at the synaptic level.

Results obtained show (ii) a predominant synaptic localization of ventral striatum A_{2A} receptors, (iii) which were downregulated upon stress exposure. Additionally, (iv) A_{2A} receptors are present in glutamatergic, GABAergic and dopaminergic nerve terminals in the ventral striatum and stress does not appear to induce any alterations in their localization. Regarding alterations in synaptic markers we observed an increased PSD-95 density after restraint stress, whereas levels of SNAP-25, synaptophysin and syntaxin remained unaltered. At last, (v) the number of glutamatergic and GABAergic nerve terminals and vGluT1 density remained unchanged upon exposure to restraint stress.

Altogether, the data obtained in this work will help to gain further knowledge about the role that A_{2A} receptors in ventral striatum may play in the pathology of depression.

Keywords: Depression, stress, adenosine A_{2A} receptors, ventral striatum.

RESUMO

O stresse crónico é um dos principais fatores de risco para o aparecimento da depressão, uma doença mental que afeta cerca de 121 milhões de pessoas em todo o mundo e que se sabe estar associada a um elevado risco de suicídio. Contudo, a neurobiologia envolvida nesta patologia ainda não é totalmente compreendida e os tratamentos disponíveis parecem ser ineficazes em certos pacientes, o que torna esta doença um grave problema de saúde pública. No entanto, sabe-se que certos aspetos da depressão parecem resultar de alterações induzidas pelo stresse em regiões específicas do cérebro, assim como nos circuitos neuronais que as interligam. Disfunção sinática e um desequilíbrio na transmissão glutamatérgica/GABAérgica são aspetos chave da depressão e consequências do stresse crónico.

Na depressão, o estriado ventral é uma das regiões afetadas. Estudos realizados em pacientes com depressão evidenciaram uma diminuição na atividade cerebral nesta área, que foi associada com o aparecimento de alguns sintomas desta doença.

Estudar os mecanismos que levam ao aparecimento da depressão é possível utilizando modelos animais como o stresse de confronto social e o stresse de imobilização. Algumas das caraterísticas observáveis no síndrome depressivo, como a anedonia, ansiedade e evitamento social, podem ser replicadas com sucesso nos animais, em laboratório.

A adenosina é uma purina que se encontra presente em todas as células. Quando estas estão expostas a condições nocivas, existe um aumento de adenosina, que irá ativar os recetores A_{2A} de adenosina. A ativação destes recetores permite controlar a excitabilidade neuronal, a libertação de neurotransmissores e a ação de alguns receptores. O consumo de cafeína – antagonista não-selectivo dos receptores A_{2A}, está inversamente correlacionado com o risco de depressão e suicídio, e a manipulação destes recetores, quer através de bloqueio ou deleção seletiva, leva à modificação de vários comportamentos associados com o humor. Além disso, o sistema adenosinérgico é capaz de modular outros sistemas, como o dopaminérgico, glutamatérgico, serotoninérgico e de corticotrofina, que se sabe estarem alterados na depressão.

Uma vez que não existe muita informação acerca dos recetores A_{2A} no estriado ventral, iremos explorar o papel destes recetores, assim como possíveis alterações induzidas pelo stresse nesta região em particular.

Os objetivos deste estudo foram: (i) a validação do modelo animal de stresse de confronto social; (ii) investigar a localização predominante dos recetores A_{2A} no estriado ventral; (iii) avaliar se a densidade de recetores A_{2A} varia após o stresse; (iv) explorar se, existindo alterações, elas ocorrem em terminais nervosos glutamatérgicos, GABAérgicos e dopaminérgicos; (v) verificar se o stresse induziu alterações em marcadores sináticos, como a proteína associada aos sinaptossomas 25 (SNAP-25), sintaxina, sinaptofisina e a proteína da densidade pós-sinática 95 (PSD-95); por último,

(vi) quisemos avaliar se existia uma alteração no equilíbrio Glutamato/GABA, avaliando os respetivos transportadores vesiculares.

O stresse de confronto social é um modelo que mimetiza alguns dos sintomas da depressão, como a anedonia, ansiedade, evitamento social e comportamentos depressivos, em murganhos. De maneira a podermos validar este modelo, realizámos testes comportamentais que nos informaram acerca da reprodutibilidade deste modelo. O stresse resultante do confronto social levou a que os murganhos evitassem o contato social, no entanto nenhuma outra caraterística chave da depressão foi observada.

Visto que não conseguimos validar este modelo (i), este objetivo foi posto de parte, e fomos estudar as alterações induzidas pelo stresse usando amostras do estriado ventral de animais sujeitos a stresse de imobilização.

No presente trabalho utilizámos sinatossomas – estruturas que incluem componentes dos terminais nervosos pré- e pós-sináticos, isolados da nossa região de interesse – o estriado ventral, de animais sujeitos a stresse de imobilização e animais controlo (não stressados), com o objetivo de verificar possíveis alterações a nível sinático.

Os resultados obtidos demonstram que (ii) os recetores A_{2A} no estriado ventral se encontram predominantemente nas sinapses (iii) e que a sua densidade diminuiu após o stresse, (iv) não alterando a sua localização em terminais nervosos glutamatérgicos, GABAérgicos e dopaminérgicos. Relativamente a alterações nos marcadores sináticos, os resultados mostram um aumento na densidade de PSD-95 com o stresse, enquanto os níveis de SNAP-25, sinaptofisina e sintaxina permaneceram inalterados.

Por fim, pudemos verificar que o número de terminais nervosos glutamatérgicos e GABAérgicos não sofreu alterações induzidas pelo stresse, tal como a densidade de vGluT1 no estriado ventral.

Resumindo, os dados obtidos neste estudo ajudarão a aprofundar o conhecimento em relação ao recetores A_{2A} no estriado ventral e o seu envolvimento na depressão.

Palavras-chave: Depressão, stresse, recetores A_{2A} de adenosina, estriado ventral.

LIST OF CONTENTS

LIST OF FIGURES AND TABLES

Figures Index

Tables index

LIST OF ABBREVIATIONS

A_1R adenosine A_1 receptors

$A_{2A}R$ adenosine A_{2A} receptors

AMP adenosine monophosphate

ANOVA analysis of variance

APS ammonium persulfate

ATP adenosine triphosphate

BCA bicinchoninic acid

BDNF brain-derived neurotrophic factor

BSA bovine serum albumin

CAPS 3-(cyclohexylamino)propane-1-sulfonic acid

CTR control

CSF cerebrospinal fluid

CUS chronic unpredictable stress

DAT dopamine transporter

DLS dorsolateral striatum

DMS dorsomedial striatum

DNA deoxyribonucleic acid

DSM-V Diagnostic Statistical Manual 5th edition

DTT dithiothreitol

ECT electroconvulsive therapy

EDTA 2-[2 [Bis(carboxymethyl)amino]ethyl} (carboxymethyl)amino]acetic acid

EPM elevated plus maze

FST forced swimming test

GABA γ-aminobutyric acid

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPA hypothalamic-pituitary-adrenal

mPFC medial prefrontal cortex

MR mineralocorticoid receptors

NAc nucleus accumbens

NMDA N-methyl-D-aspartate

OF open field

PBS phosphate buffered saline

PFC prefrontal cortex

PMSF phenylmethanesulfonyl fluoride

PSD-95 postsynaptic density protein 95

RIPA radioimmunoprecipitation assay

RST restraint stress

RT room temperature

SDS sodium dodecyl sulfate

SI social interaction

SNAP-25 synaptosomal-associated protein 25

SNARE SNAP receptor

TBS-T trizma buffered saline with tween 20

TEMED N,N,N',N'-Tetramethylethane-1,2-diamine

TST tail suspension test

vGAT vesicular GABA transporter

vGluT1 vesicular glutamate transporter 1

VS ventral striatum

CHAPTER I – INTRODUCTION

1.1 DEPRESSION

Major depressive disorder is a leading cause of disability worldwide (Kessler et al., 2005) and represents a significant public health burden (Greenberg et al., 2003). Defined as a mental disorder (Wancata and Friedrich, 2011), depression affects nearly 121 million people and twice as many women as men (Kessler et al., 1993, Lucas et al., 2011), and is highly associated with chronic illnesses or other mood disorders such as co-morbid anxiety (Evans et al., 2005).

Symptoms of depression include depressed mood, irritability, low self-esteem, feelings of hopelessness, worthlessness and guilt, low energy, fatigue, decreased interest in pleasurable activities such as social interactions, alterations in appetite, weight loss or weight gain and insomnia (Diagnostic and Statistical Manual of Mental Disorders - 5th edition). These symptoms have great impact in an individual's quality of life and more severe forms of depression can eventually lead to suicide (Wancata and Friedrich, 2011). A diagnosis is made when at least 5 symptoms are reported for longer than a 2 week period of time (DSM-V).

The neurobiology and neural circuitry underlying this disorder is not fully understood. However, there are some formulated theories that try to explain the development depression: genetic vulnerability, altered hypothalamic-pituitary-adrenal (HPA) axis activity, deficiency of monoamines, dysfunction of specific brain regions, neurotrophic processes, reduced GABAergic activity, dysregulation of glutamate system and impaired circadian rhythms (Hasler, 2010).

In most people, depression results from the interaction between a genetic predisposition (Nestler et al., 2002), which is roughly 30-40% (Sullivan et al., 2000, Hasler, 2010), and some environmental factors (Nestler et al., 2002) such as stress, trauma, viral infections and stochastic processes occurring during brain development (Akiskal, 2000, Fava and Kendler, 2000). Interplay between genes and environment may be regulated through epigenetic mechanisms such as deoxyribonucleic acid (DNA) methylation and histone modifications (Sun et al., 2013, Bagot et al., 2014). Though, not all people exposed to the mentioned external influences develop depression, whereas some that did not have any contact with the stressors do (de Kloet et al., 2005).

Depression has been defined as a stress-related disorder (Nestler et al., 2002). Under conditions of stress an increase in the production and release of glucocorticoids, corticosterone in humans and cortisol in rodents, is triggered by specific neuropeptides such as the corticotropin-releasing hormone (de Kloet et al., 2005). In healthy individuals these neuropeptides help to adapt to these unusual and unwanted situation, but during depression they fail to do so (de Kloet et al., 2005) causing profound alterations on metabolism and affecting behavior by acting on several brain regions (Nestler et al., 2002).

Clinical studies have demonstrated that in depressed individuals there is a persistent overactivation of the HPA axis and consequently a hypersecretion of corticosteroids, and a reduced negative feedback (de Kloet et al., 2005, Duman and Aghajanian, 2012, Lucassen et al., 2014).

Additionally, was shown that an imbalance in the HPA axis activity can result in depressive features (Krishnan and Nestler, 2011). Mood disorders have also been associated with altered circadian rhythms and sleep disturbances (Germain and Kupfer, 2008).

Alterations in the support conferred by neurotrophic factors, may also contribute to the pathology. Research on this topic has been focused on the brain-derived neurotrophic factor (BDNF) due to its occurrence in the adult brain. Human postmortem studies revealed decreased BDNF levels in the hippocampus of depression patients that seem to be mediated partly by stress-induced glucocorticoids and alterations in neurotransmitter systems (Nestler et al., 2002, Sun et al., 2013).

Several brain regions, major components of the limbic-reward circuitry (Russo and Nestler, 2013, Bagot et al., 2014), appear to be involved in the pathology of depression (see Figure 1). Prefrontal cortex (PFC) and hippocampus mediate some cognitive aspects of depression, such as memory impairments, feelings of worthlessness, hopelessness, guilt and suicidality (Nestler et al., 2002). Emotion, memory, reward mediating anhedonia and anxiety are regulated by the ventral striatum (VS) and the amygdala (Nestler et al., 2002, Russo and Nestler, 2013). Lastly, the hypothalamus is speculated to have a role due to some neurovegetative symptoms of depression such as too much or too little sleep and loss of appetite and energy (Nestler et al., 2002).

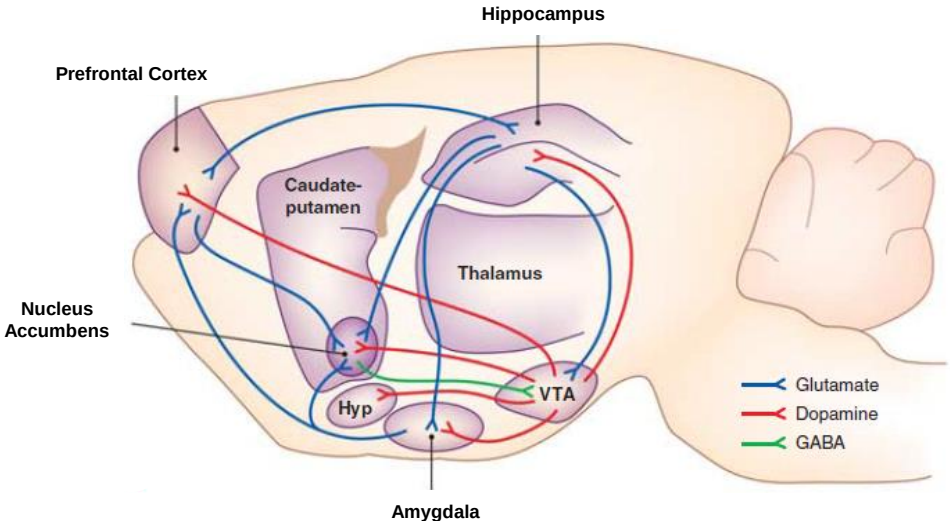

Figure 1 – Brain regions implicated in the pathophysiology of depression [adapted from (Sun et al., 2013)].

This is supported by neuroimaging and anatomical studies in depression patients that provided evidence of abnormalities in those brain regions. Changes in blood flow or glucose metabolism, and alterations in volume are consistently found in regions of prefrontal and cingulate cortex, hippocampus, striatum, amygdala and thamalus (Drevets, 2001, Manji et al., 2001). Dysfunction found in these specific regions appears to be related with neuronal atrophy, glial and synaptic loss (Table 1) (Price and Drevets, 2010, MacQueen and Frodl, 2011, Kang et al., 2012), resulting in altered circuit connectivity.

Table 1 – Neuroimaging and postmortem human studies of depression patients [adapted from (Price and Drevets, 2010, Russo and Nestler, 2013)].

Region	Alterations in Depression
Nucleus Accumbens	↓ volume
Hippocampus	↓ volume ↓ synapse density ↓ glial cell density
Basolateral Amygdala	↓ volume ↓ glial cell density
Medial Prefrontal Cortex	↓ volume ↓ dendritic branching ↓ glial cell density

Keeping a balanced inhibitory/excitatory neurotransmission is required for a proper functioning of the brain. Abnormalities in glutamatergic and GABAergic neurotransmission have been associated with depression (Krystal et al., 2002). Clinical studies reported elevated glutamate as well as reduced gamma-aminobutyric acid (GABA) levels in plasma and CSF of depressed patients (Altamura et al., 1993, Petty, 1995, Levine et al., 2000). Furthermore, glutamatergic transmission is known to be increased in limbic-thalamo-cortical circuits (Drevets et al., 2008) whereas GABAergic neurotransmission is downregulated (Sanacora et al., 1999). These alterations may be due to global changes observed in genes involved in glutamatergic and GABAergic neurotransmission (Sequeira et al., 2009).

Monoaminergic systems are known to regulate a wide variety of brain functions such as mood, attention, reward processing, sleep, appetite and cognition (Delgado, 2000). A depletion or dysfunction in dopaminergic, serotoninergic and noradrenergic systems can influence mood and ultimately lead to depression (Manji et al., 2001).

Reduction in serotonin synthesis leads to the development of depressive symptoms and abnormalities in serotonin receptors are present in several brain areas (Drevets et al., 1999) which clearly demonstrates the importance of the serotoninergic system in the development of this pathology. There's also evidence of decreased norepinephrine metabolism and transporter density in depressed subjects (Charney and Manji, 2004).

There is increasing interest in the role of the dopaminergic system. Neuroimaging studies showed reduced striatal dopamine transporter (DAT) binding and dopamine uptake in depression patients, consistent with reduced dopaminergic neurotransmission (Meyer et al., 2001). Experimentally reduced dopaminergic transmission into the ventral striatum has been associated to anhedonic-like behaviors in depressed subjects (Hasler et al., 2008). Moreover, dopamine reuptake inhibitors and receptor agonists have also been demonstrated to have antidepressant properties (Goldberg et al., 2004).

Majority of antidepressants mechanisms of action focus on rising monoamine levels (Krishnan and Nestler, 2008). Antidepressants, together with other forms of treatment such as psychotherapy and electroconvulsive therapy (ECT), represent the therapeutics available (Nemeroff and Owens, 2002). However, less than 50% of all patients show full remission in response to these treatments (Nemeroff and Owens, 2002).

New techniques have been developed, based on neuroanatomical research, such as deep brain stimulation and transcranial magnetic stimulation that target specific brain regions and have been shown to produce clinical benefits (Mayberg et al., 2005, Janicak et al., 2013) in depression patients.

Still, currently available treatments remain ineffective in a subset of patients (Trivedi et al., 2006) contributing significantly to the health burden associated with depression (Duman, 2010). Therefore there is a need in improving therapeutic options, which are dependent on the identification of more potential targets by investigating the processes that might be involved in the pathogenesis of depression.

1.1.2 Stress as a risk factor for depression

Chronic stress is considered a precipitating factor in the development of depression (Kessler, 1997, de Kloet et al., 2005). As mentioned before, stress response is highly dependent on an individual's genetic predisposition and also on the external early life factors, whose effects might accumulate overtime (de Kloet et al., 2005).

When in a stressful situation, an "alarm reaction" is activated by the autonomic nervous system which leads to the release of noradrenaline and adrenaline (de Kloet et al., 2005, Lucassen et al., 2014), immediately followed by the activation of the HPA axis. Different brain areas display different vulnerability to stress because of the distinct expression of glucocorticoid (GR) and mineralocorticoid receptors (MR) (Reul and de Kloet, 1985). An imbalance in MR/GR was demonstrated to alter negative feedback (de Kloet et al., 2005), required for an adaptive response, and eventually leading to conditions such as depression (Hasler, 2010) and anxiety (Lucassen et al., 2014). In addition, the hypercortisolaemia that results from chronic stress targets monoaminergic system in a manner similar to that seen in depression (de Kloet et al., 2005). Additionally, stress increases glutamate neurotransmission prompting excitotoxicity and neuronal damage (Jezova, 2005).

Exposure to stress can induce some deleterious effects on the brain structure and function. Animal models of stress are used in order to assess those alterations. These modifications include reduced number of spines and decreased dendritic volume in the hippocampus and PFC (Sousa et al., 2000, Wellman, 2001), alterations in synaptic plasticity and cell loss (Duman and Aghajanian, 2012). Unlike the hippocampus and PFC, chronic stress increases spine density and dendrite complexity in the NAc, amygdala and orbitofrontal cortex (Mitra et al., 2005, Schwabe and Wolf, 2009). Chronic stress also affects microglia, increasing their ramifications (Hinwood et al., 2013) and inducing a reduction in astrocyte density (Banasr and Duman, 2007).

Glucocorticoids released after chronic stress exposure affect behavior through their action on several brain regions (Nestler et al., 2002). Stress-induced alterations include anhedonia, social avoidance, anxiety and other depression-related behaviors that will be further explored below. Ongoing research using animal models that replicate those behavioral alterations have enlightened some mechanisms that imply stress as a factor in favor of the implementation of depression.

1.1.2.1 Animal models of stress

Modelling human depression has been difficult due to the complexity of the disease and subjective nature of its symptoms, but also because the current knowledge of the physiological and molecular mechanisms underlying this disease is still very limited (Nestler and Hyman, 2010).

Most popular and commonly used models merge behavioral assays with latest technological advances in molecular biology and automated video tracking (Nestler and Hyman, 2010) in order to quantify representative behaviors characteristic of human depression in rodents (Duman, 2010) (Figure 2).

In order to be considered a good and valid animal model, some requirements need to be fulfilled. The model needs to have *face*, *construct* and *predictive validity*. Replicate important anatomical, biochemical, neuropathological or behavior features of human diseases is required in an animal model to achieve *face validity* (Nestler and Hyman, 2010). Indeed, today's animal models recapitulate the features but they aren't all present simultaneously and the phenotypes generated can be transient (Krishnan and Nestler, 2010). *Construct validity* refers to a models' ability in illustrating the etiological factors or molecular insights implicated in depression (Krishnan and Nestler, 2010), which is very difficult to achieve since the mechanisms underlying depression are not fully understood (Duman, 2010). *Predictive validity* refers to a model's ability to respond to treatments that are effective in the human disease (Belzung and Lemoine, 2011). Reproducibility in a laboratory and reliability are also important features of a good animal model.

Animal models of stress can be clustered into models of acute, subchronic and chronic stress. Paradigms in which animals are exposed to acute or subchronic stress include the forced swimming test (FST), tail suspension test (TST), the learned helplessness model and imply short-term exposure to an inescapable or uncontrollable stress (Duman, 2010). The FST and TST are widely used to infer "depression-like" behavior (Nestler and Hyman, 2010). Chronic mild stress, early-life stress and social defeat are models of chronic, long-term exposure to stress (Duman, 2010) in which animals are exposed to repeated unpredictable/uncontrollable stress situations, that later are combined with assays that allow to quantify their behavior (Nestler and Hyman, 2010). These models are considered more representative of the true nature of stress exposure in humans and more accurate in simulating the processes that lead to a depressive-like state (Duman, 2010). Still, only a subset of symptoms of depression can be objectively measured in stressed animals.

Animals subjected to stress exhibit a characteristic phenotype characterized by anhedonia, anxiety and depressive-like behaviors such as increased immobility in the FST (Duman, 2010, Menard et al., 2015).

Overlapping human and rodent studies disclosed critical information that allowed developing and testing the efficacy of drugs that target currently known mechanisms of this disease.

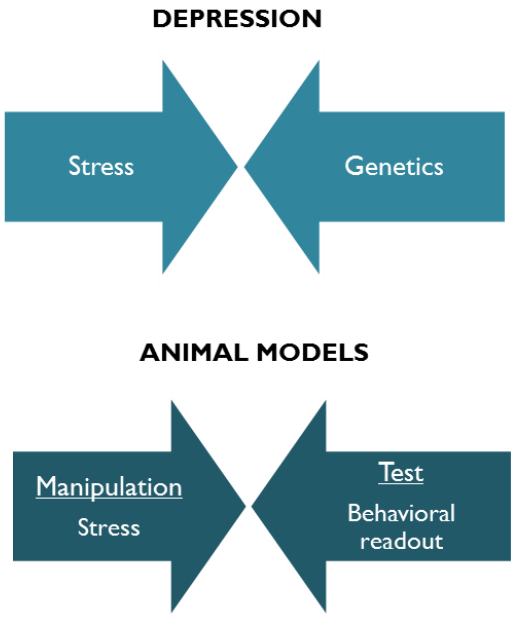

Figure 2 – Developing animal models [adapted from (Cryan and Slattery, 2007)].

I. Social Defeat Stress

Repeated exposure to social defeat generates persistent emotional stress without habituation (Tidey and Miczek, 1997) and intense and long-lasting behavioral and physiological changes along with alterations in brain neurochemistry (Blanchard et al., 2001).

Although the intensity of the stress used is more severe than seen in most humans (Nestler and Hyman, 2010), social defeat stress is an animal model widely used to study molecular mechanisms underlying depression. This model mimics several pathological dimensions of depression (Buwalda et al., 2005, Berton et al., 2006) including social avoidance, anhedonia, anxiety and depressive-like behavior (Berton et al., 2006, Tsankova et al., 2006, Krishnan et al., 2007, Hollis et al., 2010, Golden et al., 2011, Jin et al., 2015), that can be reverted by chronic administration of antidepressants (Berton et al., 2006, Tsankova et al., 2006, Covington et al., 2009, Yin et al., 2014). Social defeat stress also allows discriminate animals on depression- and anxiety-like behavioral domains (Golden et al., 2011).

In this model, C57BL/6 mice are subjected to daily agonistic encounters by CD-1 mice (Golden et al., 2011) leading to the development of some behaviors in defeated mice similar to stress-related depression in humans (Kudryavtseva et al., 1991). Since it was shown that counter-fighting while being attacked decreases the levels of stress (Meerlo et al., 1999) usually the chosen non-defeated mice have higher bodyweight and belong to a strain with high levels of aggression (Buwalda et al., 2005), to ensure the outcome of the social conflict.

This experience of defeat is not only considered a physical stressor (Hollis and Kabbaj, 2014). After each daily confrontation, C57BL/6 and CD-1 remain in the same cage until the next day defeat, only separated by a clear perforated barrier (Golden et al., 2011) that allows them to have sensory, except for tactile, contact (Blanchard et al., 2001). So, although the agonistic experience is intermittent, it allows psychogenic exposure of defeated mice to the non-defeated, aggressive mice (Blanchard et al., 2001). In fact, one important aspect of this specific stress model relies on its ability to continuously activate the HPA axis (Tornatzky and Miczek, 1993, Koolhaas et al., 1997).

In spite all positive and distinct features that make this model suitable to study stress-induced modifications, social defeat only induces avoidance of social contact and signs of a depressive-like behavior in a subset of defeated animals (Hyman, 2007, Krishnan et al., 2007) – termed susceptible, which suggests the existence of different, individual coping responses between animals of the same strain exposed to the same stressors (Krishnan et al., 2007). The remaining group of animals, unsusceptible or resilient, are not devoid of symptoms, although they fail in developing key pathological aspects of depression (Russo et al., 2012), like social avoidance (Krishnan et al., 2007), showing their ability in avoiding the deleterious consequences of chronic social stress, as it happens with humans (Russo et al., 2012).

Susceptible and resilient mice can be easily distinguished after a social defeat stress protocol through a social interaction (SI) test. In SI test defeated animals are placed in an open-field arena and allowed to interact with the non-defeated animals (Golden et al., 2011). If they interact with other mice at the same rate as control animals, they failed in developing social avoidance (Hyman, 2007), and therefore exhibit the resilient phenotype (Figure 3).

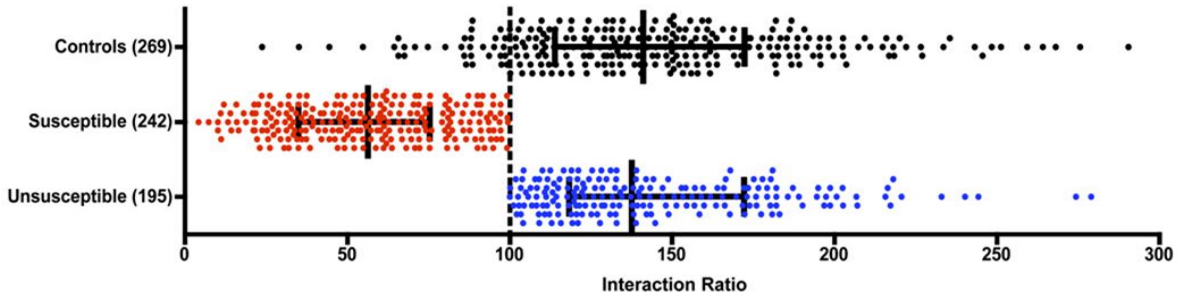

Figure 3 - Distribution of interaction ratios for control, susceptible and unsusceptible (or resilient) mice over social defeat experiments (Krishnan et al., 2007).

Susceptible mice normally exhibit a subset of physiological alterations after social defeat stress such as tachycardia and elevated blood pressure, increased adrenocorticotropic hormone and glucocorticoid activity, hyperthermia, reduction of circadian amplitudes (Tornatzky and Miczek, 1993), a significant dysregulation of the HPA axis (Hollis and Kabbaj, 2014) accompanied by enhanced serotoninergic activity (Berton et al., 1998, Blanchard et al., 2001), a metabolic syndrome and also

weight alterations (Krishnan et al., 2007, Goto et al., 2014). Additionally, the modifications in HPA axis regulation appear to be long-lasting (Buwalda et al., 1999, Ruis et al., 1999).

Although susceptible and resilient C57BL/6 mice show different depressive-like behaviors, both groups can exhibit deficits in exploratory-based behavior, interpreted as increased anxiety, stress-induced polydipsia and increased corticosterone after a stress experience (Krishnan et al., 2007).

Social defeat stress also induces morphological changes. Decreased volume and cell proliferation were observed in the mPFC and hippocampus (Czeh et al., 2001, Becker et al., 2008, Van Bokhoven et al., 2011), whereas in the NAc of susceptible mice is evident an increase in spine density, correlated with altered synaptic plasticity (Christoffel et al., 2011).

Additionally, hypofunction of dopamine systems following chronic social defeat in brain regions such as the striatum and mPFC was linked to an increased risk in developing depression (Watt et al., 2009, Novick et al., 2011).

This model has some disadvantages, like the fact that it requires a lot of space and it's labor intensive and expensive (Golden et al., 2011).

Despite its pitfalls, this animal model is considered a useful mean to investigate the mechanisms underlying depression and to aid in developing treatments (Berton et al., 2006, Golden et al., 2011).

II. Restraint Stress

Animal models that employ repeated stress represent a valuable means to study neurobiological alterations (Nestler et al., 2002) and recurrent exposure to stress has been associated with development of depression and anxiety-related disorders (Gregus et al., 2005, Kim and Han, 2006).

Restraint stress is one of the most widely used repeated stress animal models (Gregus et al., 2005) and has been used successfully in several studies to clarify the effects of chronic stress on the brain (McLaughlin et al., 2007). In this animal model, rodents are immobilized, with help of either hard-plastic containers or a wire mesh, for few hours daily and, in same circumstances, lasting several weeks (McLaughlin et al., 2007). Kim and collaborators demonstrated that a restraint protocol of 2-8 hours per day for 14 days is enough to induce anxiety- and depression-like behaviors (Kim and Han, 2006).

Restraint stress combines both psychological and physical elements, as animals are trapped and their movement restricted (McIntyre et al., 1999). This combination results in extensive morphological, neurochemical and consequently behavioral changes.

Animals subjected to restraint stress develop a depression-like phenotype characterized by reduced sucrose preference, exploratory deficits associated with increased anxiety, and increased immobility in the forced swim test, some of which can be reversed by chronic treatment with antidepressants (Conrad et al., 1999, Sandi et al., 2001, Kim and Han, 2006, Ulloa et al., 2010, Chiba et al., 2012, Lee et al., 2013).

Monoaminergic systems are quite important as stress mediators. Restraint stress has been shown to decrease norepinephrine, dopamine and serotonin levels in the hippocampus of male rats after a 21-day protocol (Sunanda et al., 2000), as well as in the mPFC of rats subjected to restraint stress for 14 consecutive days (Lee et al., 2013).

Neuronal morphology is also affected by restraint stress. Several studies evidenced dendritic atrophy and reduced cell proliferation in the rat PFC and hippocampus, respectively, (Conrad, 2006, Radley et al., 2006, Radley et al., 2008), whereas formation of new spines was observed in the amygdala (Mitra et al., 2005), following repeated restraint stress. Additionally, restraint stress appears to modify synaptic strength in NAc medium spiny neurons (Lim et al., 2012). Restraint stress disrupts the expression of several synaptic proteins (Table 2), therefore compromising synaptic plasticity and resulting in morphological and functional alterations that might play a role in the pathology of stress-related disorders (Muller et al., 2011).

Table 2 – Alterations in synaptic protein levels following restraint stress.

Synaptic protein	Restraint stress-induced alterations
SNAP-25	↓ levels in PFC (Muller et al., 2011)
Syntaxin	↑ levels in PFC (Muller et al., 2011)
Synaptophysin	↓ levels in HIPP and cerebral cortex (Thome et al., 2001, Xu et al., 2004, Cunha et al., 2006, Amin et al., 2015)
PSD-95	↓ levels in HIPP (Meng et al., 2013)

1.2 ADENOSINERGIC SYSTEM AND MODULATION OF MOOD DISORDERS IN THE VENTRAL STRIATUM

1.2.1 Adenosine

Adenosine is a purine nucleoside found in all cells that exerts important regulatory functions in the Central Nervous System, by acting in parallel as a homeostatic transcellullar messenger and as a neuromodulator (Cunha, 2001b, Fredholm et al., 2005). Adenosine exerts a crucial role in cells either when energy charge is compromised or under stressful conditions (Fredholm et al., 2005). Additionally, it is involved in several metabolism main pathways such as synthesis of purinergic nuclei acid base and aminoacid metabolism (Cunha, 2001b).

At the synaptic level, adenosine acts as a neuromodulator mediating the information flow and neuronal excitability by controlling neurotransmitter release and thus the action of some receptor systems (Cunha, 2001b, Fredholm et al., 2005, Gomes et al., 2011), independently of energy metabolism imbalance (Cunha, 2001b).

Adenosine present in extracellular milieu is very important in the modulation of synaptic transmission (Cunha, 2001b, Latini and Pedata, 2001) and can be generated through several mechanisms. The catabolism of released adenine nucleosides, mostly adenosine triphosphate (ATP), through the ectonucleotidase pathway seems to be the main source of adenosine at the synaptic level (Cunha, 2001b). Lastly, intracellular adenosine can be released to the extracellular milieu through nucleoside transporters or a reversal of the sodium gradient and synthesized extracellularly after the release of cyclic adenosine monophosphate (cAMP) (Fredholm et al., 2005).

Clearance of extracellular adenosine is made across the cells membrane or neighbor cells through nucleoside transporters (Fredholm et al., 2005). After being taken up, adenosine is either converted into its inactive metabolite inosine by adenosine deaminase, or phosphorylated by adenosine kinase into adenosine monophosphate (AMP) (Latini and Pedata, 2001, Fredholm et al., 2005).

Ischemic- and seizure-induced neuronal injury, free radical induction, metabolic poisoning, agonists of ionotropic glutamate receptors, nitric oxide and arachidonic acid are able to trigger a dramatically increase in intracellular levels of adenosine (Cunha, 2001b, Latini and Pedata, 2001, Fredholm et al., 2005). In such conditions, intracellular ATP is readily converted into adenosine, leading to an exponential increase in the intracellular concentration of adenosine (Fredholm et al., 2005). However, due to the bi-directional non-concentrative nucleoside transporters endowed in the membranes, extracellular adenosine levels will rise in parallel with the intracellular adenosine levels (Cunha, 2005), reinforcing the homeostatic role of adenosine in cells.

1.2.2 Adenosine Receptors

Adenosine, when in the extracellular milieu, acts on adenosine receptors (Cunha, 2005).

Currently there are four subtypes of adenosine receptors known and pharmacologically characterized: A_1, A_{2A}, A_{2B} and A_3. These are pleiotropic receptors that couple different G-proteins according to their localization and degree of activation (Cunha, 2005). A_1 and A_3 receptors mainly interact with the G_i family, whereas A_{2A} and A_{2B} bind to G_s (Fredholm et al., 2001).

A_1 receptors (A_1R) are the most abundant in the brain (Fastbom et al., 1987), being highly expressed in several regions such as the hippocampus, cortex and cerebellum (Cunha, 2005). Activation of A_1R in the presence of a noxious stimulus appears to decrease the rate of cell metabolism, through the inhibition of neuronal excitability and synaptic transmission (Cunha, 2005). In the brain, A_1R are also important, for instance, in the control of emotional stability (Lang et al., 2003), sleep regulation and analgesia (Chen et al., 2013).

In contrast, A_{2A} receptors ($A_{2A}R$) are mainly concentrated in basal ganglia, albeit being present throughout the brain in lower levels (Svenningsson et al., 1999). $A_{2A}R$ are mainly involved, for example, in the control of locomotion and cognition (Cunha and Agostinho, 2010).

A_{2B} receptors have low expression levels in the brain and when activated decrease A1-mediated inhibition of synaptic transmission (Goncalves et al., 2015).

Lastly, A_3 receptors appear to be poorly expressed in the brain (Cunha, 2005) and might have a role in the modulation of motor activity (Ferre et al., 1997).

In the brain adenosine acts mostly through inhibitory A_1 and facilitatory A_{2A} receptors (Cunha, 2001a), given the affinity of adenosine for its receptors ($A_1 > A_{2A} > A_{2B} > A_3$) (Dunwiddie and Masino, 2001).

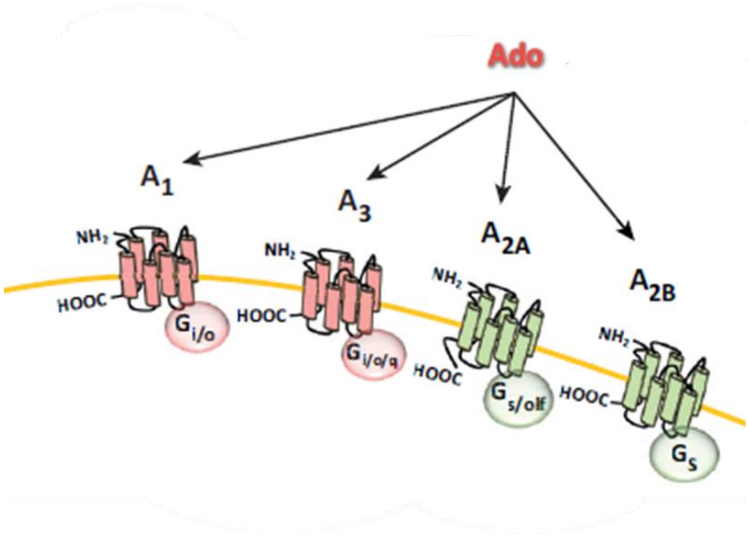

Figure 4 – Adenosine receptors are coupled to different G proteins [adapted from (Dias et al., 2013)].

1.2.3 Adenosine A_{2A} Receptors

As mentioned, A_{2A} facilitatory receptors are highly expressed in the basal ganglia (Schiffmann et al., 1991, Rosin et al., 1998, Svenningsson et al., 1999) where their density is 20 times higher than anywhere in the brain (Lopes et al., 2004). Here, they are found predominantly in dendritic spines (Rosin et al., 1998, Hettinger et al., 2001) and postsynaptic densities (Rodrigues et al., 2004, Rebola et al., 2005) of asymmetric contacts between cortico-thalamic glutamatergic projection (Rodrigues et al., 2004) and striatopallidal medium spiny GABAergic neurons (Schiffmann et al., 1991, Svenningsson et al., 1999, Hettinger et al., 2001). A minority of these A_{2A} receptors is located presynaptically (Rodrigues et al., 2004). Outside the basal ganglia, they are present in the presynaptic active zone of limbic and neocortical regions (Cunha, 2005, Rebola et al., 2005). $A_{2A}R$ are also present in astrocytes (Li et al., 2001), microglia cells (Kust et al., 1999) and blood vessels (Coney and Marshall, 1998).

Activation of $A_{2A}R$ stimulates cyclic AMP-protein kinase A pathway through coupling to G_{olf} protein in the brain, where they interact with different receptors and molecules in order to mediate motor activity, psychiatric behaviors and sleep-awake cycle, and to G_s in peripheral tissues where they modulate, for instance, inflammation and angiogenesis (Chen et al., 2013).

$A_{2A}R$ may also signal through a protein kinase C-dependent pathway (Cunha and Ribeiro, 2000) and a mitogen-activated protein kinase pathway (Fredholm et al., 2005).

$A_{2A}R$ control the release of neurotransmitters through functional interactions or the formation of heterodimers with other receptors such as A_1 (Ribeiro, 1999, Ciruela et al., 2006), dopamine D_2 (Ferre et al., 1997), group I metabotropic glutamate 5 (Tebano et al., 2005), NMDA (Ribeiro, 1999), and cannabinoid CB_1 (Carriba et al., 2007), thus triggering different signaling pathways.

1.2.4 Adenosine A_{2A} Receptors and Depression

The adenosinergic system is able to modulate mood states (Gomes et al., 2011). Nevertheless, its role in depression is rather complex.

There is evidence that some therapeutic strategies used to control mood disorders, such as ECT and sleep deprivation, result in short and long term adaptations directly associated with the adenosine modulation system (Cunha et al., 2008). Moreover, some antidepressants are able to bind to adenosine receptors, decreasing extracellular adenosine levels (Gomes et al., 2011).

Effects of caffeine, considered the most consumed psychoactive drug (Fredholm et al., 1999) and a well-known competitive antagonist of A_{2A} receptors (Fredholm et al., 2001), have been widely explored regarding modulation of mood disorders. Human studies demonstrated that consumption of low to moderate doses of caffeine decrease cognitive failures and depressive symptoms (Smith, 2009). Moreover, in stressful situations caffeine consumption increases and is inversely correlated with risk of depression (Smith, 2009, Lucas et al., 2011, Lucas et al., 2013) and the risk of suicide (Kawachi et al., 1996). In patients with panic disorder, with high susceptibility to anxiety attacks, administration of caffeine increases anxiety (Boulenger et al., 1984). Two linked polymorphisms in the A_{2A} receptor gene have been associated with increased anxiety levels after caffeine intake and the incidence of panic disorder (Alsene et al., 2003, Hamilton et al., 2004).

Nonselective activation of A_{2A} receptors by adenosine and its analogues appears to induce depressive-like behaviors in animal models of depression (Hunter et al., 2003) which can be reversed by administration of classical antidepressants (Kulkarni and Mehta, 1985). However, it was also shown that administration of adenosine can decrease the antidepressant-like effects in animal models of depression, and those effects reinstated after treatment with an A_{2A} receptor-selective antagonist (Kaster et al., 2004).

Manipulation of A_{2A} receptors has been shown to modify behavioral responses relevant for mood function in humans (Cunha et al., 2008). Both genetic depletion and A_{2A} receptors blockade results in an antidepressant-like phenotype characterized by reduced immobilization period or suppressed behavioral despair in the forced swim test and tail suspension test, respectively (El Yacoubi et al., 2001, El Yacoubi et al., 2003). Furthermore, overexpression of A_{2A} receptors leads to a depressive-like behavior (Coelho et al., 2014).

Moreover, there are evidences obtained through animal models of a stress-induced increase in A_{2A} receptors density in the hippocampus and striatum (Cunha et al., 2006, Batalha et al., 2012, Crema et al., 2013, Kaster et al., 2015). Again, their manipulation with antagonists seems to relieve the early modifications induced by stress, re-establish basal levels of corticosterone, restore HPA axis activity to basal levels and attenuate loss of synaptophysin verified after sub-chronic stress (Cunha et al., 2006, Batalha et al., 2012). Recently was also demonstrated that chronic caffeine pretreatment for 14 days and during the 10 days of social defeat stress is able to reverse social avoidance behavior and anhedonia (Yin et al., 2014).

Together, these studies highlight the interest in A_{2A}R manipulation (Cunha et al., 2006) as a novel therapeutic option to manage mood disorders.

1.2.5 Ventral striatum and Depression

The striatum is the main input structure of the basal ganglia, a neural network responsible for adaptive control of behavior by connecting different brain areas responsible for sensorimotor, motivational and cognitive actions (Ferre et al., 1997, Schiffmann et al., 2007).

This brain region can be functionally subdivided into dorsal and ventral striatum. The ventral striatum, which comprises the nucleus accumbens, ventral caudate and ventral putamen, and the olfactory tubercule (Ferre et al., 1997, Goff and Tottenham, 2014), receives glutamatergic inputs from other basal ganglia structures such as the mPFC, amygdala and hippocampus, but also dopaminergic inputs from the ventral tegmental area (VTA) (Ferre et al., 2007), that appear to facilitate information flow from limbic to motor system (Ferre et al., 1997).

This striatal region has been related to several aspects of reward learning and motivation (Goff and Tottenham, 2014). Magnetic resonance studies revealed an activation of ventral striatum in situations involving stimuli predicting rewards and during the consumption of the reward (Pujara and Koenigs, 2014). Additionally, this region was also associated with pathological risk-taking and addictive behaviors (Haber, 2011).

Hypofunctioning in the ventral striatum, mainly in mesolimbic dopamine circuits, has been correlated with depressive symptoms, mainly anhedonia, and recognized as a possible mechanism underlying this illness (Nestler and Carlezon, 2006, Dunlop and Nemeroff, 2007, Goff et al., 2013).

To support this hypothesis, there is evidence that in healthy individuals this particular brain area is highly modulated by social background (Fliessbach et al., 2007) and particularly affected following early-life stress, that leads to reduced ventral striatum activity (Dillon et al., 2009). An abnormal cerebral blood flow and elevated glucose metabolism in the ventral striatum also appear to be characteristic of depression patients (Drevets, 2001). Moreover, human postmortem studies evidenced a volume reduction in the ventral striatum, mainly in caudate, putamen and accumbens nuclei of major depression patients (Husain et al., 1991, Krishnan et al., 1992, Baumann et al., 1999). A reduction in dopaminergic transporter (DAT) levels was also demonstrated, mainly in caudate, putamen and NAc of depressed subjects, which can be correlated to blunted dopaminergic transmission (Pizzagalli, 2014).

Chronic stress, a major risk factor of depression (de Kloet et al., 2005), can cause long-term adaptations in the dopaminergic projection neurons projected from the VTA to the ventral striatum (Pittenger and Duman, 2008) and therefore may contribute to its dysregulation in depression (Nestler and Carlezon, 2006).

In rodents, stress of social nature has been shown to increase spine density and number of functional glutamatergic synapses in NAc medium spiny neurons (Christoffel et al., 2011). Despite this increased excitatory tone, stress seems to induce a further decrease in inhibitory tone as consequence of loss of inhibitory synapses (Russo and Nestler, 2013). In addition, glucocorticoid exposure in early life results in decreased NAc volume as well as decreased mesolimbic dopamine receptors density (Barrot et al., 2000, Martinez-Tellez et al., 2009) .

Since ventral striatum is tightly connected with several brain areas known to be involved in depression, this region can also be considered practical candidate for neuromodulation (Hauptman et al., 2008). Activity in the ventral striatum increases with antidepressant treatment (Willner et al., 2013) and deep brain stimulation of the ventral striatum appears to ameliorate the symptoms and reduces ahnedonia in treatment-resistant patients with major depression (Schlaepfer et al., 2008, Malone et al., 2009).

Adenosine functions as a modulator of neurotransmission in both glutamatergic and dopaminergic input neurons that converge on the dendritic spines of striatal GABAergic medium-spiny neurons (Ferre et al., 2007). In the ventral striatum, A_{2A} receptors can be found mostly in the NAc and olfactory tubercule (Rosin et al., 1998, Moreau and Huber, 1999, Svenningsson et al., 1999). In the caudate-putamen and NAc A_{2A} receptors are co-expressed with dopamine D_2 receptors (Schiffmann et al., 1991, Svenningsson et al., 1997).

Caffeine, an A_{2A} receptor antagonist, exerts effects in the striatum comparable to increased dopaminergic neurotransmission (Ferre et al., 1997). Additionally, in the ventral medial striatum of rodents, selective A_{2A} receptor antagonist 8-(3-chlorostyryl)cafeine (CSC) relieved the reserpine-induced symptoms of depression induced by pretreatment with reserpine (Hanff et al., 2010), an antipsychotic drug that acts by depleting monoamine levels.

Evidence collected using animal models and human studies suggests an involvement of A_{2A} receptors from ventral striatum in the effects of in depression.

CHAPTER 2 – AIMS

The main goal of this work was to validate the social defeat stress model in order to manipulate A_{2A} receptors in striatum sub-regions and evaluate whether they could abrogate mood changes that occur in chronic stress and depression.

Since the first aim was not successfully achieved, we pursued a parallel line of research: explore A_{2A} receptors role in the ventral striatum of rodents, as well as stress-induced alterations that could provide evidence of the contribution of ventral striatum to the pathology of depression. For that purpose, we used samples (already collected) of animals subjected to restraint stress.

Particularly, we evaluated:

- Localization of A_{2A} receptors in the ventral striatum;
- A_{2A} receptors density upon stress;
- A_{2A} receptors alterations in glutamatergic, GABAergic and dopaminergic nerve terminals;
- Changes in synaptic markers: SNAP-25, syntaxin, synaptophysin and PSD-95;
- Balance between glutamate/GABA.

CHAPTER 3 – MATERIALS AND METHODS

3.1 ANIMALS

Male C57BL/6 and CD-1 mice (Figure 5) with 14 -16 and 16 weeks, respectively, and Wistar rats (Figure 6) with, approximately, 8 weeks were purchased from Charles River (Charles River Laboratories, Barcelona, Spain) and housed under standard laboratory conditions (12h light/12h night cycles, 22–24°C, relative humidity of 55% and ad libitum access to water and food).

The experiments were done according to Union guidelines (2010/63/EU), and were approved by the Ethics Committee of the Center for Neuroscience and Cell Biology of Coimbra.

Figure 5 – CD-1 (left) and C57BL/6 (right) mice.

Image taken from: http://www.stopdown.net/sci__rudy__bio__rudy__bullied__rudy__mice__rudy__chem.gif

Figure 6 – Wistar rat.

Image taken from: http://www.scanburresearch.com/CropUp/pagewide/media/7442/Wistar-Han.jpg

3.2 REAGENTS

Table 3 – List of the reagents used.

Reagent	Supplier
(2R,3R,4S,5S,6R)-2-[(2S,3S,4S,5R)-3,4-dihydroxy-2,5-bis(hydroxymethyl)oxolan-2-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol (Sucrose)	Sigma-Aldrich
(2R,3S,4R,5R)-2,3,4,5,6-Pentahydroxyhexanal (Glucose)	Sigma-Aldrich
2-(Bis(2-hydroxyethyl)amino)acetic acid (Bicine)	Sigma-Aldrich
2,6-dibromo-4-[3-(3,5-dibromo-4-hydroxyphenyl)-1,1-dioxo-2,1λ-benzoxathiol-3-yl]phenol (Bromophenol blue)	Sigma-Aldrich
2-[2-[3,4-bis(2-hydroxyethoxy)oxolan-2-yl]-2-(2-hydroxyethoxy)ethoxy]ethyl dodecanoate (Tween 20)	Sigma-Aldrich
2-[2-[Bis(carboxymethyl)amino]ethyl} (carboxymethyl)amino]acetic acid (EDTA) tetrasodium	Sigma-Aldrich
2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol (Triton X-100)	Sigma-Aldrich
2-Amino-2-hydroxymethyl-propane-1,3-diol (Trizma base)	Sigma-Aldrich
3-(cyclohexylamino)propane-1-sulfonic acid (CAPS)	Sigma-Aldrich
30% Acrylamide/Bis solution	Bio Rad
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich
Ammonium Persulfate (APS)	Sigma-Aldrich
Bicinchoninic acid (BCA) kit	Thermo Fisher Scientific
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Cocktail of protease inhibitors	Roche
Dithiothreitol (DTT)	Sigma-Aldrich
SuperSignal ® West Pico Chemiluminescence Substrate	Thermo Fisher Scientific
Horse Serum	Invitrogen
Hydrogen Chloride (HCl)	Sigma-Aldrich
Methanol	Sigma-Aldrich
N,N,N′,N′-Tetramethylethane-1,2-diamine (TEMED)	Sigma-Aldrich
Paraformaldehyde	Sigma-Aldrich
Percoll	GE Healthcare
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich
Ponceau S	Sigma-Aldrich
Potassium Chloride (KCl)	Sigma-Aldrich
Potassium didydrogen phosphate (KH_2PO_4)	Sigma-Aldrich
Prolong Gold Antifade Reagent	Invitrogen
Glycerol	Sigma-Aldrich
Sodium Chloride (NaCl)	Sigma-Aldrich
Sodium Dodecyl Sulfate (SDS)	Bio Rad
Sodium Hydroxide (NaOH)	Merck
Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$)	Sigma-Aldrich

3.3 ANIMAL MODELS

3.3.1 Social defeat stress

Social defeat stress protocol was performed by investigators of the Purines group.

3.3.1.1 Social defeat stress protocol

Social defeat stress protocol was executed as described in *Golden et al., 2011*.

Screening of aggressive CD-1 was performed in their home cage (Figure 7) by placing in it a different naïve C57BL/6 mouse for 180 seconds, for 3 consecutive days. The criteria used for CD-1 selection was that CD-1 mouse had to attack in at least two consecutive sessions and the latency to initial aggression had to be less than 60 seconds.

Over a total of 10 days, C57BL/6 mice were exposed for 5 minutes (each day) to different CD-1 mice of those previously selected. Defeated mice were transferred to the opposite compartment of the resident (CD-1) cage until the next day confrontation, separated by a clear perforated barrier. C57BL/6 mice were exposed to different CD-1 mice each day, to prevent habituation. After daily defeats, if there were mice with open wounds (exceeding 1 cm), they were removed from the experiment and sacrificed. Control mice were paired within similar home cages and rotated daily, never being allowed to contact with their cage mates.

At this point, C57BL/6 mice were isolated in individual cages, since housing familiar male animals tends to decrease anxiety (Ruis et al., 1999).

Figure 7 – Standard home cage used in the social defeat experiments (Golden et al., 2011).

3.3.1.2 Social interaction test

On day 11, a social interaction test was performed in order to identify susceptible and resilient mice. In this test each C57BL/6 mice were placed in an open field arena (Figure 8, panel A) and their movement was recorded in two distinct sessions: one in the absence (150 seconds) and other in presence of the aggressor CD-1 mouse (150 seconds) surrounded by a wire-mesh enclosure (Figure 8, panel B). A video-tracking device and software were used to measure time mice spent in the interaction zone, in the rear corners and their total movement in the arena. The interaction ratio was calculated as (interaction time, target present)/(interaction time, target absent), multiplied by 100. In accordance with previous works, an interaction ratio of 100 was set as a cutoff: mice with score below 100 were defined as "susceptible" and those with scores equal or above 100 were defined as "unsusceptible" or "resilient" (Krishnan et al., 2007).

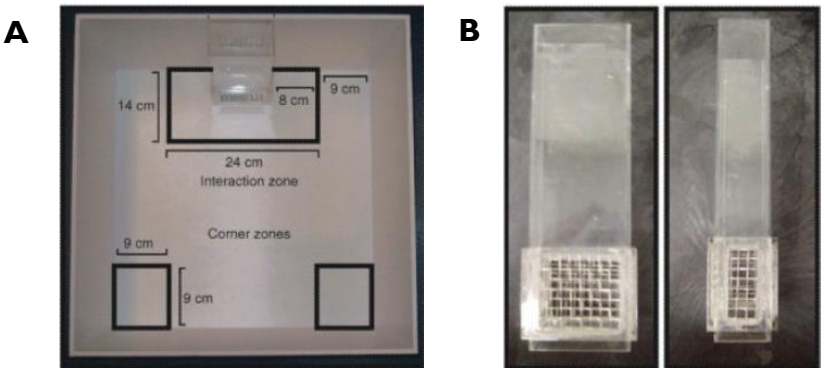

Figure 8 – Representation of the social interaction open field arena **(A)** and front-view and side view of a wire-mesh enclosure **(B)** (Golden et al., 2011).

3.3.1.3 Open field

Locomotor activity of C57BL/6 mice was monitored in an open field arena (Figure 9) and the exploratory behavior was evaluated by the total distance travelled in a 10 minutes period (Coelho et al., 2014). Activity of the animals in the open field was recorded by a video camera and software. The apparatus was cleaned with a solution of ethanol 10% between tests in order to remove animal odors or clues.

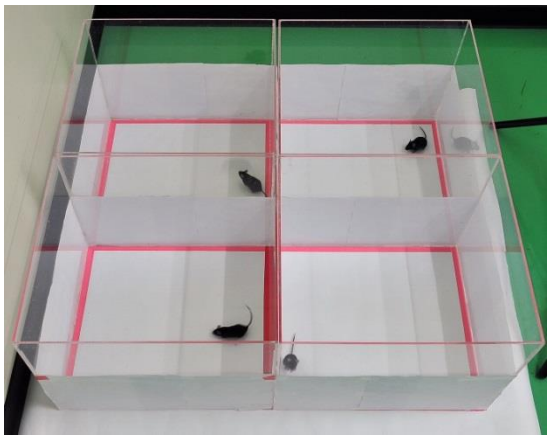

Figure 9 – Open field arena. Image taken from: http://tmc.sinica.edu.tw/images/open+field-2.jpg.

3.3.1.4 Elevated plus maze

Anxious-like behavior was assessed in the elevated plus maze test. Briefly, C57BL/6 mice were placed in the center of a plus-like elevated apparatus (Figure 10) with two opposing closed and two opposing open arms, which animals were allowed to explore freely for 5 minutes. Time mice spent in the open and closed arms and the number of entries in both arms was recorded by a video camera and software (Dawson and Tricklebank, 1995). Percentage of time mice spent in the open arms was calculated as (total time spent in the open arms)/(total time spent in open and closed arms), multiplied by 100. The apparatus was cleaned with a solution of ethanol 10% between tests in order to remove animal odors or clues.

Figure 10 – Elevated plus maze apparatus.

Image taken from: http://www.lintoninst.co.uk/Portals/0/productimages/276_0adeb.jpg

3.3.1.5 Splash test

C57BL/6 mice were placed in their home cage after the vaporization of a 10% sucrose solution on their dorsal coat (Figure 11). After being squirted with the solution, animals initiate grooming behavior (licking their fur in order to remove/taste the solution). This behavior was recorded by a video camera for 5 minutes, and later quantified by an investigator blinded to the treatments. Total time mice spent grooming and time spent grooming due to the sucrose solution were measured. These parameters were used as a measure of anhedonic behavior (Machado et al., 2012).

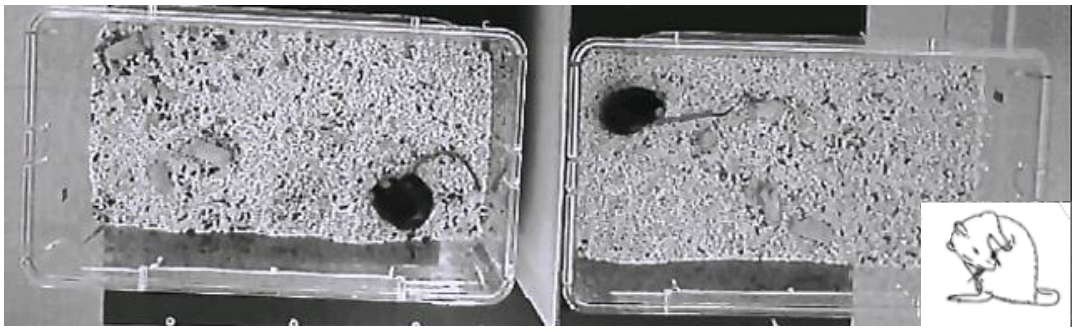

Figure 11 – Representation of splash test. C57BL/6 mice licking their fur after being sprayed with a sucrose solution [adapted from video recordings].

3.3.1.6 Forced swimming test

The FST was carried out in an open and clear cylindrical container (Figure 12) almost filled with water at approximately 22/23°C. C57BL/6 mice were placed for 6 minutes in the containers and their movement was recorded using a video camera, and later total immobility time was quantified by an investigator blinded to the experimental conditions. Immobility is defined as floating motionless in the water and making only the movements necessary to keep their heads above water, and was used as a measure of behavioral-despair (Coelho et al., 2014).

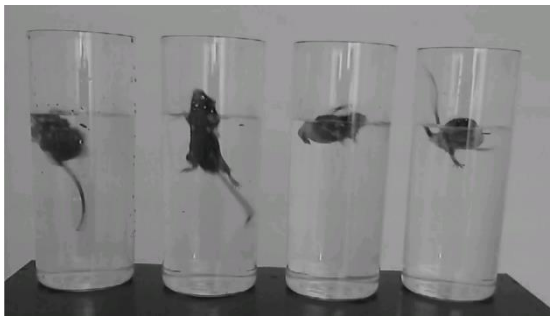

Figure 12 – Forced swimming test [adapted from video recordings].

3.3.2 Restraint stress

3.3.2.1 Restraint stress protocol and samples

Restraint stress protocol was performed by colleagues of the purines group as in previous studies (Cunha et al., 2006), although with slight alterations. Briefly, male Wistar rats were individually placed in a plastic compartment and immobilized in a plastic bottle with plastic tape on the outside and a hole at one end to allow rats to breath. Each daily restraint session lasted 4 hours and the protocol was performed in a period of 14 days. After each session, rats were returned to their home cages and left undisturbed under constant temperature and light cycles, with free access to food and water, until next day immobilization.

Tissue from the ventral striatum was obtained either by me or colleagues, in previous studies involving stress protocols or whenever there were naïve animals available belonging to experiments of other members of the Purines group, and were used for both immunocytochemistry and western blotting techniques.

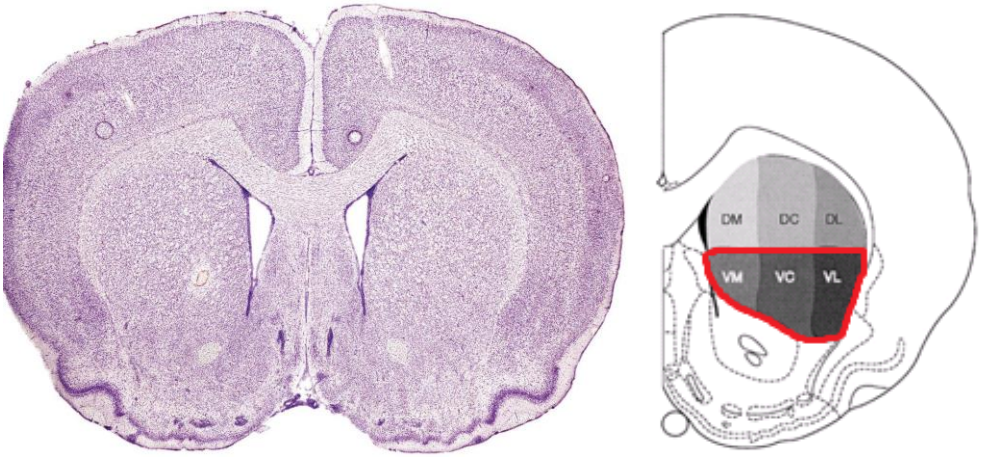

Figure 13 – Representation of a rat brain slice and the ventral striatum section collected [adapted from Paxinos and Watson – The rat brain in stereotaxic coordinates 6th edition and (Deumens et al., 2002)].

3.3.2.2 Preparation of synaptosomes and total membranes

Synaptosomes are re-sealed nerve terminals that detached from their axons and postsynaptic connections due to a homogenization of the brain tissue in an isotonic medium. They also enclose the cytoplasm, synaptic vesicles, mitochondria and the cytoskeleton, within a membranous sac (Dunkley et al., 2008).

Samples were homogenized in 8 mL of a sucrose solution [0.32 M sucrose, 1 mM EDTA-Na, 10 mM HEPES, 0.015 mM bovine serum albumin (BSA)]. After setting the volume at 10 mL, the homogenized tissue was centrifuged at 3000 g for 10 minutes (Sigma 3-18k centrifuge, rotor 12-158H) at 4°C, in order to remove the nuclei and cell debris. The supernatants were kept and divided to prepare synaptosomes and total membranes. Approximately 3 mL were used for total membranes and the remaining volume to synaptosomes.

In order to isolate total membranes, a second and single centrifugation was made at 25000 g for 1 hour, at 4°C. The supernatants were discarded and resuspended in an appropriate volume of the lysis buffer RIPA [50 mM Tris, 150 mM NaCl, 1% IGEPAL (NP-40), 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS] containing a cocktail of protease inhibitors (Roche) with 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and stored at - 20°C, until further use.

To isolate synaptosomes, the supernatant was centrifuged for 12 minutes at 14000 g at 4°C, to pellet the synaptosomes together with the plasma membranes, myelin and mitochondria. The supernatants were discarded and the pellets resuspended in 1 mL of a Percoll 45 % [45% v/v Percoll, 0.067 M NaCl, Krebs HEPES Ringer solution (140 mM NaCl, 1 mM EDTA-Na, 10 mM HEPES, 5 mM KCl, 5 mM glucose)] solution. Afterwards, the resuspended pellets were centrifuged in an Eppendorf centrifuge for 2 minutes at the maximum speed, 16000 g, and the top layer was collected and resuspended in 1 mL of KHR solution. A final centrifugation was made, also at the maximum speed and for 2 minutes, at 4°C. The resulting pellet was resuspended in the appropriate volume of RIPA containing a cocktail of protease inhibitors (Roche) with 0.1 mM PMSF (Quiroz et al., 2009).

3.3.2.3 BCA Method for protein quantification

The bicinchoninic acid (BCA) assay is a method for colorimetric detection and quantification of total protein. BSA was used as protein standard.

After incubation of the samples with the mixed reagents from the BCA protein assay kit (Pierce® BCA Protein Assay, Thermo Scientific), for 30 minutes at 37°C, absorbance was read at 570 nm in a spectrophotometer. The resulting standard curve was then used to determine the protein concentration of each sample in µg/µL.

3.3.2.4 Western blot of synaptosomes and total membranes

After protein concentration was determined, samples were prepared for Western blotting by adding sample buffer 6x [30% glycerol, 10.3% SDS, 500 mM Tris, 0.6M DTT, 0.012% bromophenol blue], followed by heating the samples at 70°C for 20 minutes. Samples were stored at -20°C until they were used.

Gel electrophoresis was carried out in a 10% polyacrylamide resolving gel with a 4% polyacrylamide stacking gel (Table 4). The proteins were transferred to nitrocellulose membranes, with a constant current of 1A, for 2 hours at, approximately, 4°C.

Membranes were incubated with Ponceau S in order to stain the protein, and then washed in distilled water, followed by a single wash in a tris buffered saline (1x) with 0.1% tween 20 (TBS-T) solution.

Prior to the incubation with the primary antibody, membranes were blocked in a solution containing 5% low-fat milk in TBS-T, for 1 hour at room temperature (RT), with agitation. Primary antibody (Table 5) was diluted in a solution of 1% milk in TBS-T and membranes immersed in it overnight, at 4°C. After washed with TBS-T, membranes were incubated in a solution containing the corresponding horseradish peroxidase conjugated secondary antibody (Table 6) diluted in 1% milk in TBS-T, for 2 hours at RT. Membranes were revealed with a SuperSignal® West Pico Chemiluminescence Substrate kit (Thermo Fisher Scientific) and then visualized in a VersaDoc system (Bio Rad).

In order to probe other proteins, such as GAPDH for protein normalization, in the same nitrocellulose membranes, reprobing was performed. Nitrocellulose membranes were blocked in a 5% milk in TBS-T solution, followed by an overnight incubation with the primary antibody. The remaining steps, from incubation with the secondary antibody to revealing the membranes, are the same as the ones mentioned above.

Table 4 – Gel formulation.

Formulation (1 gel)	Resolving Gel (10%)	Stacking Gel (4%)
Tris-buffer 1.5 M (pH 8.8)	2.5 mL	-
Tris-buffer 0.5 M (pH 6.8)	-	2.5 mL
Acrylamide 30%	3.3 mL	1.3 mL
Water	4.1 mL	6.1 mL
SDS 10 %	100 µL	100 µL
TEMED	5 µL	10 µL
APS 20 % (freshly prepared, diluted in water)	100 µL	100 µL

Table 5 – Primary antibodies for Western blot.

Antibody	Host	Supplier	Reference	Dilution	Protein Band Size (kDa)
$A_{2A}R$	Mouse	Santa Cruz Biotechnology	sc-32261	1:200	45
GAPDH	Rabbit	Abcam	ab9485	1:3000	37
PSD-95	Mouse	Sigma-Aldrich	P-246	1:10000	95
SNAP-25	Mouse	Sigma-Aldrich	S5187	1:10000	25
synaptophysin	Mouse	Sigma-Aldrich	S 5768	1:20000	38
syntaxin	Mouse	Sigma-Aldrich	S 0664	1:20000	35
vGluT1	Guinea Pig	Synaptic Systems	135 304	1:20000	55

Table 6 – Secondary antibodies for Western blot.

2ry Antibody	Host	Supplier	Reference	Dilution
Anti-Guinea pig IgG peroxidase conjugated	Rabbit	Sigma-Aldrich	A5545	1:20000
Anti-mouse IgG Horseradish Peroxidase conjugate	Goat	Thermo Scientific	31432	$A_{2A}R$ - 1:500 others -1:20000
Anti-rabbit IgG Horseradish Peroxidase conjugate	Goat	Thermo Scientific	31462	1:10000

3.3.2.5 Immunocytochemistry

I. Immunocytochemistry of synaptosomes

Synaptosomes were placed onto poly-D-lysine coated coverslips at RT for 1 hour, to make sure they adhere to their surface. Then synaptosomes were fixed with 4% paraformaldehyde [in phosphate buffered saline (PBS) medium 140 mM NaCl, 3 mM KCl, 20 mM $Na_2HPO_4 \cdot 7H2O$, 15 mM KH_2PO_4, pH 7.4] for 15 minutes at RT. Coverslips were washed twice with PBS (1x), for at least 10 minutes. This step was followed by permeabilization in which coverslips were incubated with PBS containing 0.2% Triton X-100, a non-ionic detergent, for 10 minutes at RT. After permeabilization, synaptosomes were again washed twice with PBS (10 minutes each). In order to reduce non-specific binding of the antibody, coverslips were incubated with a solution containing PBS with 3% bovine serum albumin (BSA) and 5% horse serum, blocking agents, for 1 hour at RT. This step was followed by washing twice the coverslips with PBS containing 3% BSA and then the incubation with the primary antibody (Table 7) diluted in PBS with 3% BSA was performed overnight, at 4°C.

On the second day, coverslips were washed three times with PBS with 3% BSA (10 minutes each wash) in order to remove unspecific binding of the primary antibody, and then were immunolabeled with the secondary antibody (Table 8), also diluted in PBS with 3% BSA, for 1 hour in the dark at RT. All secondary antibodies were previously centrifuged at 16000 g for 10 minutes before being applied, to avoid aggregates. Negative controls, in which the addition of the corresponding primary antibody was omitted, were made to exclude possible unspecific labelling resulting from the secondary antibody.

At last, coverslips were rinsed with PBS, dried in absorbent paper, and mounted into glass slides using Prolong Gold Anti-fading (Invitrogen), a medium that protects fluorescent dyes from fading, avoiding the quenching of the initial fluorescence signal. Mounted coverslips were allowed to dry overnight in the dark at RT, and then sealed with nail polish before the acquisition of the images with the fluorescence microscope.

Table 7 – Primary antibodies for immunocytochemistry.

Antibody	Host	Supplier	Reference	Dilution
$A_{2A}R$	guinea pig	Frontier Institute	-	1:500
DAT	rat	Millipore	MAB369	1:200
synaptophysin	mouse	Sigma-Aldrich	S 5768	1:500
synaptophysin	rabbit	Millipore	AB9272	1:1000
vGAT	rabbit	Synaptic Systems	131 003	1:500
vGluT1	mouse	Synaptic Systems	135 511	1:500

Table 8 – Secondary antibodies for immunocytochemistry.

2ry Antibody	Host	Supplier	Reference	Dilution
Alexa Fluor 594 conjugate anti-guinea pig	goat	Invitrogen	A-11076	1:1000
Alexa Fluor 594 conjugate anti-mouse	donkey	Invitrogen	A-21203	1:1000
Alexa Fluor 594 conjugate anti-rabbit	donkey	Invitrogen	A-21207	1:1000
Alexa Fluor 488 conjugate anti-mouse	donkey	Invitrogen	A-21202	1:1000
Alexa Fluor 488 conjugate anti-rabbit	donkey	Invitrogen	A-21206	1:1000
Alexa Fluor 488 conjugate anti-rat	donkey	Invitrogen	A-21208	1:1000

II. Image acquisition and quantification

The image acquisition was made using a Zeiss Axiovert 200 microscope equipped with a 63x oil objective, an AxioCam HRm camera and using Axiovision software 4.6.

Each coverslip (two to three per experiment) was analyzed by counting 5 different fields, with a total of 15 fields, and in each field analyzing a total amount of 900 individualized elements.

Quantifications of the data obtained were made using Image J software, with a macro developed in the group (Costenla et al., 2011).

3.4 STATISTICAL ANALYSIS

Data are expressed as mean ± SEM of the indicated number of independent experiments in different animals. Statistical analysis to compare the mean value of a single group with a hypothetical value was performed a one-sample t test. A Student's t test was used in order to test the significance of the difference between two independent groups. To compare two or more groups, either one-way or two-way analysis of variance (ANOVA) for independent means were used, followed by a Bonferroni post-hoc test for multiple comparisons. Statistical differences were considered significant at $p < 0.05$ and all tests were performed using the GraphPad Prism Software® (StatSoft Inc., La Jolla, CA, USA).

CHAPTER 4 – RESULTS

4.1 SOCIAL DEFEAT STRESS

4.1.1 Social interaction Test

A Social interaction test was performed in order to test the efficacy of the performed social defeat protocol.

Social interaction testing allowed separating defeated animals into susceptible, the ones that developed social avoidance and are considered to be stressed, and resilient, the ones that failed in developing social avoidance and are quite similar to control animals. Social interaction (SI) ratios were calculated as a ratio between the time mice spent in the interaction zone of the open field arena when CD-1 was present by the time mice spent there when CD-1 was absent, multiplied by 100. A SI ratio equal to 100, in which the animal spent equal time in the interaction zone when the social target was present versus absence of a social target, is defined as a threshold for separating defeated mice into susceptible and resilient (Krishnan et al., 2007).

According to the social interaction ratios obtained, in the population of defeated C57BL/6 mice were present 14 susceptible and 3 resilient mice (Figure 14). In all tests, data regarding defeated mice is presented in accordance with these results.

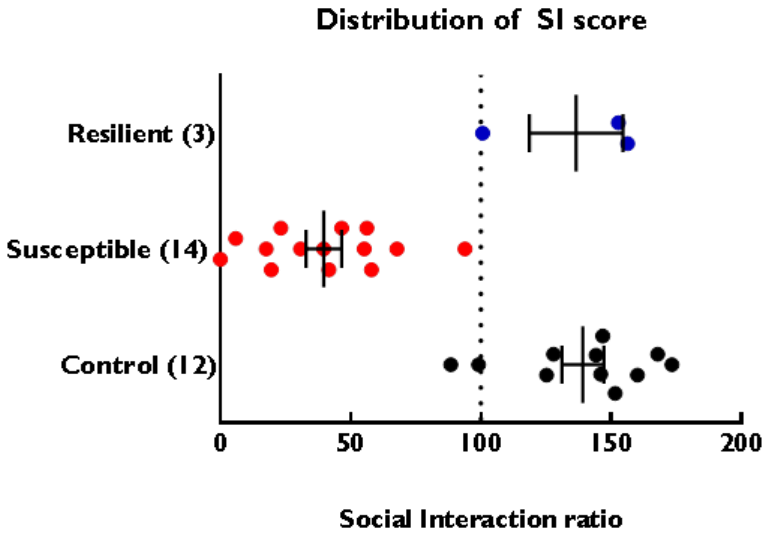

Figure 14 – Social interaction ratio allows the distribution of stressed animals in 14 susceptible and 3 resilient C57BL/6 mice. Distribution of interaction ratios for control, susceptible and resilient mice after social defeat stress. Data are presented as mean ± SEM.

In the social interaction test was measured the time control and defeated mice spent in the interaction and corner zones of the open field arena, in the presence of a CD-1 mouse, in order to establish if they developed social avoidance. Using a one-way analysis of variance (ANOVA), we compared total time spent in the interaction and corner zones between the 3 groups. To test the significance of interactions, a Newman-Keuls post hoc test was performed.

Results demonstrated an interaction between phenotype and time spent in the interaction zone, with susceptible mice spending significantly less time in the interaction zone, compared with control and resilient mice (Figure 15, Panel A). Accordingly, there's also an interaction between phenotype and time spent in the corner zones, with susceptible mice actively avoiding the target and spending more time in the corner zones, compared to control and resilient mice (Figure 15, Panel B).

Additionally was also performed a two-way ANOVA in order to see if there were differences in time each group of animals spent in the interaction zone in the presence of an awake target versus no target. Control C57BL/6 mice spent more time in the interaction zone in the presence of a target, then when the target was absent. In contrast, time susceptible mice spent in the interaction zone is decreased in the presence of a CD-1 mouse, compared to time spent there in the absence of CD-1 mouse, so it seems that a social target is required to assess if mice display social avoidance (Figure 16).

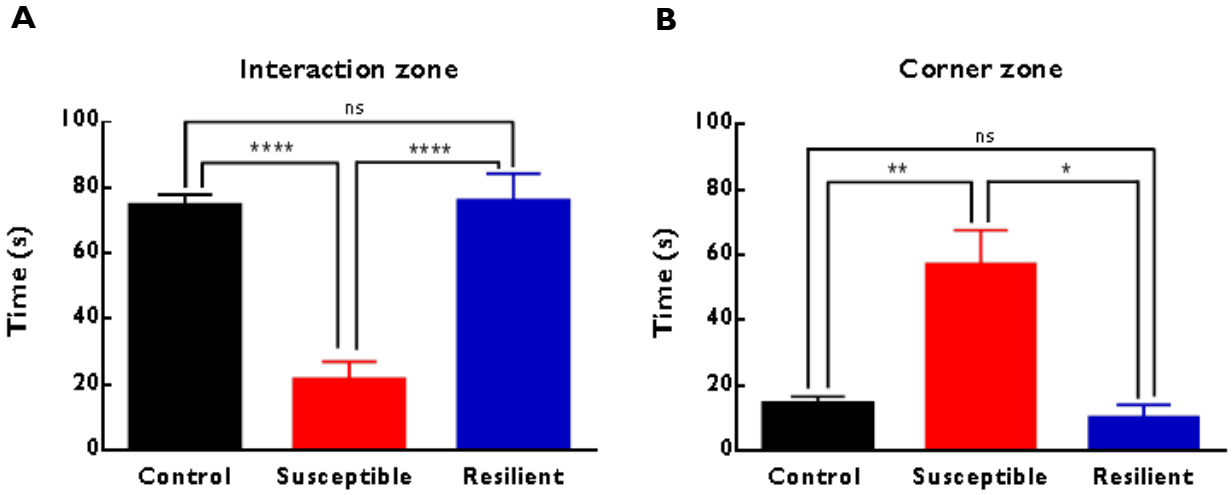

Figure 15 – Susceptible mice avoid the interaction zone and prefer the corner zones in the presence of a social target. (A) Time spent in the interaction zone. **(B)** Time spent in the corners of the open-field arena. Data are presented as mean ± SEM. **** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$; ns: non-significant, using a One-way ANOVA followed by a Bonferroni post-hoc test.

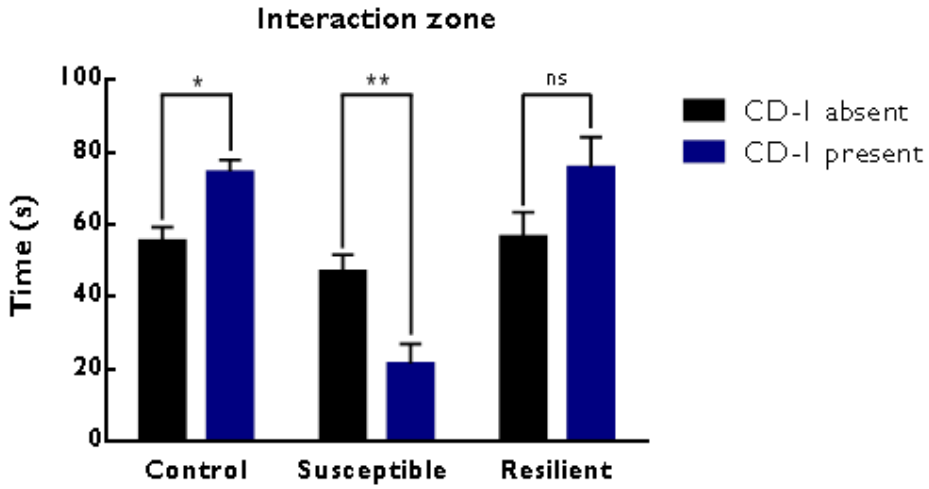

Figure 16 – A social target is required for social avoidance in susceptible mice. Time mice spent in the interaction zone in the presence and absence of an awake CD-1 mouse. Data are presented as mean ± SEM. ** $p \leq 0.01$; * $p < 0.05$; ns: non-significant, using a Two-way ANOVA followed by a Bonferroni post-hoc test.

4.1.2 Open field and elevated plus maze tests

To test whether differences observed in total time C57BL/6 control and defeated mice spent in the interaction zone could be related to motor deficits in the animals, their locomotor activity was assessed through an open field (OF) test. Besides locomotor activity, parameters measured in this test also allowed to assess anxiety in mice.

In order to assess possible locomotor changes between control and defeated mice the total distance mice travelled in the OF arena was measured, along with time they spent in the center of the arena, to assess anxious-like behaviors in mice. Results show no significant differences in both parameters measured between the three groups of animals (Figure 17 and 18, panel A).

The elevated plus maze (EPM) test was also performed to measure anxiety levels in mice, in which time mice spent in the open and closed arms was measured. In accordance with the results previously obtained in the OF test, control, susceptible and resilient mice spent the same amount of time exploring the open arms (Figure 18, panel B).

Taken together results demonstrate that locomotor activity is not related to differences in susceptibility and that defeated animals did not became anxious.

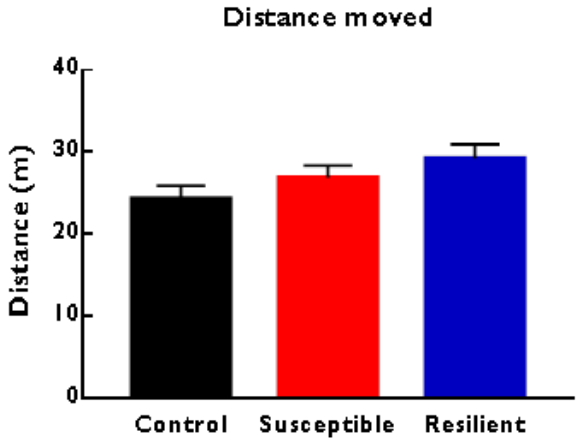

Figure 17 – Social defeat had no effect on locomotor activity. Total distance travelled by control and defeated animals in the open field test. Data are presented as mean ± SEM. ($p > 0.05$, using a One-way ANOVA).

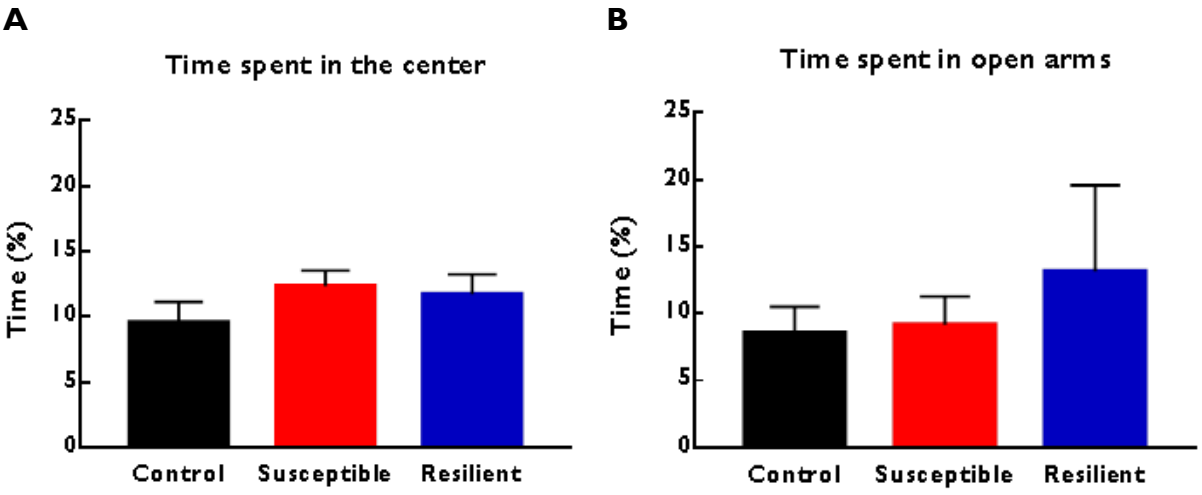

Figure 18 – Susceptible and resilient mice did not display anxiety-like behaviors. (A) Percentage of time spent in the center of the arena in the Open field test. **(B)** Percentage of time spent in the open arms of the elevated plus maze. Data are presented as mean ± SEM. ($p > 0.05$, using a One-way ANOVA).

4.1.3 Splash test

Together with anxiety and social avoidance, ahnedonia is a main feature of the depression-like phenotype resulting from chronic exposure to stress in C7BL/6 mice (Kudryavtseva et al., 1991).

For that reason, anhedonic behavior in C57BL/6 mice was assessed through the splash test in which total and sucrose grooming behavior was measured after mice being squirted with a sucrose solution on their dorsal coat.

Anhedonic symptoms are characterized by decreased time spent grooming (d'Audiffret et al., 2010). Our results show that control and defeated animals spent almost the same amount of time grooming (Figure 19), either sucrose (Panel B) or total (Panel A), meaning that defeated mice did not developed anhedonia.

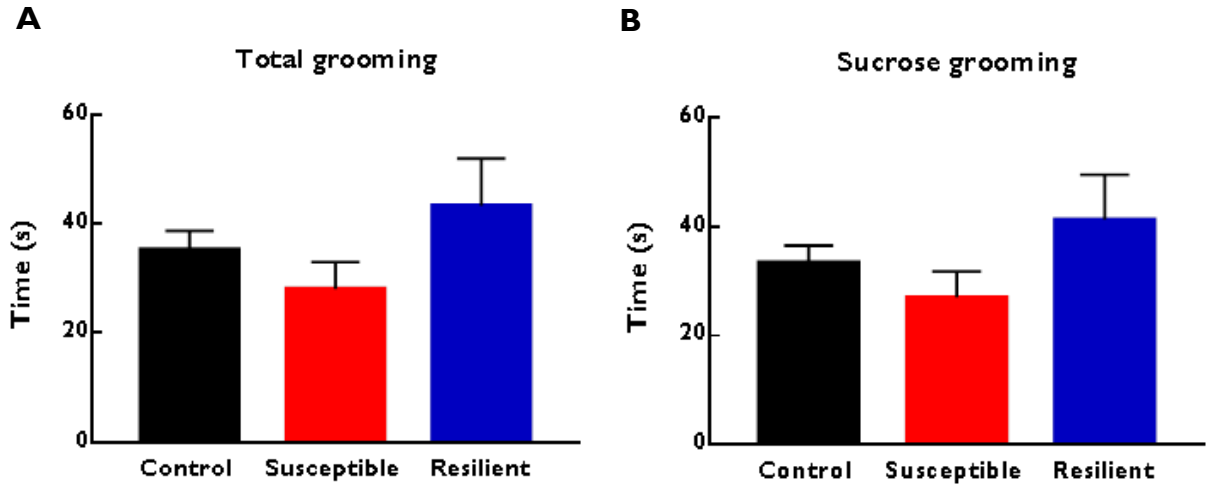

Figure 19 – Social defeat stress failed to induce anhedonia. (A) Total grooming behavior of control and defeated mice in the splash test. **(B)** Sucrose grooming time of control and defeated mice in the splash test. Data are presented as mean ± SEM. ($p > 0.05$, using a One-way ANOVA).

4.1.4 Forced swimming test

Animals' depressive-like behavior was assessed by performing the forced swimming test. Time C57BL/6 mice spent in immobility in the forced swimming test was measured. Typically, after the initial minutes of vigorous activity, animals spend a period of floating with minimum movements, considered as the time spent immobile.

Results demonstrated that control and defeated mice spent almost the same amount of time in immobility in the FST (Figure 20), suggesting that susceptible C57BL/6 mice did not display the characteristic helpless-like behavior.

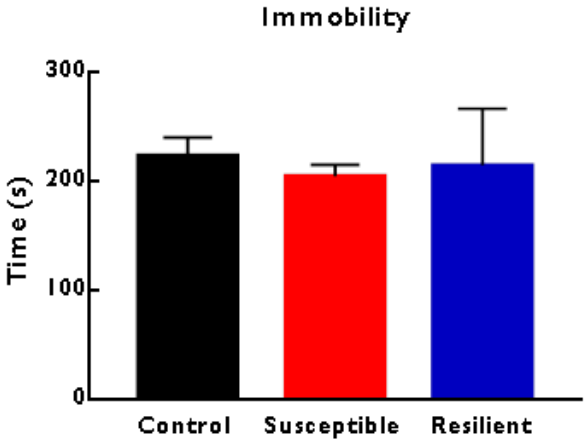

Figure 20 – Defeated mice did not exhibit a depressive-like behavior. Total immobility time of control and defeated mice. Data are presented as mean ± SEM. ($p > 0.05$, using a One-way ANOVA).

4.2 A_{2A} RECEPTORS IN THE VENTRAL STRIATUM AND STRESS-INDUCED ALTERATIONS

4.2.1 A_{2A} Receptors localization

A_{2A} receptors are concentrated in the basal ganglia (Svenningsson et al., 1999) where they can be found mainly in dendritic spines and postsynaptic densities, although a minority is also found presynaptically (Rodrigues et al., 2004, Rebola et al., 2005). However, there is still lack of information regarding A_{2A}R localization in the ventral striatum.

In order to assess A_{2A}R distribution in the ventral striatum, a Western blot was performed. Synaptosomes and total membranes were used, and A_{2A}R density compared in both preparations. GAPDH was used as control for protein loading, since was previously verified that GAPDH levels do not differ in the two different preparations (data not shown).

We observed increased A_{2A}R density in synaptosomal membranes compared with total membranes of the ventral striatum (Figure 21). Results suggest a primarily synaptic localization of A_{2A}R in the ventral striatum.

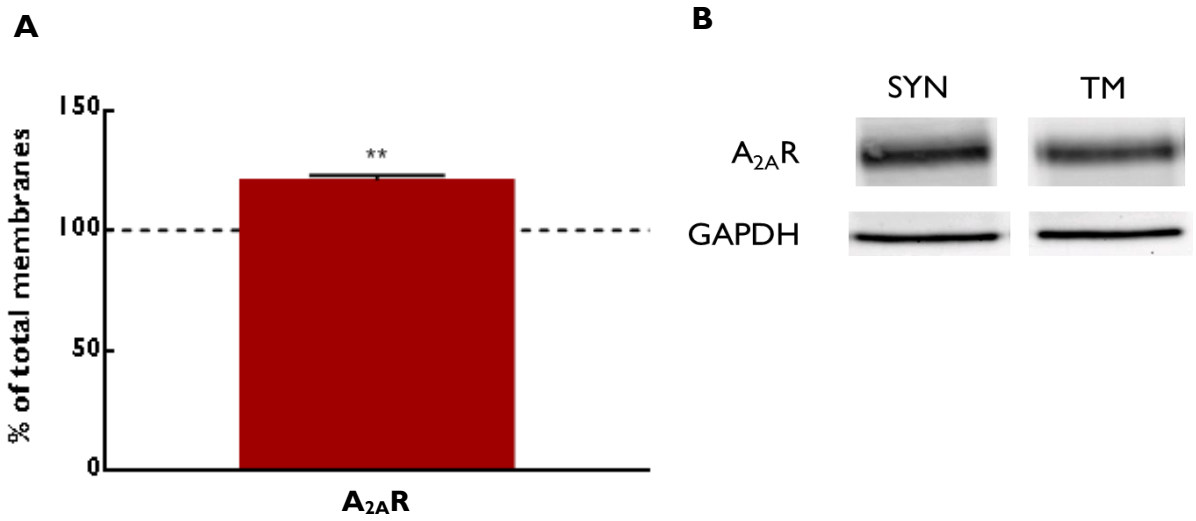

Figure 21 – Ventral Striatum A_{2A}R are enriched in synaptosomes. (A) A_{2A}R levels in synaptosomes of ventral striatum. **(B)** Representative Western blot of A_{2A}R and GADPH levels in synaptosomes (SYN) and total membranes (TM) of ventral striatum. GAPDH was used as control for protein loading. Results are presented in percentage of total membranes. Data are expressed as mean ± SEM of n=4. ** $p < 0.01$, using a one sample t-test compared with the hypothetical value of 100.

4.2.2 A_{2A} Receptors density upon stress

There is consistent evidence of a stress-induced increase in A_{2A} receptors density and binding in brain areas such as the hippocampus and striatum (Cunha et al., 2006, Batalha et al., 2012, Crema et al., 2013, Kaster et al., 2015). Though, A_{2A} receptors have not been studied in particular in the ventral striatum upon stress exposure.

Therefore assess $A_{2A}R$ density in the ventral striatum was one of the main goals of this work. A Western blot was performed in order to verify possible alterations in $A_{2A}R$ density in the ventral striatum of rats subjected to restraint stress.

Results indicate that $A_{2A}R$ density is decreased (40.37 ± 8.89%, n=3) in the ventral striatum of stressed animals, compared to nonstressed animals (Figure 22).

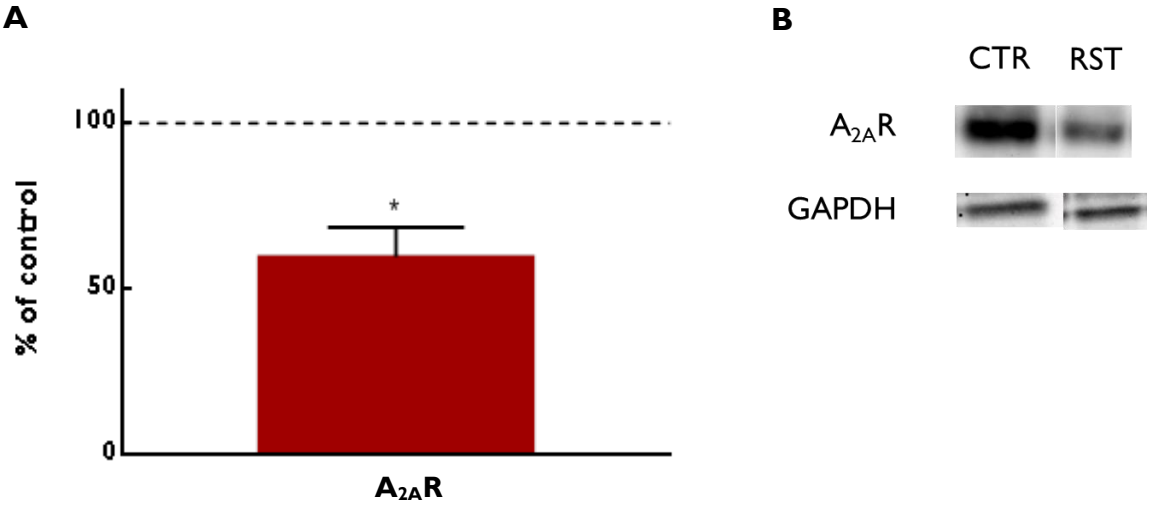

Figure 22 – Ventral striatum $A_{2A}R$ density decreased upon stress. (A) $A_{2A}R$ density in synaptosomal membranes of stressed animal's ventral striatum. **(B)** Representative Western blot of $A_{2A}R$ and GADPH levels in VS synaptosomes of control and stressed animals. GAPDH used as control for protein loading. Results are presented in percentage of control synaptosomes. Data are expressed as mean ± SEM of n=3. * $P < 0.05$, using a one sample t-test compared with the hypothetical value of 100.

4.2.3 A_{2A} Receptors in the nerve terminals sub-populations of the ventral striatum: stress-induced alterations

According to our results, A_{2A} receptors in the ventral striatum are mainly located in synapses (Figure 21) and appear to be downregulated following stress (Figure 22). Therefore we went to understand in which type of ventral striatum nerve terminals sub-populations this reduction occurs.

I. A_{2A} Receptors in glutamatergic nerve terminals

An immunocytochemistry was performed using an antibody against the vesicular glutamate transporter, vGluT1, to identify the majority of glutamatergic nerve terminals in the ventral striatum, along with an antibody against A_{2A} receptors (Table 5), to label these receptors in nerve terminal membranes.

Results show that there is no alteration of the number of nerve terminals immuno-positive for vGluT1 endowed with A_{2A}R (11.43 ± 2.43%, n=6) in the ventral striatum of animals subjected to restraint stress, when compared to control animals (12.60 ± 1.72%, n=4) (Figure 23).

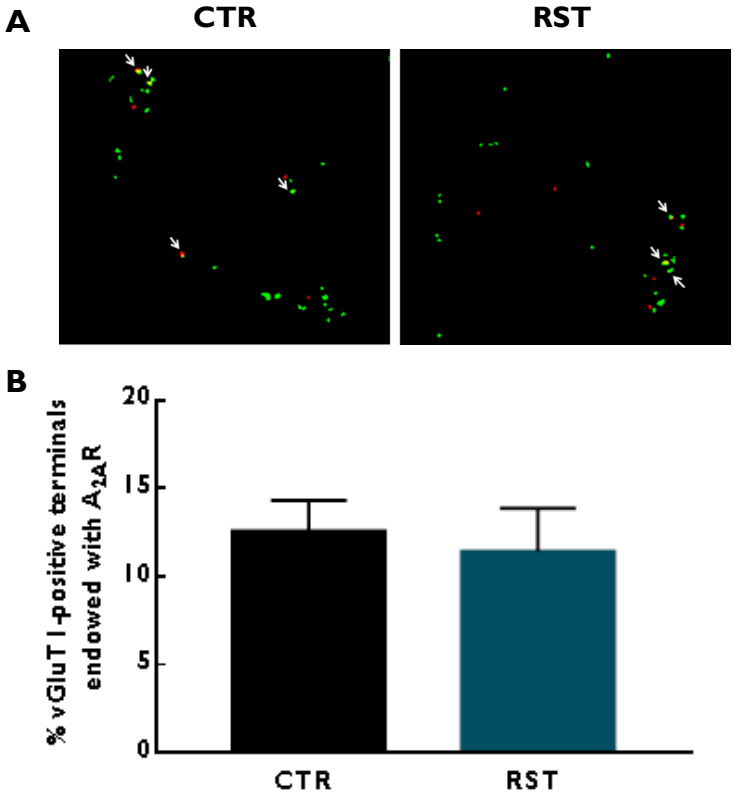

Figure 23 – Stress had no effect on the number of glutamatergic nerve terminals endowed with A_{2A}R in the ventral striatum. (A) Representative images and **(B)** immunocytochemical analysis of synaptosomal membranes from the ventral striatum of control animals (CTR) and animals subjected to restraint stress (RST) double-labelled with antibodies against **A_{2A}R** and **vGluT1**. Data are expressed as mean ± SEM of n=4-6. ($p > 0.05$, using an unpaired Student's t test).

II. A_{2A} Receptors in GABAergic nerve terminals

In order to explore possible alterations in $A_{2A}R$ population present in GABAergic nerve terminals after exposure to stress, an immunocytochemistry was performed with antibodies against $A_{2A}R$ and the vesicular GABA transporter, vGAT (Table 5).

It seems that there are no alterations in the number of nerve terminals immuno-positive for vGAT endowed with $A_{2A}R$ (14.47 ± 3.25%, n=6) in the ventral striatum of animals subjected to restraint stress, compared to control animals (16.02 ± 1.80%, n=4) (Figure 24).

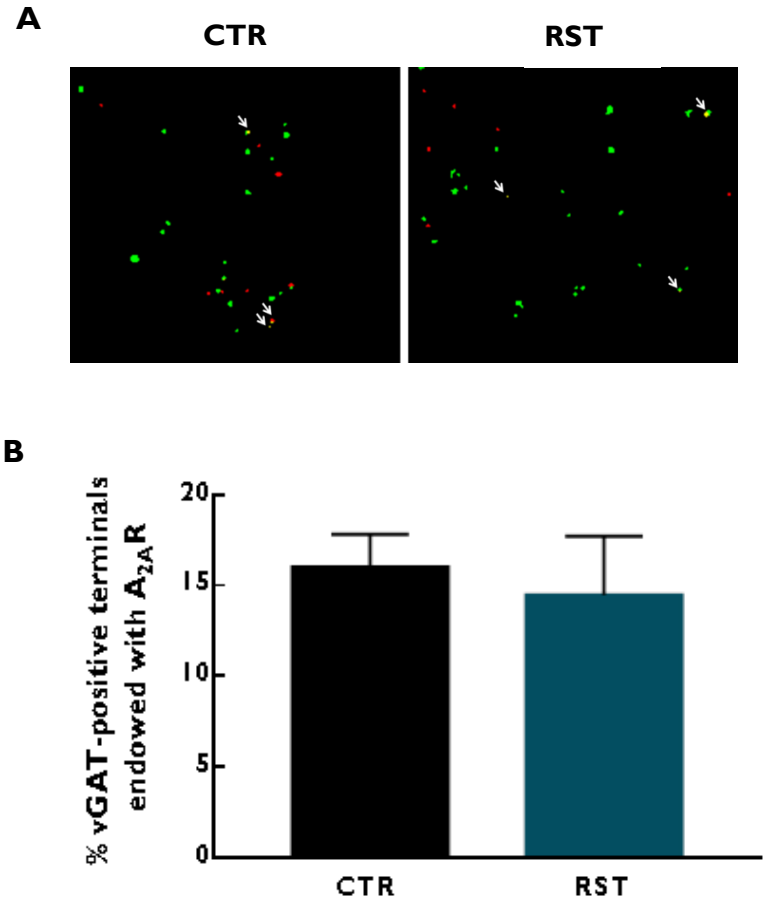

Figure 24 – Restraint stress did not induce alterations in the $A_{2A}R$ present in GABAergic nerve terminals. (A) Representative images and **(B)** immunocytochemical analysis of synaptosomal membranes isolated from ventral striatum of control animals (CTR) and animals subjected to restraint stress (RST) double-labelled with antibodies against **$A_{2A}R$** and **vGAT**. Data are expressed as mean ± SEM of n=4-6. ($p > 0.05$, using an unpaired student's t test).

III. A_{2A} Receptors in dopaminergic nerve terminals

Localization of $A_{2A}R$ in dopaminergic nerve terminals was assessed in order to determine possible alterations after restraint stress exposure. An immunocytochemistry of ventral striatum synaptosomes was performed using antibodies against $A_{2A}R$ and the dopamine transporter DAT (Table 5).

The number of nerve terminals immuno-positive for DAT endowed with $A_{2A}R$ (11.33 ± 2.17%, n=6) in ventral striatum remained apparently unchanged after restraint stress, when comparing with control (5.78 ± 1.37, n=4) (Figure 25).

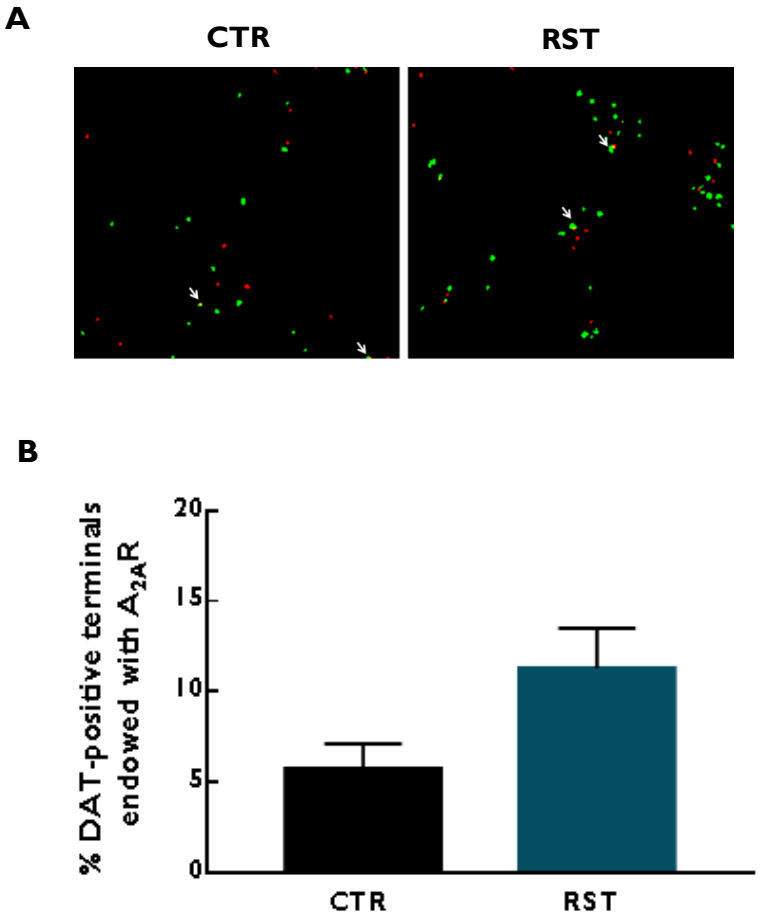

Figure 25 - $A_{2A}R$ in dopaminergic nerve terminals of the ventral striatum were not altered upon exposure to restraint stress. (A) Representative images and **(B)** immunocytochemical analysis of synaptosomal membranes isolated from ventral striatum of control animals (CTR) and animals subjected to restraint stress (RST) double-labelled with antibodies against **$A_{2A}R$** and **DAT**. Data are expressed as mean ± SEM of n=4-6. ($p > 0.05$, using an unpaired student's t test).

4.2.4 Synaptic markers

To gain further insights regarding stress-induced alterations at the synaptic level in the ventral striatum, a Western blot analysis was performed. Relative densities of presynaptic proteins, such as SNARE complex proteins SNAP-25 and syntaxin, and SNARE regulatory protein synaptophysin were assessed. Additionally, we addressed whether chronic exposure to stress affects the density of PSD-95, a general postsynaptic marker of excitatory neurons.

No significant differences were found in SNAP-25 (-10.33 ± 19.46%, n=6), syntaxin (65.90 ± 43.67%, n=6) and synaptophysin (-0.82 ± 5.97%, n=6) immunoreactivity in ventral striatum synaptosomal membranes of animals subjected to restraint stress, compared to control animals (Figure 26). In contrast, PSD-95 density (51.60 ± 9.76%, n=5) in the ventral striatum appears to be increased in stressed animals (Figure 26).

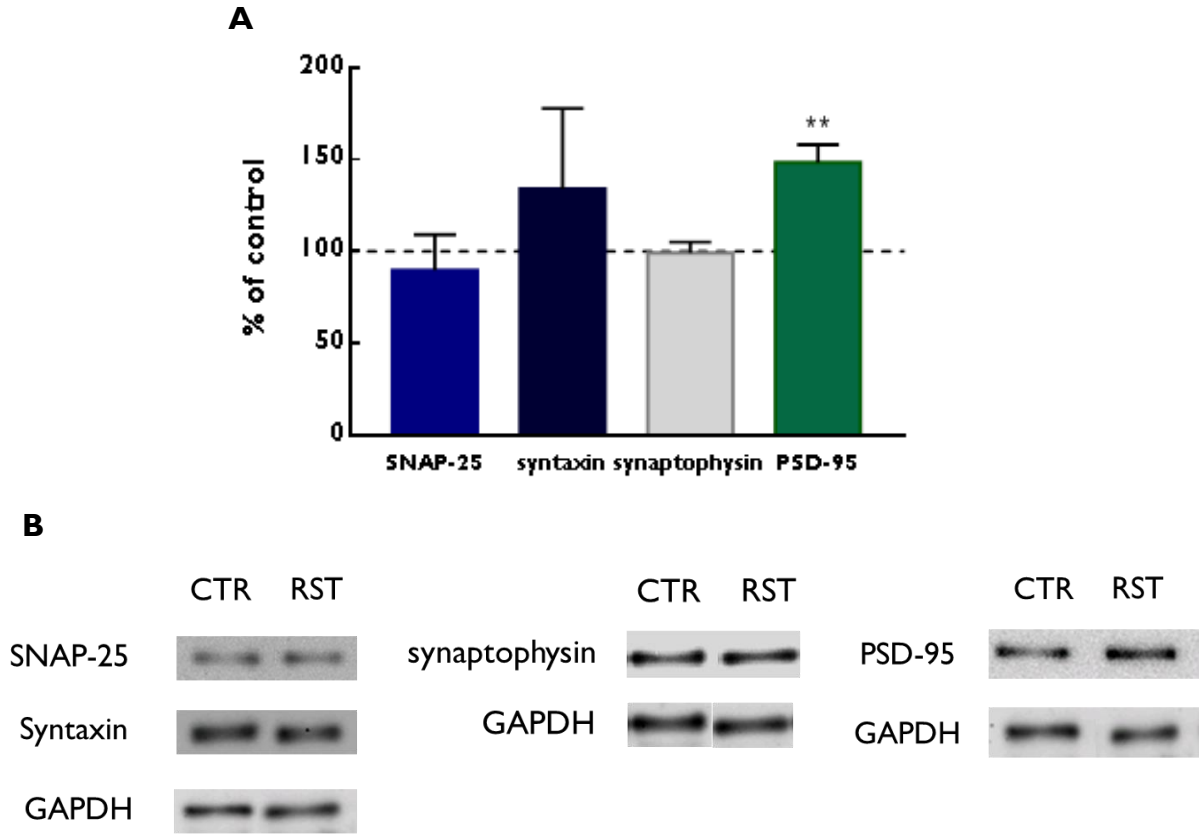

Figure 26 – PSD-95 levels are upregulated in the ventral striatum upon stress exposure. **(A)** Density of SNAP-25, syntaxin, synaptophysin and PSD-95 in nerve terminals of stressed animals' ventral striatum. **(B)** Representative Western blot of SNAP-25, syntaxin, synaptophysin, PSD-95, and GAPDH levels in synaptosomal membranes of control (CTR) animals and animals subjected to restraint stress (RST). GAPDH was used as control for protein loading. Results are presented in percentage of control synaptosomes. Data are expressed as mean ± SEM of n=5-6 per group. ** $p < 0.01$ using a one sample t-test compared with the hypothetical value of 100.

4.2.5 Glutamatergic/GABAergic balance

Stress is known to disrupt glutamatergic and GABAergic neurotransmission and to trigger loss of synapses (Sousa et al., 2000).

To further explore possible alterations in synaptic density after stress exposure, we double-labeled synaptophysin, a general marker for presynaptic nerve terminals, along with vGluT1 and vGAT (Table 6), presynaptic markers of glutamatergic and GABAergic terminals, respectively.

Results obtained show restraint stress had no effect in glutamatergic and GABAergic synaptic density in the rat ventral striatum (Figure 27).

Additionally, we assessed the relative density of vGluT1, a presynaptic marker of glutamatergic nerve terminals, to explore confirm the results obtained by immunocytochemistry.

Results demonstrate that restraint stress had no effect on vGluT1 (-19.43 ± 33.06%, n=6) levels in the rats ventral striatum (Figure 28).

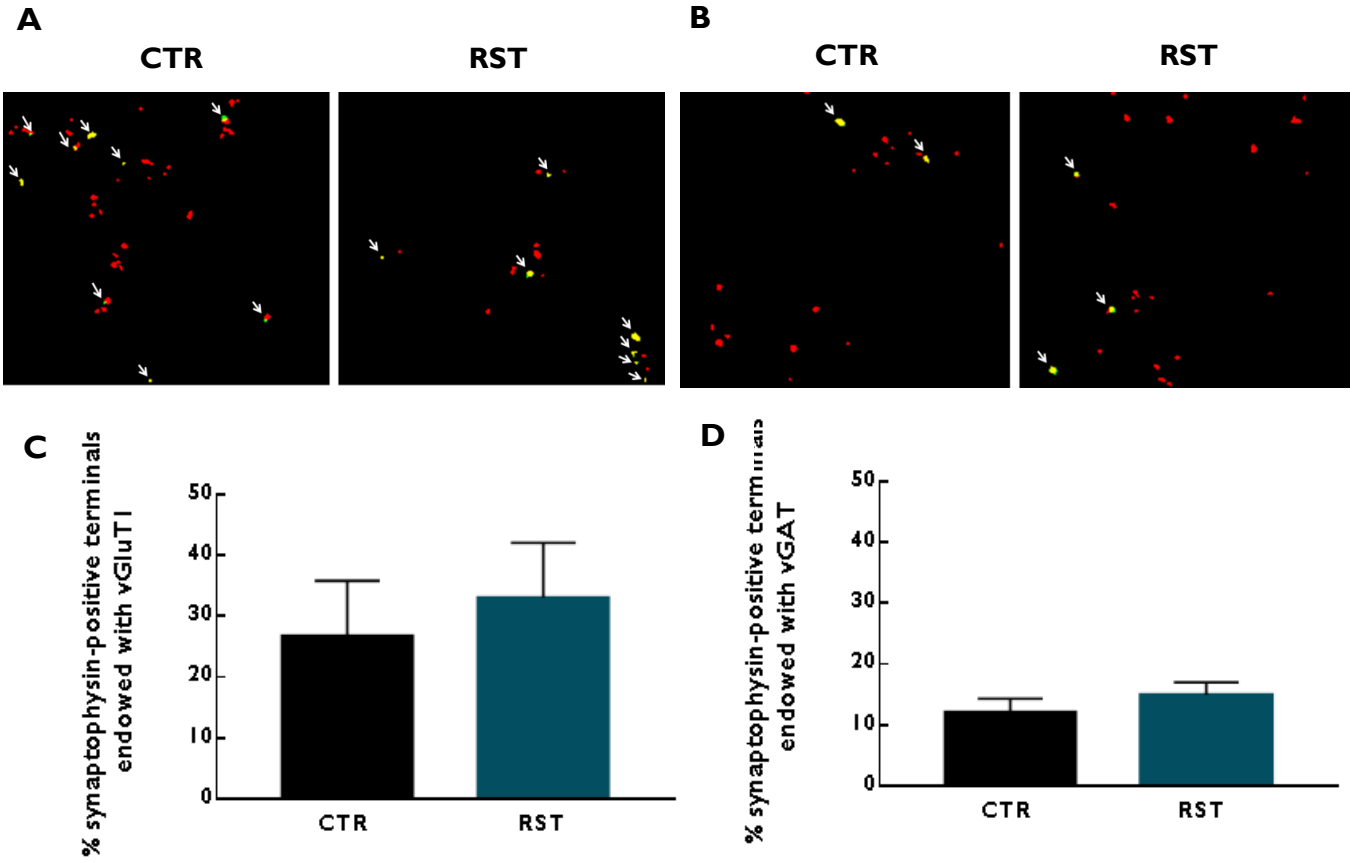

Figure 27 – Glutamatergic/GABAergic synaptic density is not altered upon stress. (A,B) Representative images and **(C,D)** immunocytochemical analysis of synaptosomal membranes isolated from ventral striatum of control animals (CTR) and animals subjected to restraint stress (RST) double-labelled with antibodies against **synaptophysin** and **vGluT1** (A,C) or **vGAT** (B,D). Data are expressed as mean ± SEM of n=4-6. ($p > 0.05$, using an unpaired student's t test).

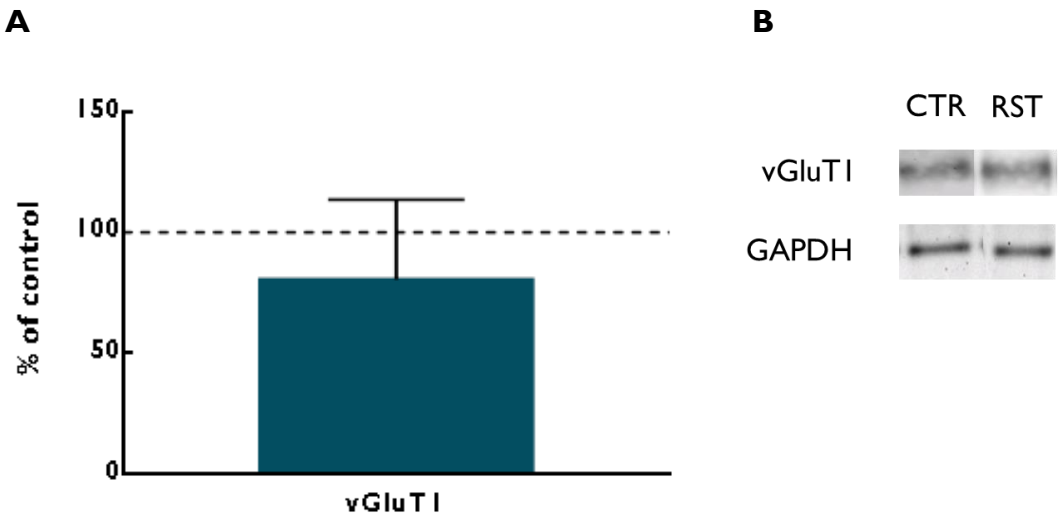

Figure 28 – Ventral striatum vGluT1 levels remained unaltered upon stress. (A) Density of vGluT1 in nerve terminals of stressed animals' ventral striatum. **(B)** Representative Western Blot of vGluT1 and GAPDH levels in synaptosomal membranes of control (CTR) animals and animals subjected to restraint stress (RST). GAPDH was used as control for protein loading. Results are presented in percentage of control synaptosomes. Data are expressed as mean ± SEM of n=6. ($p > 0.05$, using a one sample t-test compared with the hypothetical value of 100).

CHAPTER 5 – DISCUSSION

5.1 SOCIAL DEFEAT STRESS

A distinct feature of social defeat stress model is that allows separating mice subjected to stress into susceptible and resilient, that differ along many behavioral and physiological domains (Krishnan et al., 2007). This kind of stress exposure induces a depression-like phenotype in susceptible mice, characterized by anhedonia, anxiety and social-avoidance behaviors (Kudryavtseva et al., 1991), normally evaluated through behavioral tests. Ahnedonia and social avoidance can be reversed by chronic, but not acute, administration of antidepressants (Berton et al., 2006, Tsankova et al., 2006, Covington et al., 2009, Yin et al., 2014).

Social interaction ratio is a parameter measured in a behavioral test useful to understand the extent of stress-induced social avoidance in defeated mice and allows distinguish susceptible from resilient mice. The term "susceptible" refers to the animals that developed a marked social avoidance and "resilient" to the ones that failed to develop social avoidance (Krishnan et al., 2007). Normally, resilient mice tend to be approximately 40-50% of the defeated mice population (Krishnan et al., 2007). Several studies demonstrate a decrease in time susceptible mice spend in the interaction zone of the open field arena in the SI test (Berton et al., 2006, Tsankova et al., 2006, Krishnan et al., 2007, Yin et al., 2014).

According to social interactions ratios obtained, in our population of defeated C57BL/6 mice, 14 mice developed social avoidance (susceptible) and 3 failed to do so (resilient). Susceptible mice revealed a marked social avoidance as disclosed by the results obtained in the social interaction test. Susceptible mice spent less time in the interaction zone than control and resilient mice, actively avoiding the social target by spending more time in the corners of the social interaction arena. Less time spent in the interaction zone, and therefore more time in the corners, is directly associated with development of social avoidance (Golden et al., 2011).

As for locomotor behavior, assessed in the open field test, susceptible mice travelled the same distance as control and resilient mice, which is in accordance with several studies demonstrating that differences in susceptibility cannot be explained by locomotor activity (Berton et al., 2006, Kinsey et al., 2007, Krishnan et al., 2007). However, other studies established differences, with defeated mice showing significant reductions in locomotion time and distance travelled (Rygula et al., 2006, Razzoli et al., 2011, Huang et al., 2013, Jin et al., 2015).

Social defeat stress induces a behavioral syndrome that allows to differentiate animals on depression- and anxiety-like behavioral domains (Golden et al., 2011). Susceptible and resilient mice both display increased anxiety, whereas only susceptible mice display depressive-like behaviors (Krishnan et al., 2007). Increased anxiety in defeated mice has been characterized as decreased time spent in the open arms of the elevated plus maze (Krishnan et al., 2007, Kovalenko et al., 2014) and decreased time spent in the center in the open field (Huang et al., 2013).

According to our results, neither susceptible nor resilient C57BL/6 mice displayed increased anxiety levels compared to control mice, as assessed in the open field and elevated plus maze tests. Performance of susceptible mice in both tests indicated levels of anxiety similar to the ones observed in control animals when comparing percentages of time spent in the center of the arena in the OF and time spent in the open arms in the EPM.

Lack of interest in pleasurable activities – anhedonia, is considered a core symptom of depression (Rygula et al., 2005). Grooming behavior is considered to parallel with some depression symptoms such as apathetic behavior (Willner, 2005). Animals that are stressed typically spend less time grooming (d'Audiffret et al., 2010, Charney, 2013). The splash test scores grooming intensity, which is defined as a behavioral measure of a depressive-like state (Charney 2013).

Results obtained in this work suggest susceptible C57BL/6 mice did not develop anhedonic behaviors, since they spent the same amount of time grooming (sucrose and total) as control animals.

Behavioral despair tests, such as the forced swimming test, are assumed to model some symptoms of depression such as feelings of sadness and fatigue (Charney, 2013). Animals with increased emotional despair levels usually give up struggling sooner and remain immobile (Charney, 2013). Our results demonstrate similar percentages of time spent immobile in susceptible mice, compared to control, in the FST. Since it has been shown that stressed animals display increased time spent in immobility (Huang et al., 2013, Jin et al., 2015), in our case, the results suggest that susceptible C57BL/6 mice did not develop a depressive-like behavior.

Overall, results obtained indicate that implementation of social defeat stress protocol to induce stress and therefore depressive-like behaviors in C57BL/6 mice wasn't successful.

Despite development of social avoidance in susceptible mice as demonstrated in the social interaction test, in our hands execution of social defeat stress protocol failed to reproduce other key features of human depression such as anhedonia, anxiety and depressive-like behavior.

The main goal after validation of social defeat protocol was to manipulate A_{2A} receptors in striatum sub-regions areas in order to see if they could abrogate mood changes that occur in chronic stress and depression. Since we could not validate the model, this aim was set aside.

5.2 A_{2A} RECEPTORS IN THE VENTRAL STRIATUM AND ALTERATIONS UPON STRESS

In the brain, A_{2A} receptors are concentrated in the basal ganglia (Svenningsson et al., 1999), where they can be found mainly in dendritic spines and postsynaptic densities, although a minority is also found presynaptically (Rodrigues et al., 2004, Rebola et al., 2005).

Accordingly, the results we obtained using synaptosomal and total membranes preparations show that $A_{2A}R$ in the ventral striatum are enriched in synaptosomes (with both pre- and post-synaptic components) compared to total membranes, and therefore we can assume that $A_{2A}R$ are predominantly located in synapses. At the synaptic level, adenosine acts as a modulator of synaptic transmission, controlling information flow and neuronal excitability, mainly through inhibitory A_1 or facilitatory A_{2A} receptors (Fredholm et al., 2005). $A_{2A}R$ are able to control abnormal plasticity (Costenla et al., 2011, Batalha et al., 2012) and synaptotoxicity (Canas et al., 2009, Cognato et al., 2010).

According to the results obtained in this work, $A_{2A}R$ density in the ventral striatum is decreased after stress exposure. There are several studies documenting alterations regarding $A_{2A}R$ density following stress exposure using animal models. In disagreement with our results, Crema and collaborators saw an increase in $A_{2A}R$ binding in the striatum of animals subjected to chronic unpredictable stress (CUS) but not to restraint stress (Crema et al., 2013). Furthermore, in our group it was observed an increased density of hippocampal $A_{2A}R$ upon sub-chronic restraint stress (Cunha et al., 2006), as well as an increase in $A_{2A}R$ binding following CUS (Kaster et al., 2015).

A possible explanation for these conflicting results is that alterations in $A_{2A}R$ are regions specific. Additionally, when comparing our results with the study of Crema and collaborators, we get the idea of a general effect of stress in the striatum, as a whole. If we change our perspective and look at the different sub-regions separately, maybe we will be able to see different alterations in different sub-regions of the striatum, like the decrease that we observed in the ventral striatum.

The ventral striatum receives glutamatergic input from limbic and paralimbic cortices, amygdala and hippocampus and dopaminergic input from the VTA (Muller and Ferre, 2007). These inputs converge on the dendritic spines of striatal GABAergic medium spiny neurons, where there is a high expression of A_{2A} receptors (Fredholm et al., 2005).

Assessing $A_{2A}R$ localization in different nerve terminals sub-populations of the ventral striatum allowed to understand that the decrease reported for $A_{2A}R$ density did not occurred in glutamatergic, GABAergic or dopaminergic nerve terminals.

Since we did not study the presence of $A_{2A}R$ in all types of nerve terminals sub-populations existent in the ventral striatum, we cannot exclude the possibility that the decrease observed by Western blot analysis occurred in the remaining nerve terminal sub-populations, such as the

cholinergic and serotoninergic. Furthermore, we cannot rule out glial contamination in our synaptosomal preparations (Quiroz et al., 2009). Moreover, the alteration in $A_{2A}R$ can also occur in the postsynaptic density. Actually, striatal A_{2A} receptors form heteromers with dopamine D_2 receptors, glutamate mGlu5 and cannabinoid CB_1 receptors (Ferre et al., 2002, Ciruela et al., 2006, Carriba et al., 2007), mostly in the postsynaptic density, modulating synaptic changes in glutamatergic and GABAergic striatal synapses.

Synaptic dysfunction is found in several brain regions affected in depression and, as mentioned above, can be associated with loss of synaptic markers and neuronal atrophy (Price and Drevets, 2010, MacQueen and Frodl, 2011, Kang et al., 2012), also verified after stress exposure.

Syntaxin and SNAP-25 belong to the SNARE protein complex and are integral membrane proteins, involved in docking and fusion of synaptic vesicles at the presynaptic membrane. When we evaluated their density in the ventral striatum of animals subjected to stress we saw that they remained unchanged.

In agreement with our results, two studies show no alterations in the density of these two proteins in the hippocampus upon restraint stress (Gao et al., 2006, Muller et al., 2011). However, there is one study where the authors reported increased syntaxin and decreased SNAP-25 levels in the PFC (Muller et al., 2011). In another model of stress (CUS), our group documented a downregulation of both proteins in the hippocampus (Kaster et al., 2015). Also in agreement with our results, in depressed subjects were not observed changes in the levels of syntaxin or SNAP-25 (Honer et al., 2002). However, the authors show an increase in SNARE complex formation (Honer et al., 2002), so we cannot reject the hypothesis of alterations in the formation of SNARE protein complexes.

Synaptophysin is a synaptic vesicle-associated integral membrane protein that is widely used as a general marker of pre-synaptic nerve endings (Gao et al., 2006). We evaluated synaptophysin immunoreactivity in the ventral striatum of animals exposed to restraint stress and control animals and did not observe any significant alterations.

In agreement, several studies report normal synaptophysin levels in the hippocampus and prefrontal cortex after stress (Rosenbrock et al., 2005, Muller et al., 2011, Yuen et al., 2012). However, there are reports of a stress-induced decrease in synaptophysin, also in the hippocampus and cerebral cortex (Thome et al., 2001, Xu et al., 2004, Cunha et al., 2006, Meng et al., 2013, Amin et al., 2015). In patients suffering from depression, synaptophysin expression in brain areas such as the amygdala and PFC is downregulated (Gilabert-Juan et al., 2012, Varea et al., 2012).

PSD-95 is highly enriched at the excitatory postsynaptic densities, located at the head of dendritic spines (Doucet et al., 2012). This protein is widely expressed in the human brain, and particularly in regions associated with mood modulation, such as the striatum, amygdala, prefrontal cortex and hippocampus (Kristiansen and Meador-Woodruff, 2005, Toro and Deakin, 2005, Feyissa

et al., 2009, Karolewicz et al., 2009). Additionally, PSD-95 belongs to a complex composed by NMDA receptor/PSD-95/neuronal nitric oxide synthase that is important for depression, since it plays a role in synaptic plasticity and learning and memory (Doucet et al., 2012).

In this study, we evaluated whether stress altered PSD-95 levels in the ventral striatum, and we observed an increased PSD-95 density.

In agreement with our results, a recent study reported increased PSD-95 levels in the amygdala of animals subjected to prenatal chronic mild stress (Wang et al., 2015). Although, studies also report a downregulation in the hippocampus following restraint stress (Meng et al., 2013) whereas in the PFC, PSD-95 levels remained unaltered (Yuen et al., 2012).

Also in disagreement with our results, in the striatum of depression subjects PSD-95 density appears not to be altered (Kristiansen and Meador-Woodruff, 2005). Within the ventral striatum was reported a lower expression of PSD-95 in the NAC compared to caudate and putamen (Kristiansen and Meador-Woodruff, 2005). Moreover, PSD-95 levels are not altered in the hippocampus of depression patients (Kristiansen and Meador-Woodruff, 2005, Toro and Deakin, 2005) and in the PFC, PSD-95 expression levels are downregulated (Clinton and Meador-Woodruff, 2004, Feyissa et al., 2009). In the amygdala of patients suffering from depression PSD-95 levels are increased (Karolewicz et al., 2009), in accordance with the recent alterations demonstrated following stress (Wang et al., 2015) and with our results.

Increased PSD-95 levels can be associated to the maturation of synapses (El-Husseini et al., 2000) and with altered synaptic plasticity. In fact, Sebastian and colleagues reported that an increase of PSD-95 levels upon stress can be correlated with the increase of long-thin and mushroom spines in CA1 hippocampal area of stressed animals (Sebastian et al., 2013). Since we observed an increased density of PSD-95 in the ventral striatum upon stress, we can speculate that there is also an increase in the number of mature dendritic spines, however this hypothesis remains to be confirmed.

There is evidence supporting a main role of an imbalanced glutamatergic/GABAergic neurotransmission in the pathology of depression (Krystal et al., 2002) and that stress triggers synaptic loss (Sousa et al., 2000). Additionally, glutamatergic neurotransmission is known to be increased in limbic-thalamo-cortical circuits (Drevets et al., 2008) and such elevations may contribute to reductions in gray matter volume as well synaptic markers (Drevets et al., 2008).

Given these data, we decided to verify whether stress altered the balance between glutamatergic and GABAergic nerve terminals in the ventral striatum. According to our results, after restraint stress the number of glutamatergic and GABAergic nerve terminals remained unchanged.

In rodents, stress has been shown to increase the number of glutamatergic synapses in the NAc (Christoffel et al., 2011). In cortical and hippocampal areas were found decreased vGluT1 levels, following chronic mild stress (Elizalde et al., 2010). In the amygdala of patients suffering from depression, vGluT1 expression is upregulated (Varea et al., 2012). In contrast, in the PFC vGluT1

levels are reduced (Gilabert-Juan et al., 2012) in agreement with a reduction in cell number and density, accompanied by a decrease gray matter volume (Rajkowska, 2000). Furthermore, Sequeira and collaborators describe a global brain alteration of glutamate and GABA receptor subunit genes in depression, that cannot be ruled out, in this particular region and animal model (Sequeira et al., 2009).

CHAPTER 6 – FINAL REMARKS

Social defeat stress revealed not to be a good model to recapitulate several depressive-like features. Additionally, the execution of this protocol is labor intensive and expensive.

Given what we observed in this work, we are forced to question whether this is actually a model with high reproducibility in a laboratory.

In contrast, restraint stress model has proven to have reproducibility in laboratory -within the Purines group and by what is described in the literature.

We chose to use it in order to study the impact of stress in the rat ventral striatum. Our work was mainly focused in A_{2A} receptors and general alterations that are known to be present in depression, such as imbalanced glutamatergic/GABAergic transmission and synaptic dysfunction. We observed that A_{2A} receptors density decreased in ventral striatum upon stress. In this brain region, we did not observe loss of synaptic markers but instead we reported an increase of PSD-95 density, upon stress, that can be correlated with an altered synaptic plasticity. These results highlight an important role of the postsynaptic density in the ventral striatum, in stress conditions.

Moreover, the results obtained in this work are extremely helpful, since they shed some light about ventral striatum A_{2A} receptors role in depression and point us in a new direction, which is to study the role of ventral striatum post-synaptic $A_{2A}R$ in depression. As major conclusion, depending on the brain region and if there is synaptic loss in the disease model studied, $A_{2A}R$ may have a different role in trying to normalize the system.

CHAPTER 7 – FUTURE DIRECTIONS

To further complete this work:

- ✓ Study the formation and maturation of dendritic spines, as well the role of PSD-95 (Sebastian et al., 2013).

- ✓ Assess dopamine transporter (DAT) and dopamine receptors density in the ventral striatum of animals subjected to stress, by western blot analysis.

Reduced dopaminergic neurotransmission was observed in the ventral striatum and has been linked to anhedonic behaviors and therefore appears to be involved in the pathology of depression (Hasler et al., 2008). Also reduced striatal DAT binding and dopamine uptake was evidenced in depression subjects (Meyer et al., 2001, Pizzagalli, 2014).

- ✓ Assess vesicular GABA transporter (vGAT), GABA and glutamate transporters density in the ventral striatum of animals subjected to stress, by Western blot analysis.

There is evidence of decreased GABA concentrations in the plasma and CSF of depression patients (Hasler et al., 2007) as well as altered $GABA_A$ receptor signaling and downregulation of GABAergic neurotransmission (Eser et al., 2006, Feyissa et al., 2009). Also, and more importantly, alterations in gene implicated in GABAergic transmission have been found in depression patients' brains (Sequeira et al., 2009).

Future working hypothesis:

- ✓ Investigate the role of ventral striatum postsynaptic $A_{2A}R$ in depression.

- ✓ Explore the role of $A_{2A}R$ in other striatum sub-regions, such as the dorsomedial (DMS) and dorsolateral (DLS) striatum.

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