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Anna Vladímirovna Pliássova

PATHOPHYSIOLOGICAL ROLE OF ADENOSINERGIC MODULATION WITHIN THE BASOLATERAL COMPLEX OF THE AMYGDALA

Tese no âmbito do Programa Interuniversitário de Doutoramento em Envelhecimento e Doenças Crónicas, orientada pelo Professor Doutor Rodrigo Pinto Santos Antunes Cunha e pelo Professor Doutor João Miguel Seiça Bessa Peixoto e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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"O cérebro humano é o objecto mais complexo que alguma vez encontrámos no universo e o último grande enigma da ciência."

José Rodrigues dos Santos, in *A Chave de Salomão*

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LIST OF PUBLICATIONS

The following publication includes data presented in chapter 4.1:

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Other publications from the author:

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Silva, A. C., Lemos, C., Gonçalves, F. Q., **Pliássova, A. V.**, Machado, N. J., Silva, H. B., Canas, P.M., Cunha, R.A., Lopes, J.P., Agostinho, P. **(2018)** Blockade of adenosine A_{2A} receptors recovers early deficits of memory and plasticity in the triple transgenic mouse model of Alzheimer's disease. *Neurobiology of Disease* 117: 72–81; doi: 10.1016/j.nbd.2018.05.024

Pliássova, A., Canas, P.M., Xavier, A.C., da Silva, B.S., Cunha, R.A., Agostinho, P. **(2016)** Age-related changes in the synaptic density of amyloid-β protein precursor and secretases in the human cerebral cortex. *Journal of Alzheimer's Disease* 52:1209-1214; doi: 10.3233/JAD-160213

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Rodrigues, D.I., Gutierres, J., **Pliássova, A**., Cunha, R.A., Agostinho, P. **(2014)** Synaptic and sub-synaptic localization of amyloid-β protein precursor in the rat hippocampus. *Journal of Alzheimer's Disease* 40: 981–992 doi: 10.3233/JAD-132030

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LIST OF ABBREVIATIONS

A_1R – adenosine A_1 receptor subtype

A_{2A}R – adenosine A_{2A} receptor subtype

A_{2B}R – adenosine A_{2B} receptor subtype

A_3R – adenosine A_3 receptor subtype

ADA – adenosine deaminase

AK – adenosine kinase

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ATP – adenosine 5'-triphosphate

Ca^{2+}– calcium ion

cAMP – cyclic adenosine 3',5'-monophosphate

CGS21680– 4-[2-[[6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid

CNS – central nervous system

CNQX – 6-cyano-7-nitroquinoxaline-2,3-dione

CS – conditioned stimulus

CPA – N^6-cyclopentyladenosine

D_1R – dopamine receptor subtype 1

D_2R – dopamine receptor subtype 2

DL-AP5 - DL-2-amino-5-phosphonopentanoic acid

DMSO – dimethyl sulfoxide

DPCPX – 8-cyclopentyl-1,3-dipropylxanthine

EDTA – ethylenediaminetetra-acetic acid

EGTA – ethylene glycol tetra-acetic acid

EPM – elevated plus maze

EPSC – excitatory post-synaptic currents

GABA – γ-aminobutyric acid

GPCR – G-protein coupled receptor

HEPES - 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

IPSC – inhibitory postsynaptic current

K^+ – potassium ion

KO – knockout

LTD – long-term depression

LTP – long-term potentiation

MDD – major depressive disorder

mGluR – metabotropic glutamate receptors

Mg^{2+} - magnesium ion

NMDA – N-methyl-D-aspartate

NMDAR - N-methyl-D-aspartate receptor

OF – open field

PBS – phosphate buffer solution

PFC – prefrontal cortex

PLC – phospholipase C

PND – postnatal days

QX 314– N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium

RNA – ribonucleic acid

RT – room temperature

SCH58261–2-(2-furanyl)-7-(2-phenylethyl)-7***H***-pyrazolo[4,3-***e***][1,2,4]triazolo[1,5-***c***]pyrimidin-5-amine

shRNA – short hairpin RNA

US – unconditioned stimulus

vGAT – vesicular GABA transporter

vGLUT – vesicular glutamate receptor

WT – wild type

ABSTRACT

Caffeine, which is a general antagonist of adenosine receptors, as well as selective adenosine A_{2A} receptor ($A_{2A}R$) antagonists have already been described as being able to prevent and revert depressive-like symptoms. This process likely involves information processing within the amygdala, which has a central role in emotional processing and, thus, is one of the most affected brain structures upon stress exposure and depression. Furthermore, adenosine A_1 (A_1R) and $A_{2A}R$ both modulate the generation of the fear response, which is critically dependent on the amygdala and glutamate receptors, including NMDA receptors. However, the role of the adenosinergic system in the modulation of synaptic transmission within the amygdala remains poorly understood.

The present study aimed at evaluating and characterizing the involvement of A_1R and $A_{2A}R$ in the modulation of NMDA receptor-mediated responses in physiological conditions, as well as unraveling the mechanisms of altered excitatory and inhibitory synaptic transmission and their subsequent modulation by $A_{2A}R$ in synapses from the lateral nucleus (LA) to basolateral nuclei (BLA), in animals submitted to repeated restraint stress. For that purpose, whole-cell patch-clamp recordings were performed in BLA pyramidal neurons upon stimulation of the LA. In physiological conditions, in juvenile male Wistar rats, we observed that A_1R activation inhibited NMDA receptor-mediated currents. We also obtained interesting data showing that the purportedly selective $A_{2A}R$ agonist CGS21680 lacked the desired selectivity, since we observed that it decreased NMDA receptor-mediated currents in a manner which was not prevented by the selective $A_{2A}R$ antagonist SCH58261.

Thereupon, we intended to expand the study of the role of $A_{2A}R$ in amygdala circuits to an animal model of repeated restraint stress in juvenile male Wistar rats. However, not only was there no clear alteration of the phenotype after repeated restraint stress, but also we did not observe any alterations in excitatory synaptic transmission recordings and, hence, no studies regarding the role of $A_{2A}R$ were performed.

We then decided to study the role of $A_{2A}R$ in a repeated restraint stress model in adult Wistar rats, following a protocol which we had previously validated in our laboratory. Stressed animals did not exhibit changes in glutamatergic synaptic transmission but had increased evoked input/output curves of inhibitory transmission. Also, we showed, for the first time, that $A_{2A}R$ exert a tonic control on basal inhibitory transmission in the BLA, which was similar in control and in stressed animals. Curiously, the purportedly selective $A_{2A}R$ agonist CGS21680 caused opposite effects in control and in stressed animals, decreasing and increasing the amplitude of evoked inhibitory synaptic currents respectively.

Overall, our data suggest that A_1R and $A_{2A}R$ play an essential role in the regulation of the balance between excitation and inhibition within the amygdala, but possible mechanisms underlying the results which we obtained should be further explored. Since adenosine receptors are involved in the expression of amygdala-dependent behavior and in the etiology of emotional disorders, further understanding of the transduction pathways affected by repeated stress and by $A_{2A}R$ will be essential to unravel their usefulness as therapeutic targets in mood and anxiety disorders.

RESUMO

A cafeína, que é um antagonista geral dos recetores de adenosina, bem como antagonistas seletivos dos recetores A_{2A} da adenosina ($A_{2A}R$), já foram descritos como sendo capazes de prevenir e reverter sintomas depressivos. Este processo envolve, provavelmente, o processamento da informação dentro da amígdala, que tem uma função central no processamento das emoções e, portanto, é uma das regiões do cérebro mais afetadas pela exposição ao stress e depressão. Para além disso, os recetores A_1 da adenosina (A_1R) e $A_{2A}R$ já foram descritos como sendo essenciais na geração da resposta do medo, que é criticamente dependente da amígdala e de recetores de glutamato, incluindo os recetores de NMDA. No entanto, o papel do sistema adenosinérgico na modulação da transmissão sináptica dentro da amígdala permanece pouco conhecido.

O presente estudo pretendeu avaliar e caracterizar o envolvimento dos A_1R e $A_{2A}R$ na modulação da resposta mediada por recetores NMDA em condições fisiológicas, assim como explorar a alteração da transmissão sináptica excitatória e inibitória e a sua modulação pelos $A_{2A}R$ nas sinapses entre os núcleos lateral (LA) e basolateral (BLA), em modelos animais de stress de imobilização repetida. Para esse efeito, foram feitos registos de *whole-cell patch-clamp* em neurónios piramidais da BLA, com estimulação na LA. Em condições fisiológicas, em ratos machos Wistar jovens, observámos que a ativação dos A_1R inibiu as correntes mediadas por recetores de NMDA. Obtivemos, também, dados interessantes que mostram que o CGS21680, suposto agonista seletivo dos $A_{2A}R$, não tem a seletividade desejada, visto que observámos um decréscimo nas correntes mediadas por recetores de NMDA que não foi prevenido pelo antagonista seletivo dos $A_{2A}R$, SCH58261.

Expandimos, posteriormente, o estudo do papel dos $A_{2A}R$ na transmissão sináptica basal num modelo animal de stress de imobilização repetida, em ratos machos jovens. No entanto, não só o fenótipo não foi claro, como também não observámos quaisquer alterações em registos preliminares de transmissão sináptica excitatória, e, por isso, não fizemos estudos para determinar o papel dos $A_{2A}R$.

Decidimos então avançar para o estudo do papel dos $A_{2A}R$ num modelo animal de imobilização crónica repetida em ratos machos Wistar adultos, segundo um protocolo previamente validado no nosso laboratório. Os animais sujeitos a stress não exibiram quaisquer mudanças na transmissão sináptica excitatória, mas foi observado um aumento das curvas de *input/output* da transmissão sináptica. Demonstrámos, ainda, pela primeira vez, que os $A_{2A}R$ exercem um controlo tónico na transmissão sináptica basal inibitória na BLA, que foi semelhante nos animais do grupo controlo e stressados. Curiosamente, o agonista supostamente seletivo dos $A_{2A}R$, CGS21680, causou efeitos

opostos nos animais do grupo de controlo e nos stressados, diminuindo e aumentando a amplitude das correntes sinápticas inibitórias evocadas, respetivamente.

No geral, os nossos dados sugerem que os A_1R e $A_{2A}R$ desempenham um papel essencial na regulação do balanço entre a excitação e a inibição dentro da amígdala, mas os possíveis mecanismos que estão na base dos resultados que obtivemos devem ser futuramente explorados. Sendo que os recetores de adenosina estão envolvidos na expressão de comportamentos dependentes da amígdala e na etiologia de disfunções emocionais, a compreensão das vias de transdução afetadas pelo stress repetido e pelos $A_{2A}R$ será essencial para determinar o seu interesse como alvos terapêuticos em disfunções de humor e ansiedade.


I'VE DISCOVERED SOMETHING, MA'AM..
5-21
THE MORE I STUDY, THE MORE I REALIZE HOW LITTLE I KNOW...
PEANUTS by Schulz

1. INTRODUCTION

1.1. The amygdala

The term "amygdala" (*Amygdalus communis*) was coined by the German physiologist Karl Burdach in the 19th century to describe an almond-shape structure in the human anterior temporal lobe (McDonald, 2003; Tsvetkov *et al*., 2015). Since then, the description of this brain region has been largely extended and, nowadays, this structure is known as the amygdaloid complex (Sah *et al*., 2003). The amygdala has a central role in emotional processing due to its ability to encode and consolidate emotionally-arousing memories through a variety of neuroplastic mechanisms (Hermans *et al*., 2014) and, therefore, is one of the most affected brain regions in mood dysfunction. In fact, functional neuroimaging studies show that the amygdala is abnormally active in several pathologies, such as anxiety, post-traumatic stress disorder and depression (Benson *et al*., 2014; Fredrikson and Faria, 2013; Shin *et al*., 2006).

1.1.1. Amygdala organization

The amygdaloid complex is anatomically and physiologically diverse and comprises multiple nuclei, which are extensively connected and have their unique specific inputs and outputs (Sah *et al*., 2003), allowing the processing of various kinds of information. The basolateral complex integrates sensory information perceived by cortical and subcortical areas. More specifically, the lateral amygdala (LA) receives major inputs from the sensory thalamus and cortex. This sensory information is then routed through intra-amygdalar connections to the basal nucleus (BLA), which projects this information to the central area (CeA), where it may undergo additional processing (LeDoux, 2007; Pitkänen *et al*., 1997) (Figure 1). The flow of information originated in the BLA is almost unidirectionally routed into the CeA and intercalated cell masses (ITC) (Capogna, 2014). Besides its complex intrinsic projections, the amygdala shares a vast number of reciprocal connections with other brain regions implicated in the general response of the organism to the perceived stress (Millan, 2003). Since the amygdala has privileged access to primary sensory information, it is the brain structure which is usually considered to attribute positive or negative valence to emotional stimuli, leading to different behavioral outcomes, and this processing of information involves distinct circuits between different brain regions (Janak and Tye, 2015).

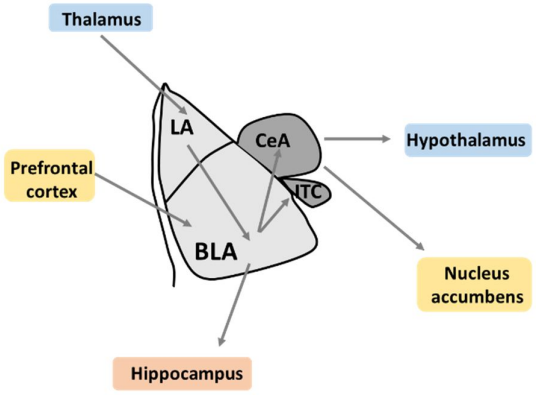


Figure 1. Schematic representation of the amygdala. The amygdala has a complex three-dimensional organization with its intrinsic neuronal projections. It also receives inputs from several brain regions, as well as it sends information through multiple projections to different areas of the brain. Within the amygdaloid complex, there are strong projections from the lateral nucleus to the basolateral one (Maren, 1996).

1.1.2. Excitatory and inhibitory synaptic transmission within the basolateral complex of the amygdala

LA and BLA neurons were first characterized according to their morphological and electrophysiological properties in 1992 (Washburn and Moises, 1992). There are two main types of cells in the basolateral complex: the principal/pyramidal-like neurons, which constitute the vast majority of the total cells and use glutamate as a neurotransmitter, and interneurons, which are smaller in size and are GABAergic (Sah *et al*., 2003; Washburn and Moises, 1992). Pyramidal neurons and interneurons can be distinguished according to their electrophysiological properties (Faber *et al*., 2001; Sah *et al*., 2003). Pyramidal neurons are characterized by broad action potentials, which are followed by a prolonged afterhyperpolarization (AHP), while interneurons present little or no spike frequency adaptation in response to a depolarizing current (Figure 2) (Sah *et al*., 2003).

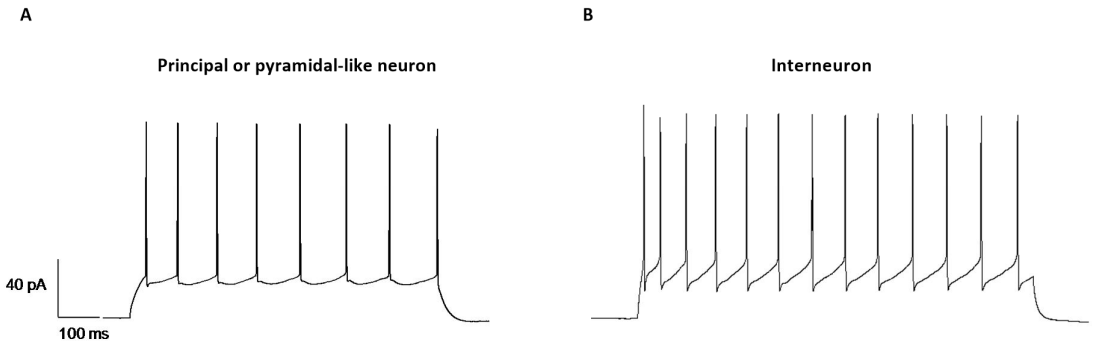


Figure 2. Principal or pyramidal-like neurons *vs.* interneurons. Principal or pyramidal-like neurons can be distinguished from interneurons due to their electrophysiological properties. **A.** Traces representing a recording of a typical principal or pyramidal-like neuron in the BLA. **B.** Traces representing a recording of a typical interneuron in the BLA.

In the early nineties of the 20th century, several studies demonstrated the existence of both excitatory and inhibitory synaptic transmission, mediated by glutamate and GABA receptors respectively, in the basolateral nucleus of the amygdala, evoked by stimulation of afferent fibers, including those of the lateral nucleus (Maren, 1996). The interplay between interneurons and pyramidal neurons defines the outcome of the overall neuronal network activity by controlling the maintenance of an adequate balance between excitation and inhibition, which determines the generation of adequate emotional responses (Muller *et al.*, 2006). The activity of pyramidal neurons is tightly controlled by synaptic excitation and synaptic inhibition, meaning that they receive both glutamatergic and GABAergic inputs, which are usually distributed asymmetrically throughout the neuron (Pouille *et al.*, 2013). Glutamatergic afferents synapse both to pyramidal neurons and interneurons, while inhibitory projections, which are originated in interneurons, project to principal neurons (Maren, 1996).

The main mediators of basal excitatory synaptic transmission in the amygdala principal neurons are glutamate ionotropic AMPA and NMDA receptors (Rainnie *et al.*, 1991; Sah *et al.*, 2003). AMPA and NMDA receptors are mainly responsible for the fast excitatory neurotransmission and readily respond to the binding of glutamate, which is released from the presynaptic nerve terminal, by opening postsynaptic ion channels and, therefore, initiating an excitatory postsynaptic current. Glutamate is the most potent co-agonist[1] of NMDA receptors endogenous to the mammalian brain, even when compared to the selective agonist NMDA. NMDA receptors have been extensively studied due to the increasing amount of evidence which shows that they are involved in the pathophysiology of several

[1] NMDA receptor activation, in addition to the binding of glutamate, also requires the binding of glycine. For that reason, glutamate is not considered an agonist, since neither of them alone can open the ion channel.

neuropsychiatric disorders. For instance, their over-activation can lead to excitotoxicity as a result of excessive glutamate release, thereby killing target neurons (Iacobucci and Popescu, 2017). In fact, glutamatergic synaptic transmission is affected in depressive-like states (Popoli *et al*., 2013) and it has been stated that there is an increase in extracellular glutamate levels following acute stress exposure in the BLA, which is reverted with antidepressant treatment (Reznikov *et al*., 2007). Therefore, the abnormal functioning of glutamate AMPA and NMDA receptors is likely involved in the onset of emotional dysfunction-related pathologies. Indeed, it has recently been shown that chronic stress induces changes in glutamatergic transmission in the rat amygdala through alterations in AMPA (Yi *et al.*, 2017; Zhou *et al*., 2018) and NMDA receptors (Zhou *et al*., 2018). AMPA receptors undergo a change in their localization after stress by migrating from dendritic shafts to dendritic spines, and this is associated to alterations in the excitatory synaptic transmission, confirmed by an increase in the frequency of miniature excitatory postsynaptic currents (mEPSC) (Hubert *et al*., 2014). NMDA receptors were first described to be involved in the processes of changes that occur in anxiety-like behavior more than twenty years ago (Adamec *et al.*, 1998a, 1998b). It is now known that they participate in synaptic plasticity mechanisms related to anxiety-like behavior at both a functional level, including their role in the acquisition of fear in the LA and BLA (Kim *et al*., 2007; Sotres-Bayon *et al*., 2007), and at a structural level, being also involved in the remodeling of synapses following stress and essential for the delayed strengthening of synaptic connectivity observed in the BLA (Yasmin *et al.*, 2016).

GABA receptors are the main mediators of inhibitory synaptic transmission and are divided in two major classes: $GABA_A$, which are coupled to an integral Cl^- channel and are blocked by bicuculline, and $GABA_B$, which belong to the family of G-protein-coupled receptors (Olsen, 1991). Inhibitory neurons are responsible for the GABAergic transmission, are crucial in shaping the flow of information within the amygdala circuit and regulate neuronal activity levels and behavioral output. More specifically, BLA, ITC and CeA interneurons reduce the excitatory output mediated by pyramidal cells, thereby modulating the behavioral anxiety response (Babaev *et al*., 2018). In the BLA, although interneurons constitute a small fraction of the overall population of cells, they tightly regulate the excitability of pyramidal neurons: a single interneuron is even able to block entirely the activity of a pyramidal neuron (Spampanato *et al*., 2011). Interneurons are able to shape signal propagation in several ways, amongst which are feedforward inhibition and feedback inhibition (Figure 3) (Tremblay *et al*., 2016). In feedforward inhibition, an excitatory synaptic input projects to both pyramidal neurons and interneurons, but the activity of the pyramidal cells is limited by the inhibition caused by the interneuron population (Figure 3A). In feedback inhibition, there is also an interaction between excitatory and inhibitory populations of neurons, but the activity is generated within the pyramidal

cells, which excite other pyramidal cells, as well as within interneurons, which provide synaptic inhibition back to them (Figure 3B) (Fröhlich, 2016; Hull, 2017).

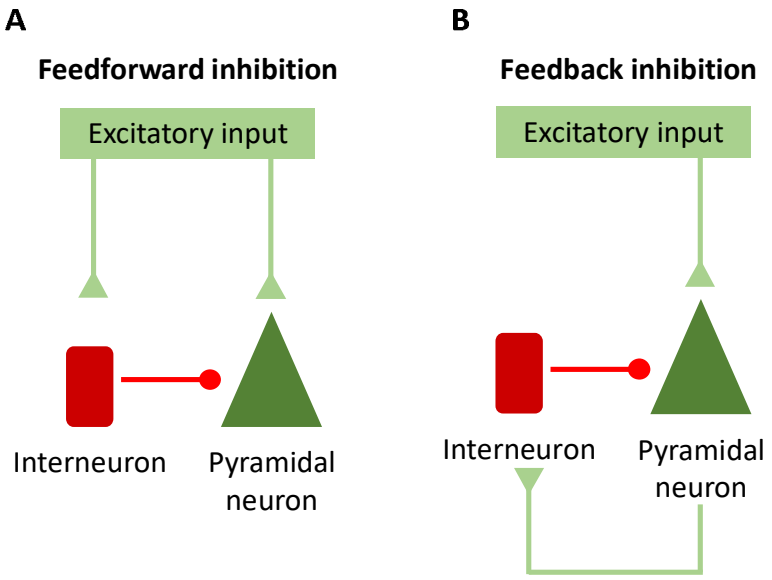


Figure 3. Schematic representation of the mechanisms of feedforward (A) and feedback (B) synaptic inhibition.

Interneurons can be classified based on their morphology, calcium-binding proteins or electrophysiological properties. Amygdala GABAergic interneurons resemble those existing in the cerebral cortex and may contain calcium-binding proteins, such as parvalbumin, calbindin or calretinin, and neuropeptides, such as somatostatin, cholecystokinin or neuropeptide Y (McDonald and Mascagni, 2001; Muller *et al*., 2006). This diversity of interneurons correlates with the postsynaptic targets which they innervate (Spampanato *et al*., 2011). Parvalbumin-containing (PV^+) interneurons are virtually all GABAergic, account for approximately half of the inhibitory neurons within the BLA and can control excitatory pyramidal neurons through their perisomatic innervation, along with other interneurons (Mascagni *et al*., 2009; Rainnie *et al*., 2006). PV^+ interneurons commonly co-express calbindin (CB), but not calretinin (CR) (Mascagni *et al*., 2009). Calretinin-immunopositive (CR^+) interneurons constitute about 25–30% of BLA GABAergic interneurons and primarily project to other interneurons (Capogna, 2014; McDonald and Mascagni, 2001; Muller *et al*. 2006). This high diversity of interneurons accounts for the neuronal network flexibility, since all of them release GABA on their postsynaptic targets, but differ in the subcellular domain which they target, connectivity and intrinsic membrane properties, resulting in highly specific inhibitory control

of the activity of principal neurons and local networks (Tremblay *et al*., 2016). Each interneuron population in the BLA exhibits distinct properties and forms unique connections, having, as a result, a peculiar role in the intrinsic amygdalar circuitry (Pinard *et al*., 2008). For instance, PV^+ interneurons in the BLA are part of a complex neuronal circuit which regulates anxiety-states (Hale *et al*., 2010). Therefore, the functional consequences of an impaired GABAergic transmission depend on the type of the affected interneurons. Indeed, when the activity of the interneurons is compromised, which includes persistent increases in their firing, a situation of hyperexcitability of glutamatergic neurons might emerge and it is typically associated to anxiety states (Babaev *et al*., 2018; Marín, 2012). Due to its impairment in anxiety conditions, the GABAergic inhibitory system is one of the main targets of therapeutic treatments. For example, benzodiazepines are widely used in clinics, because they allosterically enhance the postsynaptic actions of GABA at the $GABA_A$ receptor, leading to a sedative effect (Prager *et al*., 2016).

1.1.3. Synaptic plasticity in the amygdala

The mechanisms underlying formation of emotional memories have intrigued scientists for decades and most authors consider that the learning process which eventually leads to the storage of new, experience-dependent, information relies on synaptic plasticity of the CNS (Maren and Quirk, 2004). Synaptic plasticity is the process by which specific patterns of synaptic activity lead to alterations in synaptic strength, either enhancing or diminishing it, and, since those changes modify synaptic transmission, synaptic plasticity is thought to be the main mechanism of learning and memory. When the postsynaptic electrical response is augmented for a relatively long period of time as a result of high-frequency electrical stimulation, the process is called long-term potentiation (LTP); when the postsynaptic response is reduced upon low-frequency stimulation and this effect is maintained for a certain time, the phenomenon is designated long-term depression (LTD) (Citri and Malenka, 2008). Both processes exist in several brain regions, including the amygdala, where it has been described for the first time by Racine and his colleagues (Racine and Milgram, 1983). Some years later, Maren and Fanselow described both short- and long-term plasticity *in vivo* in the BLA upon high-frequency stimulation of the hippocampal afferents (Maren and Fanselow, 1995). The events responsible for LTP in the basolateral complex vary, especially according to the afferents which are being stimulated, and even if the recordings are performed *ex vivo* or *in vivo*. For instance, when the thalamic input is stimulated, at least two different forms of LTP can be activated in the LA *ex vivo*, one which depends on NMDA receptors and another depending on voltage-gated calcium channels (VGCC). *In vivo,* both forms of LTP contribute to the encoding of fear memories (Weisskopf *et al*., 1999). In the BLA, LTP

is also dependent on NMDA receptors (Drephal *et al*., 2006) and on VGCC (Weisskopf *et al*., 1999) upon stimulation of both cortical and thalamic afferents, along with the stimulation of the lateral nucleus. Amygdalar synaptic plasticity processes are considered to be the main mechanisms responsible for the triggering, processing and extinction of conditioned fear, as well as for the coordination of the most adequate response to stress (Millan, 2003). The fear conditioning paradigm was created by the Russian physiologist Ivan Pavlov in the 20th century (Pavlov, 1927) and has been used ever since, with a variety of experimental adaptations, to create animal models of conditioned fear by studying the associative learning between a threatening stimulus and a non-threatening context, which is later able to elicit defensive responses, commonly in the form of fear (Fanselow and Sterlace, 2014). The main principle of every protocol is, however, quite simple: a neutral stimulus (conditional stimulus, CS), such as a tone, is paired with an aversive stimulus (unconditional stimulus, US), such as an electrical footshock. After several presentations of both stimuli (conditioning), the CS alone begins to induce a learned fear response (Maren and Quirk, 2004). It is also possible to diminish the conditioned fear response through a process known as extinction, in which the animal is presented with several re-exposures of the CS, in the absence of the US. Depending on the time interval between the acquisition of the learned fear and its extinction, different neural circuits and mechanisms are recruited (Myers *et al*., 2006).

Both LA and BLA are involved in fear learning, since they are converging sites of multiple sensory inputs and the LA is the major source of intra-amygdaloid projections to the BLA (Sah *et al*., 2003). The LA synaptic plasticity is crucial for the acquisition of conditioned fear and it is one of the processes that underlie the association between CS and US (Johansen *et al*., 2011; LeDoux *et al*., 1990). Besides, it has already been demonstrated that lesions in the LA impair the acquisition of fear (Maren and Quirk, 2004). BLA has a role in the consolidation and storage of fear-related memories (Maren, 2001), where the pyramidal neurons assign affective value to the perceived sensory information (Tye *et al*., 2011). Several forms of synaptic plasticity have been described in the LA-BLA pathway, including LTP (Dityatev and Bolshakov, 2005; Rammes *et al*., 2000).

There are several ways to induce long-term synaptic plasticity, including LTP. High-frequency stimulation has been the most commonly used stimulation protocol to induce LTP, since it was described for the first time in 1973 (Bliss and Gardner-Medwin, 1973). Theta-burst stimulation was described some years later, in a study where the authors claimed that repetitive bursts of high frequency stimulation are more effective in inducing LTP than a single set of them (Larson *et al*., 1986). Also, this paradigm is thought to better mimic the spike discharges which occur in neurons in physiological conditions (Larson and Munkácsy, 2015). In the LA, it is common to induce LTP by pairing a weak presynaptic stimulation with strong postsynaptic depolarization, since it mimics the activity patterns

and the underlying mechanisms of association involved in the fear conditioning paradigm, in which presynaptic stimulation can be compared to the CS, while postsynaptic depolarization represents the US (Dityatev and Bolshakov, 2005; Sigurdsson *et al*., 2007).

Most of the studies found in the literature focus on LTP upon stimulation of glutamatergic afferent to principal cells in the basolateral complex, due to their abundance, and neglect the role of inhibitory interneurons, which constitute approximately 10-20% of the total population of neurons (Dityatev and Bolshakov, 2005; Spampanato *et al*., 2011). Yet, LTP also exists in interneurons upon stimulation of glutamatergic afferents and it has been argued that, upon tetanic stimulation, in the LA it depends solely on AMPA receptors (Mahanty and Sah, 1998). It has also been shown that LTP in the LA interneurons may underlie the processes related to fear conditioning and even might potentiate the inhibitory circuits more than the excitatory ones (Bauer and LeDoux, 2004). Now it is also known that there is a specific population of interneurons in the LA, which mediates synaptic plasticity upon stimulation of cortical afferents by recruiting AMPA receptors and by providing a feedforward inhibition to principal neurons, thus limiting their excitability (Polepalli *et al*., 2010).

1.1.4. Stress, depression and the amygdala

"Stress is a scientific concept which has received the mixed blessing of being too well known and too little understood" - Hans Selye

Stress can be generally defined as an actual or anticipated threat for the well-being and homeostasis of an organism, but it is essential to survival through adaptation to constantly changing stressful conditions (McEwen, 2007; McEwen and Akil, 2020). Stress is part of our everyday life, due to increasing demands of the modern world we live in. However, although the term is usually associated to a negative stimulus, only severe stress is a risk factor for the development of mood dysfunctions, such as Major Depressive Disorder (MDD), due to a maladaptive response of the individual (Lucassen *et al*., 2014; Sousa, 2016). This illness is highly prevalent in nowadays society and it is defined by the World Health Organization (WHO) as the mental disorder which constitutes the leading cause of disability at a global scale. Some of the main features characterizing depression include depressed mood, lack of interest and pleasure in routine activities, anxiety and, in severe cases, it is associated to cognitive impairment (Belzung *et al*., 2015). It is now accepted that, besides the environmental factors involved in the etiology of depression, there is also a disruption in synaptic connections which underlie mood and emotion circuitry (Duman and Aghajanian, 2012). Therefore, it is critical to understand how this synaptic mal-functioning could be ameliorated in order to reverse the symptomatology of the disease.

1.1.4.1. Animal models of depression

Some of the biggest limitations in understanding the molecular mechanisms underlying the development of depression are the heterogeneity of this disease and the existence of overlapping features with other mental illnesses (Krishnan and Nestler, 2008). Animal models allow the study of neural circuitry and the signal transduction pathways which may be implicated in the pathogenesis of depression and have been extensively used in research (Wang *et al*., 2017). However, some studies still often consider the disease from a monosymptomatic perspective. It is of utmost importance to assess the animal models which mimic the behavioral aspects of depression by evaluating at least three dimensions: their face validity (the degree of similarity of the core depressive symptom between the animal model and the human disease), their mechanistic or construct validity (the degree of similarity between the molecular mechanisms involved in the onset and development of the disease in the animal model and to what is presumed to happen in human disease) and their predictive validity (the degree of reversion of the symptoms resulting from the use of drugs with antidepressive properties) (Belzung

and Lemoine, 2011; Bessa *et al*., 2009). Furthermore, in order to better simulate the human pathology, it is important to evaluate behavioral parameters indicating alterations in mood, anxiety and cognition, which are known to be affected in depression (Dotson *et al*., 2014; Gotlib and Joorman, 2010), by using the appropriate animal behavioral tests.

There is a wide variety of stress paradigms used in animal models and the choice of the appropriate model requires a clear definition of the research objectives and endpoints, which might be quite diverse: behavioral, metabolic and neurochemical, amongst others (Patchev and Patchev, 2006).

1.1.4.2. Stress effect on the amygdala

The brain is one of the most affected organs in a stressful environment, since it is responsible for the integration of the information, as well as for the behavioral and systemic responses (McEwen, 2007), but, luckily, it is also capable of adaptive neural plasticity through life (McEwen and Akil, 2020). However, upon chronic stress, the brain activity suffers profound changes when compared to the pattern of brain activity in healthy individuals and several regions are morphologically and functionally altered (Sousa, 2016).

The amygdala is one of the most affected brain regions in emotional dysfunctions, since it attributes affective and emotional valence to the different aspects of cognition which the brain perceives (Lucassen *et al*., 2014). In anxiety-like conditions, the occurrence of dendritic hypertrophy of BLA pyramidal neurons is well characterized, in contrast to the hippocampus, where a neuronal atrophy is usually observed upon chronic unpredictable stress, chronic immobilization stress and acute immobilization stress (Mitra *et al*., 2005; Vyas *et al*., 2002, 2004). Besides the structural plasticity and morphological changes, exposure to stress affects both excitatory (Popoli *et al*., 2013) and inhibitory (Rodríguez Manzanares *et al*., 2005) amygdalar synaptic transmission, causing an imbalance between glutamatergic and GABAergic systems (Wang *et al*., 2016). Upon stress, the amygdala is typically associated to a state of hyperactivity and hyperresponsiveness of glutamatergic neurons, as a consequence of the removal of the strong inhibitory control that is typical in physiological conditions in this brain structure (Zhang *et al*., 2018). LA hyperexcitability of pyramidal neurons upon chronic restraint stress is linked to alterations in the intrinsic membrane properties of the neurons, which depend on calcium-activated potassium channels (Rosenkranz *et al*., 2010). It is important to outline that the hyperexcitability of the amygdala basolateral complex largely contributes to the observed behavioral changes, which are usually associated to an increase in anxiety, emotional dysregulation or even seizure activity (Prager *et al*., 2016). For this reason, one of the main therapeutic approaches to

treat anxiety-like conditions includes the administration of benzodiazepines, which are agonists of the $GABA_A$ receptors (Haefely, 1984; Starcevic, 2014).

Although a wide range of molecular mechanisms is altered upon a stress-induced state, it is noteworthy that an upregulation of adenosine A_{2A} receptors ($A_{2A}R$) (Cunha *et al.*, 2006) has been described. It has also been recently shown that the expression of the $A_{2A}R$ gene is enhanced in the basolateral complex of female rats exposed to a stress protocol of feeding restriction (Micioni Di Bonaventura *et al.*, 2019). Interestingly, in addition to that, $A_{2A}R$ antagonism has been described to be beneficial, since it prevents synaptic, mood and memory dysfunction induced by repeated stress (Kaster *et al.*, 2015). Therefore, the adenosinergic system (which is described in detail in the next section) and the alterations which it suffers in depressive-like conditions are the main focus of this work.

1.2. The adenosinergic system

1.2.1. Adenosine and adenosine receptors

Adenosine is an ubiquitous purine nucleoside, present in all cells which depend on this molecule to perform key metabolic processes which ensure their survival (Cunha, 2016). Adenosine exerts its functions through adenosine receptors, which are highly expressed in the brain when compared to other organs (Cunha *et al*., 2008). The first suggestion that adenosine receptors exist in the CNS dates to the 70's decade of the 20th century, when Sattin and Rall reported the ability of theophylline to prevent the increase of cAMP mediated by adenosine (Sattin and Rall, 1970). Some years later, Geoffrey Burnstock proposed, for the first time, that there exists a family of so called purinergic receptors which can be subdivided in two classes - P_1 and P_2 (Burnstock, 1978). Now it is considered that four types of adenosine receptors can be accounted for - A_1, A_{2A}, A_{2B} and A_3 - and all of them are G-protein coupled (Fredholm *et al*., 1994, 2005). A_1 and A_{2A} receptors are the most abundant in the brain and possess the highest affinity for adenosine (Burnstock *et al*., 2011), but are differentially involved in the modulation of synaptic transmission: adenosine A_1 receptors (A_1R) are inhibitory, whereas adenosine A_{2A} receptors ($A_{2A}R$) are facilitatory and adenosine modulation mostly depends upon a balance of their activation (Cunha, 2001).

Adenosine plays a dual role in the CNS: not only does it act as a transcellular messenger, but also as a neuromodulator by regulating neurotransmitters release and synaptic transmission pre-, post- and extrasynaptically. More specifically, adenosine can facilitate or inhibit neurotransmitters release to the synaptic cleft, affect other neurotransmitters and alter the intrinsic excitability of neurons (Cunha, 2001). Adenosine does not exclusively act in synapses (in the same way as it does not solely transfers information from the pre- to the postsynaptic nerve terminal) and it is not stored in vesicles (Chen *et al*., 2014; Sheth *et al*., 2014).

Adenosine can go across the plasma membrane through selective transporters responsible for equilibrating the levels of adenosine inside and outside of the cell (Choi et al., 2004). These transporters exist in almost all cells and maintain adenosine extracellular and intracellular levels similar (although some of them are concentrative transporters which drive adenosine into the cell through a concentration gradient) (Young *et al*., 2008). Alternatively, adenosine can be produced extracellularly from membrane–anchored enzymes which catabolize AMP into adenosine through ecto-5'-nucleotidades (CD73) (Gonçalves *et al*., 2019; Zimmermann *et al*., 1998). Adenosine levels are also controlled by an effective clearance system in the brain: it can be either phosphorylated into AMP via adenosine kinase or metabolized into inosine through the action of adenosine deaminase

(Burnstock *et al*., 2011). In order to act on its receptors, adenosine has to be released into the extracellular space and it usually results either from the cleavage of adenine nucleotides by ecto-nucleotidases or from the transport of adenosine to the extracellular space from inside the cell (Zimmermann, 2000).

All adenosine receptors have seven transmembrane domains and have preferential signal transduction mechanisms, but, since A_1R and $A_{2A}R$ are the most abundant in the brain and are responsible for the tonic effect of endogenous adenosine (Fredholm *et al*., 2005), they will constitute the main focus of this work. The effects which adenosine exerts depend on the concentration of extracellular adenosine, as well as on the density of the adenosine receptors in a given cell type or brain region (Chen *et al*., 2014).

A_1R are the most abundant in the brain and are extensively located throughout several brain regions (Fredholm *et al*., 2005), including the neurons of cortex, hippocampus and cerebellum, where their highest density is observed, likewise in other structures, including the amygdala, where they are unevenly distributed, with high labeling in the lateral and basal nuclei, in both rat and human brain (Fastbom *et al*., 1987; Svenningsson *et al*., 1997; Trussell and Jackson, 1987). $A_{2A}R$ are found at the highest levels in the striatum, nucleus accumbens and olfactory tubercles, but they also exist in other brain regions, such as hippocampus, cortex and the amygdala (Burnstock *et al*., 2011; Cunha *et al*., 1994; Cunha *et al*., 1995). $A_{2A}R$, although not abundant, present higher densities in the amygdala when compared to the hippocampus (Rosin *et al.,* 1998). Both A_1R and $A_{2A}R$ exist in synapses (Rebola *et al*., 2003; Rebola *et al*., 2005), being typically more abundant in glutamatergic ones (Rebola *et al*., 2005; Simões *et al*., 2016; Tebano *et al.,* 2005), but they have also been observed in GABAergic synapses (Cristóvão-Ferreira *et al*., 2009; Cunha and Ribeiro, 2000; Rombo, 2015).

In normal physiological conditions, adenosine acts in the brain as a predominantly inhibitory neuromodulator by regulating the activity of both neurons and glia (Cunha, 2001). The resting state concentration of extracellular adenosine ranges between 30 and 200 nmol/L, which is enough to activate both A_1R and $A_{2A}R$, depending on their relative abundance in a specific brain region (Ballarin *et al*., 1991; Fredholm, 2014). In basal experimental and physiological conditions, an A_1R-mediated inhibition is mostly present (Cunha, 2005; Dunwiddie and Masino, 2001). Nevertheless, whenever there is an imbalance between the formation of ATP and its cleavage, the extracellular levels of ATP rise and lead to augmentation in adenosine levels in sufficient amount to activate the $A_{2A}R$ (Fredholm, 2014). This increase in adenosine extracellular concentration might also occur upon high-frequency nerve stimulation, which triggers long-term potentiation (Costenla *et al*., 2011; Rebola *et al*., 2008), or under extreme or pathological conditions, which are metabolically stressful, such as intense physical exercise or hypoxia (Fredholm, 2014).

1.2.2. A_1R and $A_{2A}R$ main signaling pathways

The signaling mechanisms recruited by any receptor depend on the conditions of the surrounding environment, and adenosine receptors appear to modulate neuronal activity via a diverse array of signal transduction pathways. We would like the reader to take into consideration that this section focuses on the most well-known signaling pathways in which A_1R and $A_{2A}R$ are involved.

A_1R are crucial players in the control of synaptic transmission inhibition, since they control glutamate release in synaptic nerve terminals (Lupica *et al*., 1992; Proctor and Dunwiddie, 1987; Tetzlaff *et al*., 1987). Adenosine receptors are G-protein coupled receptors (GPCR): A_1R typically interact with G_i and G_o proteins, inhibiting adenyl cyclase (AC) and leading to a decrease in cyclic adenosine monophosphate (cAMP) levels, whereas $A_{2A}R$ bind to G_s proteins, stimulating AC activity and resulting in an increase of cAMP levels (Cunha, 2016). Interestingly, the inverse process is also true: if AC is activated, the concentration of extracellular adenosine rises and $A_{2A}R$ are activated (Pleli *et al*., 2018). Intracellular cAMP can then diminish (if A_1R are activated) or enhance protein kinase A (PKA) activity (if $A_{2A}R$ are activated) (Chen *et al*., 2014; Fredholm *et al.*, 2007). PKA is important in a variety of biological processes, including regulation of cell growth and division, together with gene transcription (Grönholm *et al.*, 2003), and one of its key targets is the cAMP-response element binding protein (CREB), which is localized in the nucleus and acts as a transcription factor (Alberini, 2009; Josselyn and Nguyen, 2005). Hence, CREB transcriptional activation may be reduced, when A_1R are activated, and increased, when $A_{2A}R$ are stimulated (Merighi *et al*., 2018). Since adenosine receptors act as part of a complex neuronal network, they can also be involved in mitogenic signaling (Rozengurt, 2007), commonly designated as mitogen-activated protein kinase (MAPK) cascade. This signaling pathway was initially described to mediate cell division mechanisms, but now it is known that it is crucial for neuronal processes, such as synaptic plasticity. The MAPK includes a family of proteins composed by three main classes: the extracellular signal-regulated protein kinases (ERK), the stress-activated protein kinases (SAPK) p38 and the SAPK c-jun N-terminal kinases (JNK) (Schulte and Fredholm, 2003; Sweatt, 2001). Ultimately, MAPK phosphorylates CREB, making this protein a point of convergence of both cAMP/PKA and MAPK pathways. Moreover, the MAPK cascade may be preferred in situations where modulation of $A_{2A}R$ confers neuroprotection. Particularly, it has been shown that an impaired neuronal outgrowth can be rescued through $A_{2A}R$ activation with its selective agonist (CGS21680), in a process which integrates the MAPK pathway (Chen *et al*., 2002). On the other hand, both selective and genetic blockade of $A_{2A}R$ prevent the synaptotoxicity in a model of Alzheimer's disease, through control of the MAPK pathway, rather than of the cAMP/PKA one (Canas *et al*., 2009). It is noteworthy that $A_{2A}R$ may even switch pathways, according to the extracellular

levels of glutamate: if the glutamate levels are low, $A_{2A}R$ signaling may occur through the canonical cAMP/PKA cascade; if the glutamate concentration increases drastically, the $A_{2A}R$ signaling may be shifted towards the PKC/MAPK pathway (Dai *et al*., 2010).

A_1R and $A_{2A}R$ can also be involved in other signaling mechanisms. A_1R are able to induce hyperpolarization of the cell membrane by activating K^+ channels (Heurteaux *et al*., 1995; Rotermund *et al*., 2018) and to inhibit calcium channel activity through voltage-gated calcium channels (VGCC) (Yamashiro *et al*., 2017). Stimulation of A_1R can also activate the phospholipase C (PLC) pathway, in which the degradation of phosphatidylinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3) occurs. These metabolites are also important: DAG acts as an activator of protein kinase C (PKC) and IP_3 binds to the endoplasmic reticulum (ER) and allows the release of Ca^{2+}, thereby augmenting its intracellular levels, also activating PKC (Rogel *et al*., 2006). The activation of PKC in the CNS is involved in the regulation of several processes, namely, neurotransmitter release, ion channels activity and neuronal plasticity (Huang, 1989) and its signal transduction depends on both A_1R and $A_{2A}R$ activation (Cunha and Ribeiro, 2000; Marala and Mustafa, 1995). The increase in Ca^{2+} levels is also modulated by calmodulin-kinases (CaMKs), which participate in a cascade critical to neuronal development, plasticity and behavior (Wayman *et al*., 2008), and are likewise able to phosphorylate CREB (Alberini, 2009).

The development of *optoA$_{2A}$R*, which is a chimeric rhodopsin-$A_{2A}R$ protein, has leaded to a better understanding of $A_{2A}R$-mediated signaling in certain cells, since it allows the researcher to control the $A_{2A}R$ activation with light stimulation in a precise spatiotemporal manner (Li *et al*., 2015). Indeed, the authors of the study elegantly showed that *optoA$_{2A}$R* triggered different molecular responses in distinct brain regions. More specifically, in the hippocampus, the cAMP/PKA cascade was activated, while, in the nucleus accumbens, the preferred pathway was the MAPK (Li *et al*., 2015).

A_1R and $A_{2A}R$ are, thus, involved in multiple signaling pathways, accounting for their complex and diverse functions throughout the cells. Some of the above mentioned mechanisms are represented schematically in Figure 4.

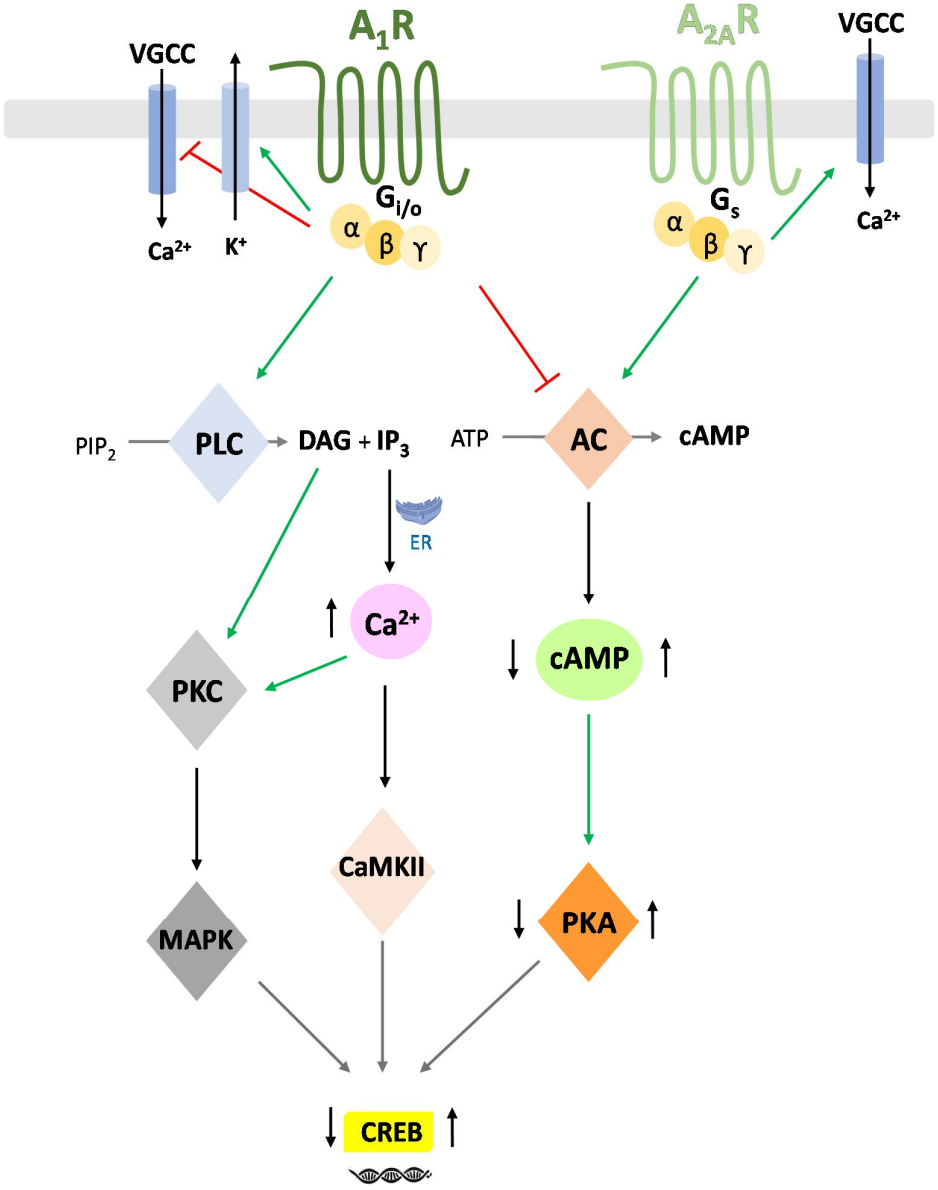


Figure 4. A_1R and $A_{2A}R$ and their main signaling pathways. A_1R are usually coupled to the $G_{i/o}$ family of G proteins, which inhibit the adenyl cyclase (AC), thereby lowering the intracellular levels of cAMP. On the other hand, they can activate phospholipase C (PLC), which promotes the cleavage of phosphatidylinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). The latter, in its turn, binds to the endoplasmic reticulum (ER) and allows the release of Ca^{2+}, hence augmenting its intracellular levels and further recruiting calmodulin kinase II (CaMKII) or mitogen-activated protein kinase (MAPK), which ultimately phosphorylate cAMP responsive element binding (CREB). A_1R also activate inwardly rectifying K^+ channels and inhibit voltage-gated calcium channels

(VGCC). $A_{2A}R$ are bound to the G_s family of G proteins, which activate AC, thus increasing cAMP levels. This enhancement in cAMP levels leads to the activation of phosphokinase A (PKA), which initiates several molecular pathways, including (CREB). $A_{2A}R$ also activate VGCC.

1.2.3. Heteromerization of A_1R and $A_{2A}R$

The existence of GPCR heteromers, in which two or more functional receptor units form a macrocomplex which exhibits distinct biochemical, pharmacological and pathophysiological features from the separate monomers, is now broadly accepted (Ferré *et al*., 2009). A variety of studies suggest that A_1R and $A_{2A}R$ are able to physically associate between themselves or with other GPCRs (Ferré and Ciruela, 2019).

A_1R-$A_{2A}R$ heteromers functional association is not surprising, as these receptors co-exist in glutamatergic nerve terminals (Rebola *et al*., 2005) and, in the striatum, the A_1R-$A_{2A}R$ complex is located both pre- and postsynaptically (Ciruela *et al*., 2006a). It is interesting to outline that neither of these receptors loses its affinity for the respective agonist (Ciruela *et al*., 2006a). Noteworthy, in the A_1R-$A_{2A}R$ complex, when $A_{2A}R$ is activated, the affinity of A_1R for its agonist decreases, while the opposite is not true (Ciruela *et al*., 2006b). Thus, the regulation of glutamate release by the A_1R-$A_{2A}R$ may switch from inhibition to facilitation, according to the extracellular levels of adenosine (Ciruela *et al*., 2006b). The oligomerization of GPCRs and, in particular, of adenosine receptors, may provide safer therapeutic targets, but it is also challenging, since the pharmacological complexity of possible modulators is also increased (Vecchio *et al*., 2018).

1.2.4. Control of excitatory and inhibitory synaptic transmission by A_1R and $A_{2A}R$

A_1R and $A_{2A}R$ have already been reported to directly affect NMDA and AMPA receptors function in the hippocampus. More specifically, in the excitatory synapses of CA1 neurons, the activation of A_1R inhibits NMDAR- and AMPAR-mediated currents and, hence, the release of glutamate in several brain regions (de Mendonça *et al*., 1995; Manita *et al*., 2004; Marchi *et al*., 2002; Yum *et al*., 2008). In contrast, the activation of $A_{2A}R$ enhances both AMPAR- and NMDAR-mediated currents through PKA-dependent pathways (Dias *et al*., 2012; Mouro *et al*., 2018; Rebola *et al*., 2008; Temido-Ferreira *et al*., 2018). In addition to that, it has also been shown that $A_{2A}R$ facilitate long-term potentiation throughout the brain, including the amygdala (Kerkhofs *et al*., 2018; Rebola *et al*., 2008; Simões *et al*., 2016). Furthermore, $A_{2A}R$ has been shown to play an important role in excitatory synaptic transmission onto fast spiking interneurons (Kerkhofs *et al*., 2018) and GABAergic transmission

between feedforward interneurons (Rombo *et al*., 2014). More specifically, in the PFC, $A_{2A}R$ is necessary for LTP in excitatory synapses onto fast-spiking interneurons (Kerkhofs *et al*., 2018). It has been shown that, in the hippocampus, $A_{2A}R$ activation increased inhibitory monosynaptic inputs from PV^+ interneurons to other interneurons, resulting in decreased IPSC, i.e., a disinhibition of pyramidal cells (Rombo *et al*., 2014). On the other hand, the same authors showed that A_1R reduce tonic, rather than phasic, GABAergic transmission, in hippocampal pyramidal neurons and in CB_1R^+ interneurons (Rombo *et al*., 2016a). It has also been demonstrated that $A_{2A}R$ are involved in the regulation of inhibitory synaptic transmission in striatal neurons, in a study where the activation of presynaptic $A_{2A}R$ diminished the GABAergic-mediated currents (Mori *et al*., 1996).

In the amygdala, it has been shown that astrocytic release of ATP/adenosine activates neuronal A_1R and $A_{2A}R$ in the CeM, which is mainly composed by interneurons, and then the activation of A_1R diminishes excitatory currents in BLA-CeM synapses, whereas $A_{2A}R$ activation enhances inhibitory currents in CeL-CeM synapses (Martin-Fernandez *et al*., 2017). The synaptic actions of A_1R and $A_{2A}R$ on excitatory and inhibitory synaptic transmission within the basolateral complex of the amygdala will be explored in more detail in the next section of the present work, although there is scarce information on that matter.

1.2.5. Role of adenosine receptors in the amygdala and amygdala-dependent behavior

Since the amygdala is highly affected in anxiety disorders (Fredrikson and Faria, 2013) and mood dysfunction pathologies (Benson *et al*., 2014), and the modulation of synaptic transmission is one of the possible strategies to normalize pathological alterations, adenosine emerges as a prime neuromodulator, and adenosine receptors, particularly A_1R and $A_{2A}R$, as candidate therapeutic targets for the treatment of these conditions (Dunwiddie and Masino, 2001). A_1R have a heterogeneous distribution in the amygdala, with high density in the lateral and basal nuclei, in both rat and human brains (Fastbom *et al*., 1987; Svenningsson *et al*., 1997; Trussell and Jackson, 1987). At the same time, $A_{2A}R$ have higher density levels in the amygdala when compared to the hippocampus, but, nevertheless, they are not abundant (Rosin *et al*., 1998). In the BLA, A_1R are located presynaptically and, in basal conditions, function similarly to what is described in other brain regions, since their activation with the selective agonist CPA inhibits glutamate release without affecting the intrinsic properties of pyramidal neurons and leads to a decrease in AMPA-mediated synaptic transmission (Rau *et al*., 2014). On the other hand, BLA $A_{2A}R$ appear to be located postsynaptically and their activation with the selective agonist CGS21680 increases the firing rate of pyramidal neurons, in a manner similar to what happens in anxiety-like states, through a mechanism which depends on PKA activity, involved in the canonical signal transduction pathway of adenosine signaling (Rau *et al*.,

2015). In non-basal conditions, such as in extracellular synaptic plasticity recordings, a facilitatory role of $A_{2A}R$ has been shown in the induction of LTP in the LA through stimulation of cortical afferents (Simões *et al*., 2016).

The involvement of the adenosinergic system in amygdala-related processes has been demonstrated in several studies. The majority of the show that caffeine, which is an antagonist of all four types of adenosine receptors without substantial selectivity (Ribeiro and Sebastião, 2010), impairs some stages of the fear conditioning paradigm, which is critically dependent on the amygdala (Maren, 1999), proving that adenosine receptors are involved in the generation of fear response (Corodimas and Tomita, 2001; Dubroqua *et al*., 2015). Indeed, striatal $A_{2A}R$ are involved in the regulation of fear conditioning, since their genetic deletion increased the freezing response to the tone and context, an effect which was normalized when $A_{2A}R$ deletion was extended to the prefrontal cortex (Wei *et al*., 2014). On the other hand, systemic activation of A_1R impairs the acquisition of fear (Corodimas *et al*., 2001). Moreover, $A_{2A}R$ have been described in glutamatergic synapses of the amygdala and they participate in the control of synaptic plasticity in the LA upon high-frequency cortical stimulation. Furthermore, the authors of the study elegantly showed that $A_{2A}R$ are essential in the formation of the long-term memory of fear, by selectively silencing those receptors with shRNA (Simões *et al*., 2016). In addition to the research mentioned above, it has been proven that selective activation of A_1R decreases anxiety-like behavior in both naïve mice and mice suffering from ethanol withdrawal, which is *per se* anxiogenic (Jain *et al*., 1995; Prediger *et al*., 2006). Besides neuronal involvement, astrocytes also play a functional role in the amygdala, by establishing a bidirectional communication with neurons. More importantly, the amygdalar astrocytes regulate the inhibitory activity in the BLA through A_1R and $A_{2A}R$-dependent mechanisms (Martin-Fernandez *et al*., 2017). All these data suggest that the adenosinergic system is involved in the amygdala physiological and pathological signaling, likely through A_1R and $A_{2A}R$-mediated modulation.

1.2.6. Adenosine receptors as therapeutic targets in depression

Most of the alterations emerging in neuronal circuits and eventually leading to pathological conditions are associated to mysfunction in modulation systems (Cunha, 2016a). Since adenosine's primary function is to act as a neuromodulator, increasing evidence supports the idea that adenosine receptors are promising targets in the treatment of several pathologies of the CNS (Gomes *et al*., 2011; Lopes *et al*., 2011), including those of psychiatric nature, such as depression (Cieślak *et al*., 2016; Krügel, 2016), in which it is typical to observe increased levels of extracellular adenosine (Cunha *et al*., 2001) and an upregulation and aberrant signaling of $A_{2A}R$ (Chen *et al*., 2013; Coelho *et al*., 2014;

Cunha and Agostinho, 2010; Temido-Ferreira *et al*., 2019). Adenosine receptors have, thus, been extensively studied as possible therapeutic targets, since adenosine exists in all synapses ever studied and exhibits a wide variety of functions across the CNS (Dunwiddie and Masino, 2001). Adenosine receptors might emerge as good pharmacological targets in two situations: either there is an excess of adenosine and an antagonist of those receptors could be used, or adenosine receptors activation and subsequent increase of endogenous adenosine levels would be beneficial, and the application of an agonist presents a solution (Fredholm, 2014). The most known antagonist of adenosine receptors is caffeine (Ribeiro and Sebastião, 2010), which is widely consumed around the world as a psychostimulant drug (Ferré, 2016). It is important to outline that caffeine is an effective antagonist of A_1, A_{2A} and A_{2B} receptors only at low doses (Fredholm *et al*., 2017) and $A_{2A}R$ antagonism has been proved to be neuroprotective in several disease models, primarily through blockade of excitotoxic effects mediated by an uncontrolled glutamate release (Canas *et al*., 2018; da Silva *et al*., 2016; Silva *et al*., 2018).

A wide variety of clinical investigations and pharmacological studies have shown that adenosine neuromodulation is implicated in the etiology and regulation of anxiety- and depressive-like states in both rodents and humans (Kaster *et al*., 2015; Simões *et al*., 2016; Yamada *et al*., 2014). As mentioned before, most of the data emerges from research based on the use of caffeine, since it is a non-selective antagonist of all four adenosine receptors, which has been shown to be effective in the prevention and treatment of the altered mood in MDD (Alsene *et al*., 2003; Cunha *et al*., 2008; López-Cruz *et al*., 2018). Moreover, in the brain, adenosine acts mainly through receptors with opposite functions: on inhibitory A_1R and facilitatory $A_{2A}R$ (Cunha *et al*., 2008), and it is generally accepted that increased activity of A_1R is linked to fast antidepressant effects, while $A_{2A}R$ over-activation is typically associated with induction of depressive symptoms (Coelho *et al*., 2014; Kaster *et al*., 2004; Krügel, 2016; van Calker *et al*., 2019; Van Calker and Biber, 2005), since they are prevented by the selective blockade of $A_{2A}R$ upon a chronic unpredictable stress paradigm (Kaster *et al*., 2015). $A_{2A}R$ antagonists were initially suggested to exert antidepressant-like effect in 1990 and confirmed by several studies in both pharmacological and genetic knock-out mice models for the $A_{2A}R$ (El Yacoubi *et al*., 2001). Besides the studies in which caffeine was used, the involvement of the adenosinergic system has also been demonstrated with the amelioration of symptoms in patients who were subjected to electroconvulsive therapy aimed to control mood-dysfunction (Van Calker and Biber, 2005). In rodents, the research is quite controversial. In animal models of despair, adenosine and its analogues were shown to induce a depressive-like response (Hunter *et al*., 2003; Minor *et al*., 1994b) and its antagonist to revert the acquired impairment (Minor *et al*., 2001, 1994a). However, other studies point to an antidepressant effect of A_1R activation (Kaster *et al*., 2004), which has been confirmed by other

authors, at least in the prefrontal cortex, where the upregulation of A_1R is mediated by an immediate early gene, Homer1a, the overactivation of which is usually linked to the administration of antidepressant treatment (Serchov *et al*., 2015). Recently, the same researchers have demonstrated that this mechanism also leads to an upregulation of AMPA receptors and, consequently, to an enhancement of glutamatergic synaptic transmission, at least in the forebrain (Holz *et al*., 2019).

Despite the diversity of existing data, an inverse correlation between the intake of caffeine and the incidence of mood disorders definitely exists (Lucas *et al*., 2011; Kaster *et al*., 2015). Also, since $A_{2A}R$ blockade prevents and reverts mood alterations upon chronic stress (Kaster *et al*., 2015), and rats overexpressing $A_{2A}R$ in the forebrain display depressive-like behavior (Coelho *et al*., 2014), $A_{2A}R$ are undoubtedly important players in the pathophysiology of depression. In fact, a possible key role of $A_{2A}R$ in depression would imply that the over-activation of $A_{2A}R$ is actually sufficient to cause mood alterations, which remains to be established. In this respect, our preliminary data suggest that administration only in the amygdala of lentivirus expressing sh-RNA to downregulate $A_{2A}R$, is sufficient to revert mood alterations in rodents subjected to repeated restraint stress (unpublished data).

Taking these data into account, the major goal of this work was to explore changes in modulation by $A_{2A}R$ within the basolateral complex of the amygdala in a rat model of depression.

2. AIMS

The overall aim of this dissertation was to assess the role of adenosine A_1R and $A_{2A}R$ in the BLA in physiological conditions and in an animal model of depression.

More specifically, the aims were:

- to evaluate the modulation of synaptically evoked NMDAR currents by A_1R and $A_{2A}R$ within the local BLA circuitry

- to assess the role of $A_{2A}R$ modulation on changes in excitatory and inhibitory synaptic transmission in animals submitted to repeated restraint stress

"- These creatures you call mice, you see, they are not quite as they appear. They are merely the protrusion into our dimension of vast hyperintelligent pandimensional beings. The whole business with the cheese and the squeaking is just a front.
The old man paused, and with a sympathetic frown continued.
- They've been experimenting on you, I'm afraid."

Douglas Adams, in *The Hitchhiker's Guide to the Galaxy*

3. METHODOLOGY

3.1. Animals

The definition of the adolescence age span in animal models is not consensual among literature, but the most accepted one in rats comprises the period from 28 to 42 postnatal days, while adulthood is usually considered to initiate at approximately 63 postnatal days (Spear and Brake, 1983; Sengupta, 2013). Male adolescent Wistar rats (4-5 weeks) were obtained from the CNC animal house and adult Wistar (8-12 weeks old) rats were obtained from Charles River (Barcelona, Spain). All animals were handled according to the principles and procedures outlined as "3Rs" in the FELASA, ARRIVE, and European Union guidelines (86/609/EEC), were approved by the Animal Welfare Committee of the CNC (ORBEA 138-2016/15072016 and ORBEA_238_2019/14102019). The rats were maintained under controlled environment (23 ± 2 °C, 12-h light/dark cycle, *ad libitum* access to food and water) and group-housed until used in electrophysiological studies.

3.2. Experimental procedures

3.2.1. Patch-clamp recordings

"There is a common impression that electrophysiologists can be quite picky when preparing and running an experiment. Indeed, you may have felt the fixed gaze of vigilance when moving close to an electrophysiology setup. […] Electrophysiologists are made, not born, and in the process of learning the tricks of the trade they realize how small changes within an experimental setup can have huge consequences, that may take weeks to fix. This is the reason that, once they believe their setup is optimized, they become precious about letting others near it."

Isabel Plasencia-Fernández and Jimena Pérez-Sánchez

The patch-clamp technique is an electrophysiological tool developed during the 70's decade of the 20th century by Erwin Neher and Bert Sakmann, who were able to characterize, for the first time, single-channel currents in the neuromuscular junction (Neher and ASakmann, 1976; Sakmann and Neher, 1984). Nowadays, patch-clamp recordings are most frequently used to study electrical currents in entire cells and are particularly useful for neurobiological studies, where they are usually applied *in vitro* to brain slices, freshly dissociated neurons and cell cultures (Sontheimer and Olsen, 198; Segev *et al*., 2016). The results of the experiments presented in this study were performed in brain slices, since this method has several advantages, in particular, the relatively good preservation of neural circuits, which allows a more similar comparison to i*n vivo* conditions (Segev *et al*., 2016).

To better understand the patch-clamp technique, it is important to comprehend the basics of communication between neurons. Synaptic transmission is a process involving signal transduction initiated by the release of neurotransmitters from a presynaptic terminal to the synaptic cleft, which then bind to and activate postsynaptic receptors, thereby causing alterations of the electrical and biochemical properties of the cell. Neurotransmitters are stored in synaptic vesicles, which are released from the presynaptic terminal when an action potential arrives and activates voltage-dependent calcium channels, leading to an increase in the intracellular levels of Ca^{2+}. After the release and activation of postsynaptic receptors, the excess of neurotransmitters is re-uptaken either by the presynaptic terminal or by glial cells (Holz and Fisher, 1999) (Figure 5).

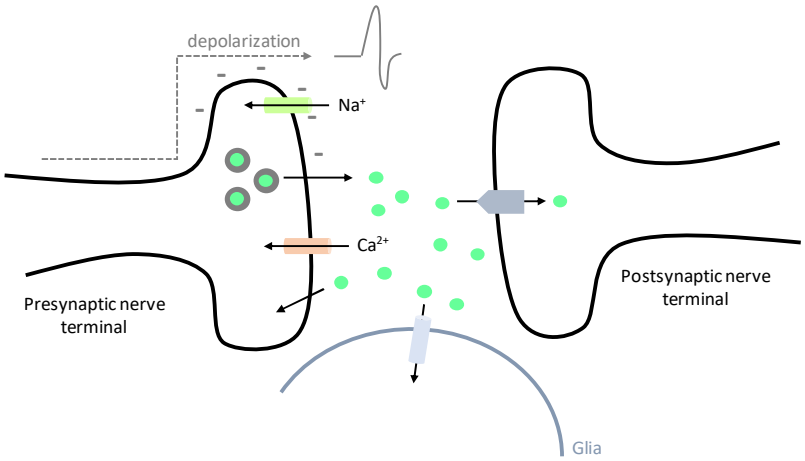


Figure 5. Schematic representation of a simplistic synaptic transmission mechanism.

Neurons are excitable cells, meaning that they can detect an external stimulus, convert it to an electrical signal through a change in the membrane potential (V_m), and pass it on to the next cell (Nelson and Cox, 2008). Therefore, it is possible to take advantage of the neuronal electrical properties and compare them to electrical circuits. In simple terms, the cell membrane, which is formed by lipids, can be considered a capacitor (since it is able to store charge), which has a high resistance (since ions can pass through the membrane, although with some difficulty, needing ion channels to do it); both sides of it are surrounded by saline solutions which act as conductors. Specific ions flow through the plasma membrane and their relative permeability and distribution define the membrane potential of the cell. The membrane potential changes according to the redistribution of ion charges on both sides of the membrane: it can either become depolarized and achieve positive values (if there is an influx of

positively charged ions or an efflux of negatively charged ones) or hyperpolarized with more negative potential values (if there is an efflux of positive charges)[2] (Molleman, 2002) (Figure 6).

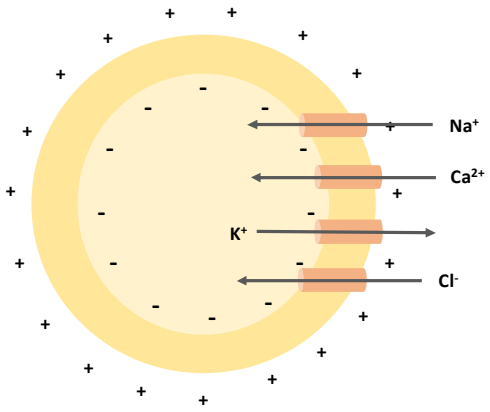


Figure 6. Schematic representation of the ions that define the electrical potential of a cell. The electrical potential of a cell is maintained due to specific neuronal membrane properties: it has differential permeability to ions and these ions are unequally distributed across the membrane. In the resting state, neurons are usually highly permeable to K^+, moderately permeable to Cl^- and slightly permeable to Na^+ and Ca^{2+}. The intracellular space commonly contains high levels of K^+ and low concentrations of Na^+, Cl^- and Ca^{2+} and their ionic gradients are maintained due to ion-specific pumps. Besides, the interior of a neuron also contains negatively charged proteins that do not exist in the extracellular space.

Most of the patch-clamp recordings are performed in voltage-clamp configuration, allowing the experimenter to control the membrane potential (V_m) and record the membrane current (I), which depends on the membrane resistance, according to the Ohm's Law: Voltage (V) = Resistance (R) x Current (I) (Molleman, 2002).

Every patch-clamp experiment begins with a cell-attached configuration, in which the electrode makes contact with the cell membrane (Figure 7A-L), followed by the formation of a giga-seal (Sakmann and Neher, 1984) and by the rupture of the membrane patch under the electrode, providing direct access to the cell cytoplasm (Molleman, 2002; Sontheimer and Olsen, 1981). Immediately after rupturing the membrane, the cell's actual resting potential can be estimated. Within minutes of establishing a whole-cell configuration, the intrapipette solution equilibrates with the cell's cytoplasm

[2] By convention, V_m is considered negative when the inside of a cell is more negative than the outside and positive when the inside of a cell is positive relatively to the outside.

and changes the membrane potential to an artificial one, established by the experimenter (Sontheimer and Olsen, 1981). A summary of the procedure described above is represented in Figure 7.

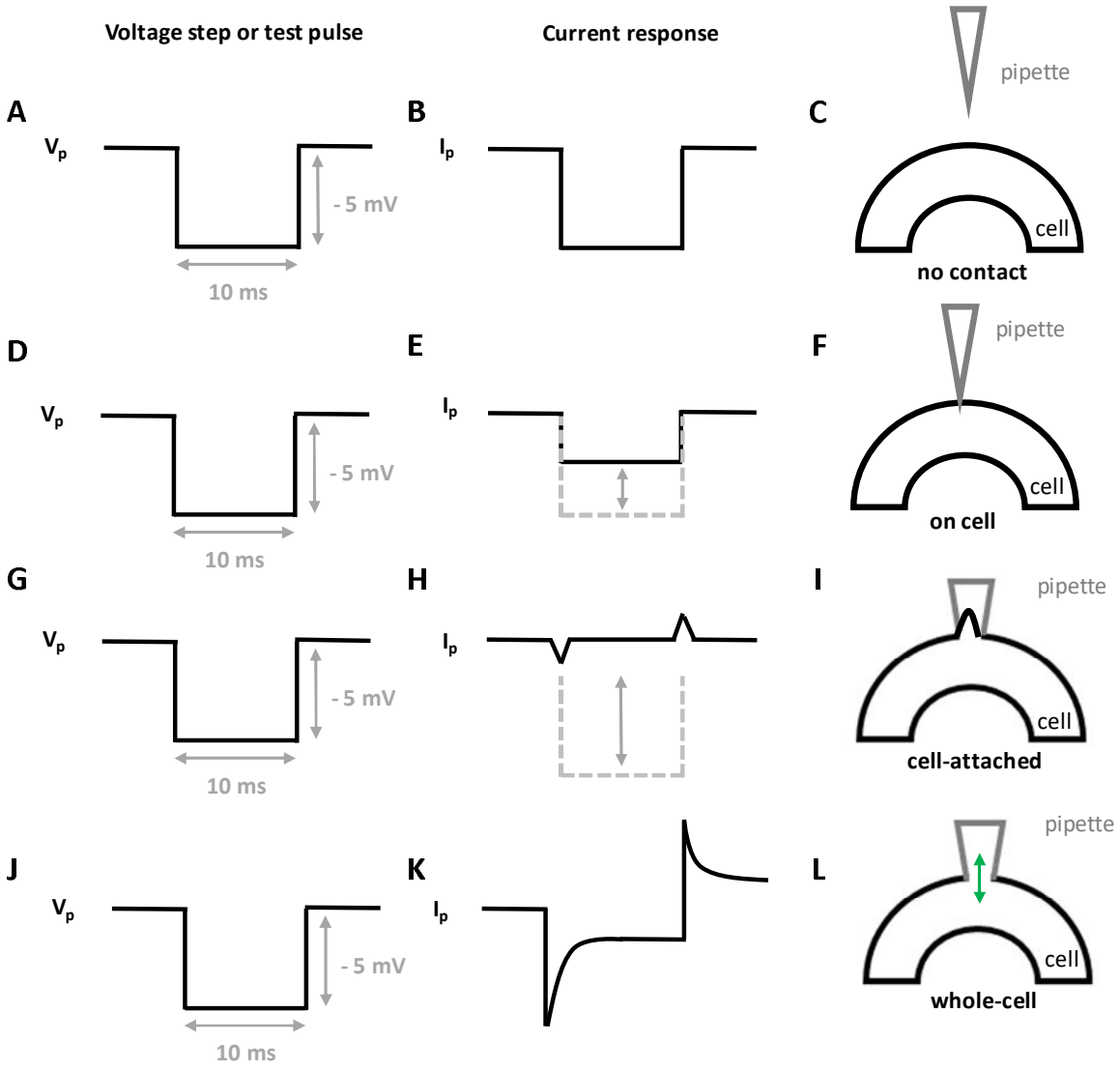


Figure 7. Schematic representation of the beginning of a whole-cell patch-clamp experiment. A-C. Pipette in the bath. In this configuration, the current response (**B**) depends solely on the pipette resistance. **D-F.** Pipette on the cell. When the pipette approaches the cell and touches its membrane, the current response (**E**) decreases, due to the increase in the electrode resistance. **G-I.** Cell-attached mode. When negative pressure is applied, a small portion of the cell membrane is sealed onto the pipette glass and the measured current flows uniquely through that seal, and, thus, its value becomes negligible due to an increase in resistance in the range of GΩ ("giga-ohm seal"). **J-L**. Whole-cell mode. When the experimenter ruptures the cell membrane within the pipette, the pipette's content becomes directly coupled to the cell's cytoplasm. In this configuration, almost all the current flows through the cell membrane and reflects the activity inside the cell, and, therefore, the response

to a voltage test pulse changes. More specifically, the initial jump and the subsequent decay reflect the flow of charge through the pipette and through the membrane.

Other variations besides the whole-cell mode are also used, such as perforated patch, which also records macro-currents, but overcomes the problem of cytosolic components washout; this is carried out by establishing electrical access between the cell and the patch pipette using pore-forming antibiotics, like nystatin or amphotericin B. These pores are only permeable to small monovalent ions, providing a physical barrier to larger molecules (Linley, 2013).

The patch-clamp technique in brain slices is a valuable tool, since it allows the experimenter to assess alterations of neuronal function resulting from dynamic synaptic activity and, thus, this method is particularly important for the study of changes which occur in the brain upon any form of experience, namely learning, acute and chronic stress or even in pathological conditions (Segev *et al.*, 2016). Moreover, this electrophysiological approach also allows studying the impact of cell receptors on information processed in neuronal networks, by application of known receptors agonist and antagonists, which may also be tested for their efficacy as potential therapeutic strategies.

3.2.2. Electrophysiology

3.2.2.1. Acute brain slices preparation

In any patch-clamp recording in brain slices, the main goal is to best preserve the neurons located both superficially and deeply within the slice. Therefore, we had to optimize the ideal experimental conditions to obtain healthy slices, with good-quality cells. Since we used groups of animals with different ages, the process of slice preparation was also distinct, especially because adult brain tissue is far more prone to be damaged during the slicing procedure than the juvenile animal brain. It is important to outline that the procedure also depends on the brain region being studied. First of all, to reduce damage to the tissue and to prevent oxygen deprivation, it is particularly important to remove the brain as rapidly as possible by immersing the decapitated head in ice-cold saline solution. Secondly, the total time of the slicing procedure should be fast and ideally take less than 15 minutes. Finally, one of the most important strategies to diminish the possible insults to the adult brain during slicing is to use specific solutions, which are usually modified formulations of the commonly used artificial cerebral spinal fluid (aCSF). The composition of aCSF is critical for an adequate control of the extracellular environment during *ex vivo* recordings, which affects the activity of the neurons and their properties, such as their firing rate and resting membrane potential (An *et al.*, 2008). The regular aCSF usually contains a high concentration of NaCl and its typical composition, which is used in our laboratory, is shown in Table 1.

Table 1. Composition of the resting and recording aCSF

Compound	Concentration (mM)
NaCl	124
KCl	3
NaH_2PO_4	1.25
$NaHCO_3$	26
glucose	10
$MgSO_4$	1
$CaCl_2$	2
Osmolarity: ± 300 mOsm pH 7.4	

For juvenile animals, the composition of the aCSF was slightly changed for the dissection and slicing steps, in which the concentrations of glucose and Mg^{2+} were increased to 25 and 3 mM, respectively, thereby increasing neuroprotection, as well as reducing excitability and burst firing (An *et al*., 2008; Fonseca, 2013; Wang *et al*., 2004).

For adult animal models of depressive-like behavior we used a methodology adapted from Ting and colleagues, who optimized a procedure which allows the preparation of healthy brain slices from mature animals, with a higher degree of neuronal preservation when compared to other described methods (Ting *et al*., 2018). A *N*-methyl-*D*-glucamine (NMDG)-based solution (Table 2) was used for the brain dissection, preparation of slices and their recovery. Also, in those experiments, the animals were perfused transcardially with ice-cold NMDG-based solution prior to decapitation. NMDG acts as a better alternative to NaCl, sucrose or choline, due to the fact that glutamine is an essential aminoacid in the cerebral spinal fluid, which acts as precursor of glutamate and GABA synthesis and also serves as an energy substrate (An *et al*., 2008). Among the other components of the solution, some are particularly important: HEPES is added in order to provide increased pH buffering and, thus, prevent excessive edema; a high Mg^{2+}/Ca^{2+} ratio diminishes neural excitability; pyruvate ensures an additional energy supply and ascorbate and thiourea act as antioxidants (Ting *et al*., 2018).

Table 2. Composition of the NMDG-based solution

NMDG	
Compound	**Concentration (mM)**
NMDG	92
KCl	2.5
NaH_2PO_4	1.25
$NaHCO_3$	30
glucose	25
$MgSO_4$	2
$CaCl_2$	2
HEPES	20
thiourea	2
ascorbate	5
pyruvate	3
Osmolarity: ± 300 mOsm pH 7.4	

The optimal slice orientation is critical to preserve the contacts between neurons within the slices. This cutting orientation varies according to the neurons and brain region being studied and the individual experimental goal. We used both coronal and horizontal amygdala slices in our experiments and the recordings were performed in BLA pyramidal neurons upon stimulation of the LA (Figure 8). The majority of studies described in the literature use coronal amygdala slices to perform the experiments; however, the use of horizontal amygdala slices was described by Drephal and his colleagues, who were able to induce LTP in the LA by high-frequency stimulation of the external capsule and of intranuclear connections, in the absence of GABA receptors antagonists, which is more difficult to achieve in coronal slices. In the horizontal orientation most of the connections between the amygdala, the hippocampus, the entorhinal and perirhinal cortex are preserved (Drephal *et al*., 2006), while in coronal slices the amygdala connects with the external capsule, *stria terminalis*, perirhinal cortex, the hippocampus and the intercalated nuclei (Sah *et al*., 2003). Therefore, the preferred slices orientation is selected according to the researcher experimental hypothesis.

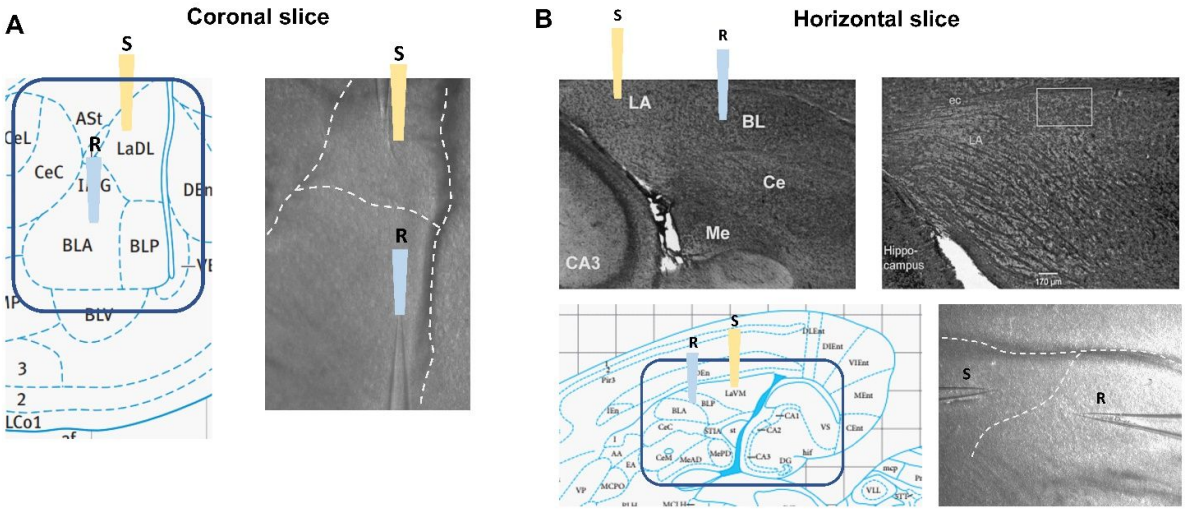


Figure 8. Schematic representation of amygdala coronal (A) and horizontal (B) slices. A. Images of coronal amygdala slices with the stimulus electrode placed in the LA (S) and the recording electrode positioned in the BLA (R). **B.** Images of horizontal amygdala slices with the stimulus electrode placed in the LA (S) and the recording electrode positioned in the BLA (R). (Adapted from Paxinos and Watson, 2007; Von Bohlen Und Halbach and Albrecht, 1998). The stimulation electrode was positioned in the LA, because it is the major source of intra-amygdaloid projections to the BLA (Krettek and Price, 1978).

3.2.2.2. Excitatory synaptic transmission in adolescent animals

Excitatory postsynaptic currents (EPSCs) were evoked, using a patch pipette filled with aCSF positioned in the LA. Whole-cell recordings were made at room temperature from visually identified BLA pyramidal neurons with borosilicate glass capillaries with pipette resistance which ranged from 3 MΩ to 6 MΩ, with an internal solution containing (in mM): 140 $CsMeSO_3$, 4 NaCl, 10 HEPES, 0.2 EGTA, 2 Na_2ATP, 0.3 NaGTP, 5 phosphocreatine, 2 $MgCl_2$, 5 QX-314 chloride, pH adjusted to 7.2 with CsOH. In these experiments, cesium was used as the major component of the internal solution, since it reduces the cell membrane's potassium currents and a better uniformity of the space-clamp is obtained. The bigger size of the cesium ion does not allow it to pass through the pore, as it would for potassium. Due to this, once the whole cell patching is achieved, there is an increasing blockage of potassium channels which will cause the cell to depolarize. Traditionally, potassium-based intracellular solutions are used to record the firing pattern and to measure synaptic activity, but cesium-based intracellular solutions are preferred by several experimenters for the latter as, by blocking K^+ channels, it improves space clamp and allows more stable recording at a more depolarized membrane potential.

The stimulus intensity was set to yield EPSC amplitudes from 50 pA to 200 pA. To record evoked NMDA-EPSC, neurons were voltage-clamped at +40 mV holding potential and isolated with 20 μM CNQX, an AMPA receptor antagonist. All EPSCs were recorded in the BLA in the presence of 10 μM (+)-bicuculline, a $GABA_A$ receptor antagonist, to suppress $GABA_A$ receptor-mediated currents.

Both series and input resistance were monitored throughout the experiment by delivering 5 mV voltage steps. If the series resistance changed in more than 20% during the course of an experiment, the data were discarded.

3.2.3. Animal models of depression - repeated restraint stress

In order to study the role of stress in depression, several animal models have been developed, allowing researchers to evaluate neurobiological features of this disease. In the present work we used repeated restraint stress to induce depressive-like behavior in Wistar Han rats.

3.2.3.1. Adolescent animals

Adolescent male Wistar rats (bred at CNC animal house) were used and they were housed 2 or 3 per cage with *ad libitum* access to food and water and maintained on a 12 h light/dark cycle (light cycle from 7:00 a.m. to 7:00 p.m.). They were habituated to and handled in the facility before starting the restraint or control handling protocol, which began at PND 28 (the approximate body weight was of

95 g) and included the subsequent 9 days. Adolescent rats were used at PND 37 for the elevated-plus maze (EPM) and electrophysiology, PND 38 for the open field (OF) and PND 39 and 40 for the fear conditioning with an approximate body weight of 190 g on the day of fear conditioning. The animals' weight was monitored daily since PND 27 until the day they were sacrificed.

A 7-day repeated restraint protocol was adapted to model the effects of chronic stress (Zhang and Rosenkranz, 2013; Zhang *et al.*, 2014). This protocol is one of the few found in the literature which is applied to adolescent animals and which has been validated by the authors to induce changes in amygdala neurons. This stress paradigm was used in order to avoid habituation to the restraint stress between sessions, which frequently occurs when the stress pattern is the same for a long period of time (Jean Kant *et al.*, 1985; Melia *et al.*, 1994; Zhang and Rosenkranz, 2013). Also, it has already been reported that a 7-day immobilization stress protocol is sufficient to cause an imbalance in the $A_1R/A_{2A}R$ ratio, in which the A_1R density is diminished, while $A_{2A}R$ are upregulated (Cunha *et al.*, 2006). The animals were randomly assigned into the control and the stressed (repeated restraint) groups. The rats in the stressed group were placed into a disposable decapicone immobilization bag for 20 min, once a day, for 7 out of 9 days, in the procedure room. Each bag was secured around the tail, and the animals were placed in clean cages for the duration of the stress. The rats in the control group were maintained in their home cages, but without access to food and water for 20 min, once a day, for 7 out of 9 days (Figure 9). All the procedures were performed between 9:00 a.m. and 5:00 p.m., during the light phase of the light/dark cycle.

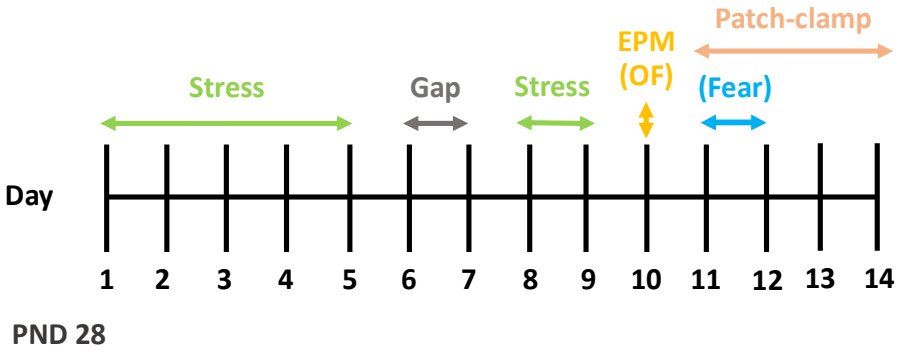


Figure 9. Schematic representation of the experimental design. The rats were exposed to restraint stress or control handling for 5 days, followed by 2 days with minimal manipulation, and other 2 days of restraint stress or control handling. The behavioral tests were performed on the following two days after the last restraint or control handling session. The animals that were not used for electrophysiology experiments were subjected to fear conditioning, which was performed on the next day after the last behavioral test. The control rats that did

not exhibit an anxiety-like phenotype and the stressed rats that exhibited anxiety-like behavior in the EPM were used for further electrophysiology experiments.

3.2.3.2. Adults animals

Chronic restraint stress is known to induce anxiety-like behavior, as well as cognitive impairment, in adult male Wistar rats, and to cause dendritic hypertrophy of the amygdala neurons (Guedri *et al*., 2017; Suvrathan *et al*., 2014; Vyas *et al*., 2002). It is based on imposed physical immobility, which is a purely psychological stressor, mostly prevalent in humans (Hammen, 2005), and does not involve pain or physical exhaustion of the animals. We chose to adapt the protocol to increase its severity, and young adults Wistar male rats (Charles River) (8 weeks old) were placed in Decapicone immobilization bags (Braintree Scientific) for 4 h every day, for 15 days (9 a.m. – 5 p.m.). Each bag was secured around the tail and the animals were placed in clean cages for the duration of the stress. The unstressed animals were handled daily (Figure 10).

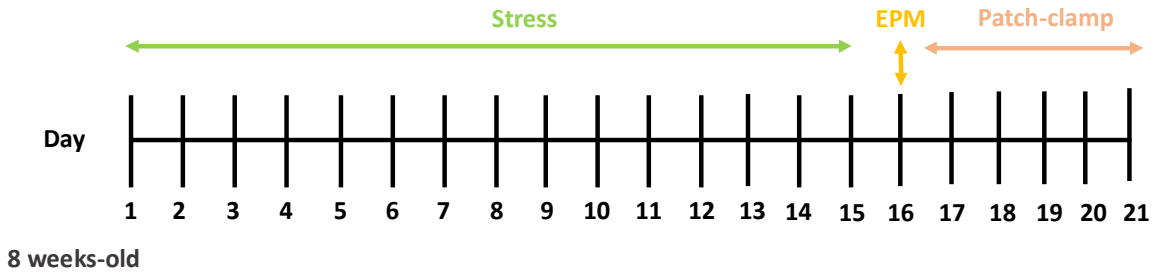


Figure 10. **A. Schematic representation of the experimental design.** The rats were exposed to restraint stress or control handling for 15 days, for 4 hours a day. The behavioral tests were performed on the following day after the last restraint or control handling session. The control rats which did not exhibit an anxiety-like phenotype and the stressed rats which exhibited anxiety-like behavior in the EPM were used for further electrophysiology experiments.

3.2.4. Behavioral tests

All the behavioral tests were performed between 10 a.m. and 6 p.m., under dim red light (3-12 lux). The analysis was performed using an automated tracking system based on video feed (ANY-maze v.6 software, Stoelting, Ireland, paired with a Logitech webcam). After each trial, the apparatus was cleaned with 70% ethanol solution and dried with paper wipes to remove and disperse olfactory, tactile and visual clues.

3.2.4.1. Elevated plus maze (EPM)

The EPM is commonly used to evaluate anxiety-like behavior in rodents and it is based on their natural curiosity to explore new environments, as well as on their innate fear of open spaces (Montgomery, 1955; Pellow *et al*., 1985; Sousa *et al*., 2006). The test is easy to perform, since it does not involve prior training or the use of aversive stimuli, and the apparatus consists of two closed arms perpendicular to two open arms, elevated from the ground. The rats were placed in the center of the four arms, facing the open arm opposite to the experimenter, and were, therefore, confronted with an avoidance-approach conflict, in which they had to choose between staying protected in the enclosed arms or investigate novel, but unprotected open arms (Rodgers and Dalvi, 1997). The level of anxiety was inversely proportional to the time spent in the open arms, based on the observation made by Montgomery, who showed that the avoidance of open spaces is related to increased fear and stress level (Montgomery, 1955).

The animals were tested in the EPM one day after the final restraint/control handling session. The behavior of the animals was recorded for 5 min and analyzed using a personal computer running a video-tracking software (Any-Maze). Several parameters were calculated: the time spent on open arms and the number of entries were used as an index of anxiety-like behavior, the travelled distance was utilized as an indicator of locomotor activity and the number of head dips, rears and stretch-attend posture were used as measures of risk assessment (Walf and Frye, 2007).

3.2.4.2. Open field (OF)

The open-field test is an arena initially designed by Calvin Hall to assess the level of rodents' timidity based on the number of fecal pellets they had left during the test (Hall, 1934). Since then, with a few variations, it has become extremely popular in studies of animal behavior, as not only is the apparatus very simple, but also because the experimenter is able to evaluate several parameters, including locomotion and exploratory behavior (Walsh and Cummins, 1976). The open field arena is usually divided into virtual zones, such as the center and the borders, and the animals which spend more time in the inner space are commonly considered to be less anxious (Sousa *et al*., 2006).

The adolescent animals were placed in the center of an open field arena (width × length × wall height: 50 cm x 50 cm x 1 m), which they were allowed to explore for the duration of the test. All the tests lasted for 30 min. Locomotion was assessed by measuring the total distance travelled, and the anxiety-like behavior was measured considering the time spent, as well as the distance travelled in the center (as assessed using the ANY-maze software).

3.2.5. Fear conditioning

Fear conditioning tests are usually used to study the acquisition, consolidation and extinction of aversive memories and, since the amygdala is considered to be the main locus for these processes to occur (LeDoux, 2000), it is possible to validate whether a stress paradigm affects this region by performing fear conditioning.

Fear conditioning was performed on adolescent animals in order to validate the involvement of the amygdala in the repeated restraint stress protocol and consisted of a two-day procedure, in an attempt to reproduce the results described by Zhang and his colleagues (Zhang and Rosenkranz, 2013). Conditioning and testing were conducted in the same chamber, but using distinct contexts (wall pattern, color and texture, odors and flooring) to minimize contextual freezing. The chamber was enclosed by a sound-attenuating cabinet. Mounted inside the cabinet were a source of light, as well as an audio speaker and a Logitech webcam, both connected to personal computers running the sound and video-tracking software (Any-Maze). Conditioning was performed on the first day and consisted of 2 min of habituation, followed by 5 pairings of a neutral tone (10 s, 1500 Hz, 85 dB) with a footshock (1 s, 0.4 mA) which co-terminated with the tone. Conditioning trials were presented at 60 s intertrial intervals. The rats remained in the chamber for 1 min after the end of the last conditioning trial and were then returned to their home cage. The next day, conditioned freezing and its within session extinction were tested in a contextually distinct chamber. The testing consisted of a 2 min habituation, followed by 15 trials of tone presentation (20 s, 1500 Hz, 85 dB) at a 60 s intertrial interval. No footshock was presented during testing trials. After testing, the animals were returned to their home

cage. After each trial, the apparatus was cleaned with 1% acetic acid solution instead of 70% ethanol solution to provide a new olfactory context. Freezing was defined as behavioral immobility, for at least 2 s, except for movement associated with respiration, and was quantified manually. Total freezing during each trial (entire 60 s) was used as an index of conditioned fear and converted to a percentage ([time of freezing/60 s] × 100) for analysis.

3.2.6. Adrenal glands weight assessment

One of the main systems activated upon a stressor is the hypothalamus-pituitary-adrenal axis (HPA), which readily acts by coordinating the activity of different brain structures to generate behavioral, emotional and hormonal responses to the perceived aversive inputs (Lucassen *et al*., 2014). The paraventricular nucleus of the hypothalamus is responsible for the release of the corticotropin-releasing hormone (CRH), the anterior pituitary gland for the release of adrenocorticotropin (ACTH) and adrenal glands for the release of glucocorticoids (corticosterone in rodents and cortisol in human beings) (Sousa, 2016). Since adrenal glands are altered upon stress and usually increase in size (Hernandez *et al*., 2013), the assessment of their weight is a valuable measure of the effectiveness of the used stress paradigm.

The monitoring of the adrenal glands weight was performed only in adolescent animals, which were anesthetized with halothane before sacrifice. Their adrenal glands were excised and weighed while wet. Adrenal gland weight was normalized to body weight (mg/g of body weight).

3.2.7. Synaptic transmission in animal models of depression

3.2.7.1. Adolescent animals

It has been largely reported that amygdala glutamatergic synaptic transmission is altered upon different stress paradigms, including changes in the firing rate of the neurons, NMDA to AMPA ratio and in the frequency and amplitude of spontaneous neuronal activity (Duman and Aghajanian, 2012; Rau *et al*., 2015; Suvrathan *et al*., 2014; Zhang and Rosenkranz, 2012)

In order to assess whether the excitatory synaptic transmission was altered in these animals, several parameters were evaluated.

- *Firing patterns:* Firing patterns of the cells were obtained in current-clamp mode, immediately after achieving the whole-cell configuration, through the injection of a series of hyperpolarizing and depolarizing steps, in order to evaluate the spontaneous firing rate of the BLA pyramidal neurons (Figure 2). These recordings were made at room temperature from visually identified

BLA pyramidal neurons with borosilicate glass capillaries with pipette resistance ranging from 3 MΩ to 6 MΩ, with an internal solution containing (in mM): 125 K-gluconate, 11 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 2 Na_2ATP, 0.3 NaGTP, 10 phosphocreatine. The osmolarity of the solution above was adjusted to 290 ± 10 mOsm.

The following recordings were made with borosilicate glass capillaries with pipette resistance ranging from 3 MΩ to 6 MΩ, with an internal solution containing (in mM): 140 $CsMeSO_3$, 4 NaCl, 10 HEPES, 0.2 EGTA, 2 Na_2ATP, 0.3 NaGTP, 5 phosphocreatine, 2 $MgCl_2$, 5 QX-314 chloride, pH adjusted to 7.2 with CsOH, osmolarity 290 ± 10 mOsm.

- *Spontaneous excitatory postsynaptic currents (sEPSC)*: sEPSC were recorded at room temperature from visually identified BLA pyramidal neurons, which were voltage-clamped at -60 mV holding potential. The events were recorded with a sampling rate of 5 kHz.

- *Evoked NMDA to AMPA ratio*: NMDA to AMPA ratio is used to assess the predisposition for synaptic potentiation (Kauer and Malenka, 2007). Excitatory postsynaptic currents (EPSCs) were evoked using a patch pipette filled with aCSF positioned in the LA and whole-cell recordings were made at room temperature from visually identified BLA pyramidal neurons, which were voltage-clamped at -60 mV to record AMPAR-mediated currents and at +40 mV holding potential, in the presence of CNQX (20 μM), to isolate NMDAR-mediated currents.

In all of the above experiments, both series and input resistance were monitored throughout the experiment by delivering 5 mV voltage steps. If the series resistance changed in more than 20% during the course of an experiment, the data were discarded.

3.2.7.2. Adult animals

Besides the reported changes in amygdala excitatory synaptic transmission, inhibitory connections have also been proved to be affected upon stress (Jie *et al*., 2018; Masneuf *et al*., 2014; Wang *et al*., 2016). In our adult animal model of depression, we evaluated whether both excitatory and inhibitory transmission were affected, and several measurements were performed.

The following recordings were made with borosilicate glass capillaries with pipette resistance ranged from 3 MΩ to 6 MΩ, with an internal solution containing (in mM): 140 $CsMeSO_3$, 4 NaCl, 10 HEPES, 0.2 EGTA, 2 Na_2ATP, 0.3 NaGTP, 5 phosphocreatine, 2 $MgCl_2$, 5 QX-314 chloride, pH adjusted to 7.2 with CsOH, osmolarity 290 ± 10 mOsm.
Evoked NMDA to AMPA ratio was recorded as described above.

- *Evoked excitatory postsynaptic currents (eEPSC)*: EPSC were evoked using a bipolar electrode placed in the LA and were recorded at room temperature from visually identified BLA pyramidal neurons, which were voltage-clamped at -60 mV holding potential.

- *Evoked inhibitory postsynaptic currents (eIPSC*): IPSC were evoked using a bipolar electrode placed in the LA and were recorded at room temperature from visually identified BLA pyramidal neurons, which were voltage-clamped at 0 mV holding potential.

- *Input-output curves (I/O):* for both eEPSC and eIPSC, I/O curves, which are markers for synaptic strength, were recorded. eEPSC and eIPSC were recorded as described above, but in response to an increasing stimulus intensity. These responses represent the increase in the number of active excitatory and inhibitory synapses.

Both eEPSC and eIPSC were recorded at the same neuron, by adjusting the holding potential from -60 mV to 0 mV, respectively.

3.2.8. Drugs

Unless otherwise stated, drugs were added via the superfusion solution, at a superfusion speed of 2 mL/min, and their final concentration diluted from concentrated stocks. Stock solutions of bicuculline, CGS21680, CPA, DPCPX and SCH58261 were prepared in dimethylsulfoxide (Sigma) and dilutions were prepared in ACSF, controlling for the impact of the residual amount of dimethylsulfoxide. The complete list of drugs used in this study can be viewed in Table 4.

Table 4. List of pharmacological tools used in the electrophysiology experiments.

DRUG	SUPPLIER	BIOLOGICAL ACTIVITY	FINAL CONCENTRATION
Bicuculline	Tocris	$GABA_AR$ antagonist	1 µM; 10 µM
CGS21680	Tocris	$A_{2A}R$ agonist	30 nM
CNQX	Tocris	AMPA/ kainate receptors antagonist	20 µM
CPA	Tocris	A_1R agonist	30 nM
D-AP5	Tocris	NMDAR antagonist	50 µM
DPCPX	Tocris	A_1R antagonist	100 nM
Regadenoson	-	$A_{2A}R$ agonist	300 nM
SCH58261	Tocris	$A_{2A}R$ antagonist	50 nM
QX 314-chloride	Tocris	Na^+ channel blocker	5 mM

3.2.9. Data treatment and statistical analysis

All electrophysiological data were analyzed with Clampfit 10.7 software (Molecular Devices). All statistical analyses were performed with Prism6 GraphPad Software. Values are presented as mean ± S.E.M. in the text and figure legends. Unpaired Student's and paired Student's *t*-tests, multiple *t*-tests, a one-sample Student's *t* test compared with a hypothetical value of 100, an one-way ANOVA or two-way repeated measures ANOVA were performed. Statistical differences were considered significant at $P < 0.05$.

"Alice: This is impossible.
The Mad Hatter: Only if you believe it is."

Lewis Carroll, in *Alice in the Wonderland*

4. RESULTS AND DISCUSSION

4.1. Impact of A_1R and $A_{2A}R$ on NDMA receptor-mediated excitatory synaptic transmission in the intra-amygdalar circuit

The basolateral complex of the amygdala integrates sensory information perceived by cortical and subcortical areas, mostly through the LA; the projections which originate and terminate in the LA are mainly excitatory (Sah *et al*., 2003; Savander *et al*., 1997) and the majority of neurons in the BLA are pyramidal-like and glutamatergic. The LA receives major inputs from the sensory thalamus and cortex and this sensory information is routed through intra-amygdalar connections to the BLA. The BLA, in its turn projects this information almost unidirectionally to the CeA and ITC (Capogna, 2014; LeDoux, 2007; Pitkänen *et al*, 1997; Pitkänen *et al*., 1998). The amygdala is deeply involved in processes related to anxiety and fear memory, in which glutamatergic transmission is known to be dysregulated (Rau *et al*., 2014). For instance, chronic stress induces changes in glutamatergic transmission in the rat amygdala through alterations in AMPA (Yi *et al*., 2017; Zhou *et al*., 2018) and NMDA receptors (Zhou *et al*., 2018).

Since one of the possible strategies to normalize pathological modifications is to modulate synaptic transmission, adenosine emerges as a prime choice due to its role as a neuromodulator of glutamatergic synaptic transmission throughout the brain (Dunwiddie and Masino, 2001). However, there is scarce information on its synaptic actions within the BLA: it is known that A_1R activation inhibits spontaneous and evoked AMPA-mediated synaptic transmission (Rau *et al*., 2014) and $A_{2A}R$ increase the firing rate of pyramidal neurons (Rau *et al*., 2015). Furthermore, it has been shown, in extracellular recordings, that $A_{2A}R$ facilitate the induction of LTP in the LA, through stimulation of cortical afferents, and their silencing diminishes the expression of fear memory (Simões *et al*., 2016). On the other hand, systemic activation of A_1R impairs the acquisition and expression of fear (Corodimas and Tomita, 2001). It is also well described that fear memory depends significantly on NMDA receptors in the LA and BLA (Sotres-Bayon *et al*., 2007) and, since both A_1R and $A_{2A}R$ are known to directly affect synaptic NMDA receptor function in the hippocampus (de Mendonça *et al*., 1995; Dias *et al*., 2012; Mouro *et al*., 2018; Rebola *et al*., 2008; Temido-Ferreira *et al*., 2018), the aim of this part of the work was to assess whether A_1R and $A_{2A}R$ are involved in the control of NMDA receptors in BLA pyramidal neurons, in LA to BLA synapses.

4.1.1. A_1R inhibit evoked NMDA-EPSC in BLA pyramidal neurons

We first tested the effects of the A_1R selective agonist CPA (30 nM) and the A_1R selective antagonist DPCPX (100 nM) on NMDA receptor-mediated EPSCs (NMDA-EPSC) in BLA pyramidal neurons clamped at +40 mV upon stimulation of the LA (Figure 11A-B).

The blockade of A_1R with DPCPX (100 nM) did not alter the amplitude of NMDA-EPSC when compared to the baseline (108.40 ± 12.67% of baseline; n=11; p=0.5237; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 11C-E), suggesting that there is no tonic control of NMDA receptor-mediated currents by A_1R in amygdala slices.

The exogenous activation of A_1R with CPA (30 nM) significantly decreased the amplitude of NMDA-EPSC when compared to the baseline (47.93 ± 6.38 % of baseline; n=10; p<0.0001; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 11F-H) in all recorded cells. There was no effect of CPA in the presence of DPCPX (100 nM) (76.70 ± 9.46 % of DPCPX-baseline; n=5; p=0.0695; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 11I-K).

Our results show that A_1R inhibit evoked NMDA receptors-mediated responses in BLA pyramidal neurons, an effect which has been described for hippocampal NMDA currents in isolated rat neurons (de Mendonça *et al*., 1995).

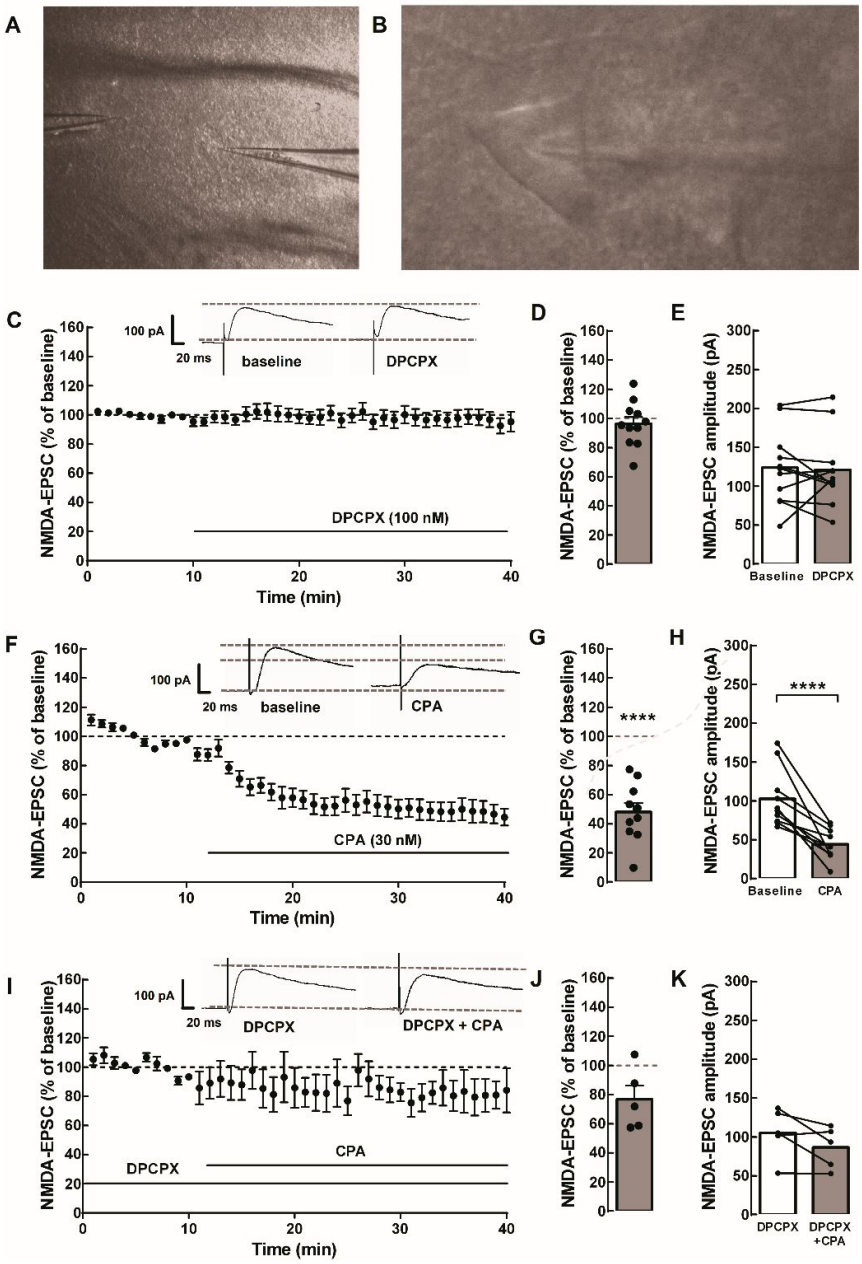


Figure 11. A_1R inhibit evoked NMDA-EPSCs in BLA pyramidal neurons in basal conditions. A. Photograph of a horizontal amygdala slice with the stimulus electrode placed in the LA (S) and the recording electrode positioned in the BLA (R). **B.** Photograph of a BLA principal neuron being patched. **C.** Time course of the effect mediated by the selective A_1R antagonist DPCPX (100 nM) on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of DPCPX (100 nM) are shown in the inset. **D.** Dispersion of the impact of DPCPX (100 nM) during the last 10 minutes of the application of DPCPX (100 nM). **E.** Amplitude (pA) of the evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of DPCPX (100 nM). **F.** Time course of the effect mediated by the selective A_1R agonist CPA (30 nM) on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of CPA (30 nM) are shown in

the inset. **G.** Dispersion of the effect of CPA (30 nM) during the last 10 minutes of the application of DPCPX (100 nM)**. H.** Amplitude (pA) of the evoked NMDA-EPSC during baseline and during the last 10 minutes of CPA (30 nM). **I.** Time course of the effect mediated by the selective A_1R agonist CPA (30 nM) in the presence of the selective A_1R antagonist DPCPX (100 nM) on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC during application of DPCPX (100 nM) and during the last 10 minutes of the simultaneous application of DPCPX (100 nM) and CPA (30 nM) are shown in the inset. **J.** Dispersion of the effect of the simultaneous application of the DPCPX (100 nM) and CPA (30 nM) during the last 10 minutes of the application of DPCPX (100 nM). **K.** Amplitude (pA) of the evoked NMDA-EPSC during application of DPCPX (100 nM) and during the last 10 minutes of the simultaneous application of DPCPX (100 nM) and CPA (30 nM). **** $p<0.0001$ using a one-sample *t*-test with a hypothetical value of 100 for comparison (**G**) and a paired Student's t-test (**H**).

4.1.2. $A_{2A}R$ do not control evoked NMDA-EPSC in BLA pyramidal neurons

We focused on the effects of the $A_{2A}R$ selective agonist CGS21680 (30 nM) and the $A_{2A}R$ selective antagonist SCH58261 (50 nM) on NMDAR-mediated EPSCs (NMDA-EPSC) in BLA pyramidal neurons clamped at +40 mV upon stimulation of the LA (Figure 11A-B).

The selective $A_{2A}R$ antagonist SCH58261 (50 nM) did not affect the amplitude of NMDA-EPSC when compared to the baseline (95.83 ± 8.57 % of baseline; n=13, p=0.6356 one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 12A-C), implying that synaptically evoked NMDA currents in the LA-BLA pathway are not tonically modulated by $A_{2A}R$.

Surprisingly, the selective $A_{2A}R$ agonist CGS21680 (30 nM) significantly decreased the amplitude of evoked NMDA-EPSC when compared to the baseline (86.36 ± 3.98% of baseline, n=12, p=0.0056; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 12D-F).

The CGS21680-induced decrease of NMDA-EPSC was still observed in the presence of the selective $A_{2A}R$ antagonist, SCH58261 (50 nM), as in the last 10 min of the recordings, the decrease in the amplitude of NMDA-EPSC was still present (81.74 ± 7.42 % of SCH-baseline, n=9, p=0.0394; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 12G-I).

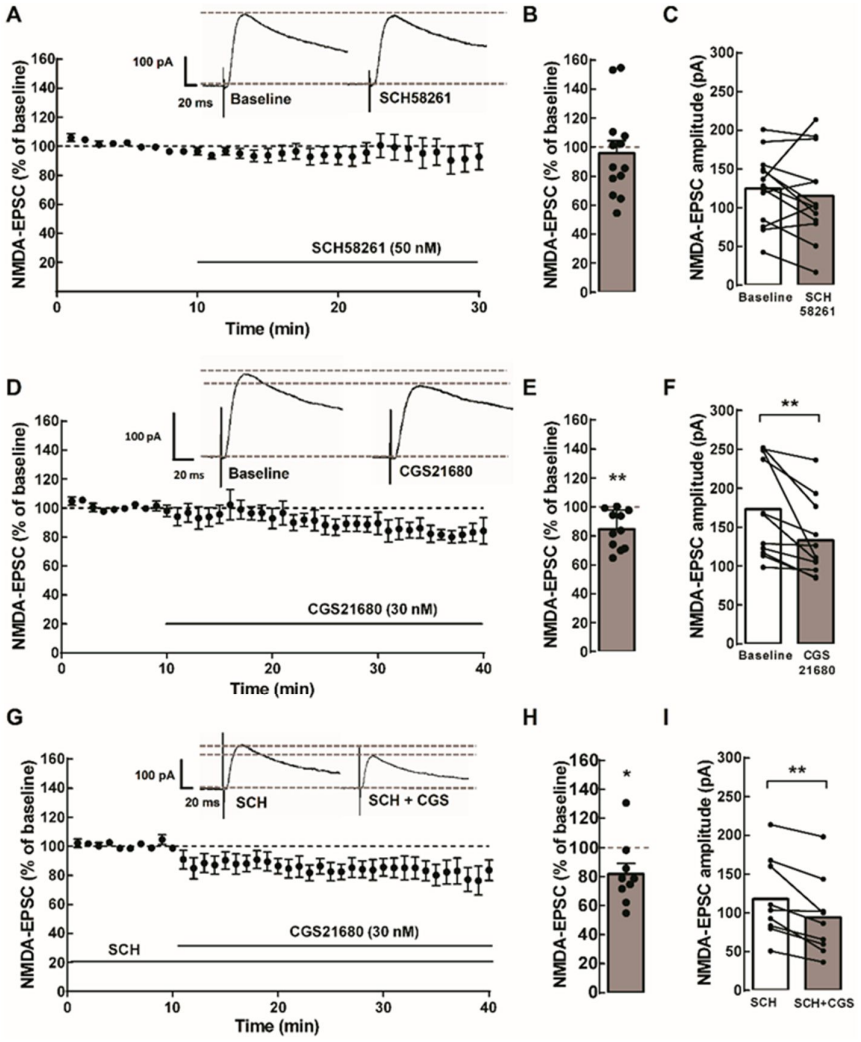


Figure 12. Effect of $A_{2A}R$ on the amplitude of evoked NMDA-EPSCs in BLA pyramidal neurons upon stimulation of the LA. A. Time course of the effect of the selective $A_{2A}R$ antagonist, SCH58261 (50 nM), on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of SCH58261 (50 nM) are shown in the inset. **B.** Dispersion of the effect of SCH58261 (50 nM) during the last 10 minutes of the application of DPCPX (100 nM). **C.** Amplitude (pA) of the evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of SCH58261 (50 nM). **D.** Time course of the decrease mediated by the selective $A_{2A}R$ agonist, CGS21680 (30 nM), on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC illustrating the decrease of the amplitude of the currents with the application of CGS21680 (30 nM) are shown in the inset. **E.** Dispersion of the effect of CGS21680 (30 nM) during the last 10 minutes of the application of DPCPX (100 nM). **F.** Amplitude (pA) of the evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of CGS21680 (30 nM). **G.** Time course of the decrease mediated by CGS21680 (30 nM) in the presence of SCH58261 (50 nM) on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC illustrating the decrease mediated by CGS21680 (30 nM) in the presence of SCH58261 (50 nM) are shown in the inset. **H.** Dispersion of the effect

of the simultaneous application of the SCH58261 (50 nM) and CGS21680 (30 nM) during the last 10 minutes of the application of DPCPX (100 nM). **I.** Amplitude (pA) of the evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of CGS21680 (30 nM) in the presence of SCH58261 (50 nM)**.** * $p<0.05$, ** $p<0.01$ using a one-sample *t*-test with a hypothetical value of 100 for comparison (**E**, **H**) and $p<0.01$ using paired Student's *t*-test (**F**, **I**).

We next evaluated if CGS21680 was having a non-selective effect through the activation of the inhibitory A_1R. There was no effect of CGS21680 (30 nM) in the presence of the selective antagonist A_1R DPCPX (100 nM), (89.77 ± 6.01 % of DPCPX-baseline; n=13; p=0.1145; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 13A-C), which *per se* was devoid of effect (Figure 11C-E)

We also tested if the effect of CGS21680 (30 nM) was still observed following exogenous activation of A_1R with the selective agonist CPA (30 nM): indeed, CGS21680 still decreased the amplitude of the NMDA-EPSC (72.62 ± 1.61 % of CPA-baseline; n=4; p=0.0004; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 14D-F), in the presence of CGS21680 (30 nM).

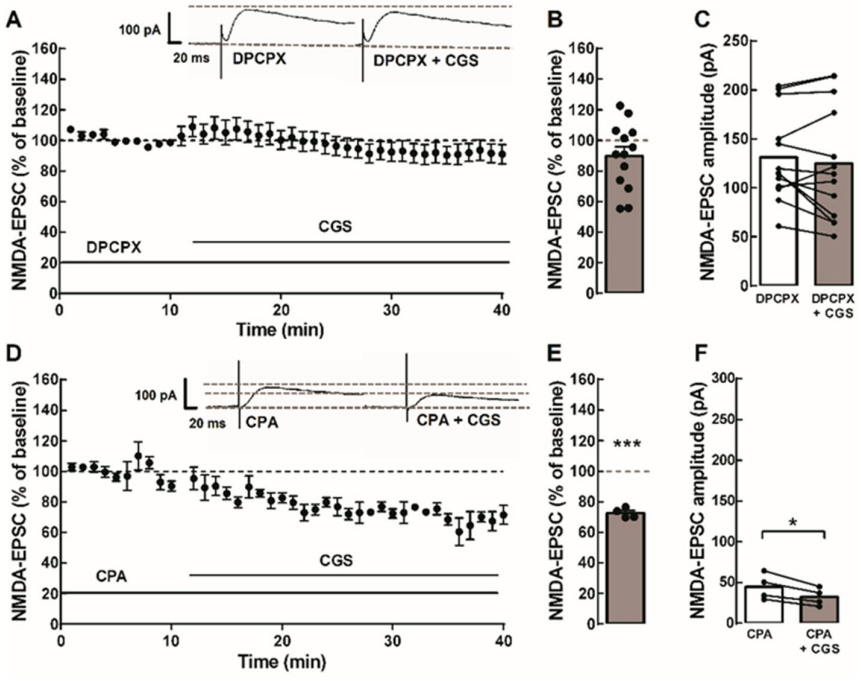


Figure 13. CGS21680-mediated decrease of evoked NMDA-EPSCs in BLA pyramidal neurons is prevented by A_1R blockade, while not occluded by A_1R activation. A. Time course of the prevention of the effect mediated by the selective $A_{2A}R$ agonist, CGS21680 (30 nM), by the selective A_1R antagonist, DPCPX (100 nM), on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC illustrating the prevention

by DPCPX (100 nM) of the decrease mediated by CGS21680 (30 nM) are shown in the inset. **B.** Dispersion of the effect of the simultaneous application of DPCPX (100 nM) and CGS21680 (30 nM) during the last 10 minutes of the application of DPCPX (100 nM). **C.** Amplitude (pA) of the evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of CGS21680 (30 nM) in the presence of DPCPX (100 nM). **D.** Time course of the effect mediated by CGS21680 (30 nM) in the presence of the selective A_1R agonist CPA (30 nM) on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC representing the effect of the simultaneous application of CPA (30 nM) and CGS21680 (30 nM) are shown in the inset. **E.** Dispersion of the effect of the simultaneous application of CPA (30 nM) and CGS21680 (30 nM) during the last 10 minutes of the application of DPCPX (100 nM). **F.** Amplitude (pA) of the evoked NMDA-EPSC during application of CPA (30 nM) and during the last 10 minutes of the simultaneous application of CPA (30 nM) and CGS21680 (30 nM). *** $p<0.001$ using one-sample *t*-test with a hypothetical value of 100 for comparison (**E**) and * $p<0.05$ using paired Student's *t*-test (**F**).

We further tested the effect of another $A_{2A}R$ agonist, regadenoson (CVT-3146). This compound was primarily designed to be used in myocardial perfusion imaging, in order to provide coronary vasodilation with minimal side effects (Palani and Ananthasubramaniam, 2013), but it has also been considered for evaluation in clinical trials for the treatment of other diseases (Jacobson *et al*., 2019). Like CGS21680, regadenoson (300 nM) significantly decreased the amplitude of evoked NMDA-EPSC when compared to the baseline (79.40 ± 8.17 % of baseline, n=7, p=0.00452; n=number of recorded cells; Figure 14A-C). Regadenoson (300 nM) still decreased the amplitude of evoked NMDA-EPSC in the presence of the $A_{2A}R$ antagonist SCH58261 (50 nM) (79.80 ± 4.46 % of baseline, n=5, p=0.0106; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 14D-F).

In contrast to the effect of CGS21680, the decrease mediated by regadenoson (300 nM) was still observed in the presence of the selective A_1R antagonist, DPCPX (100 nM) (79.91±8.085% of DPCPX-baseline; n=9; p=0.0378; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 14G-I).

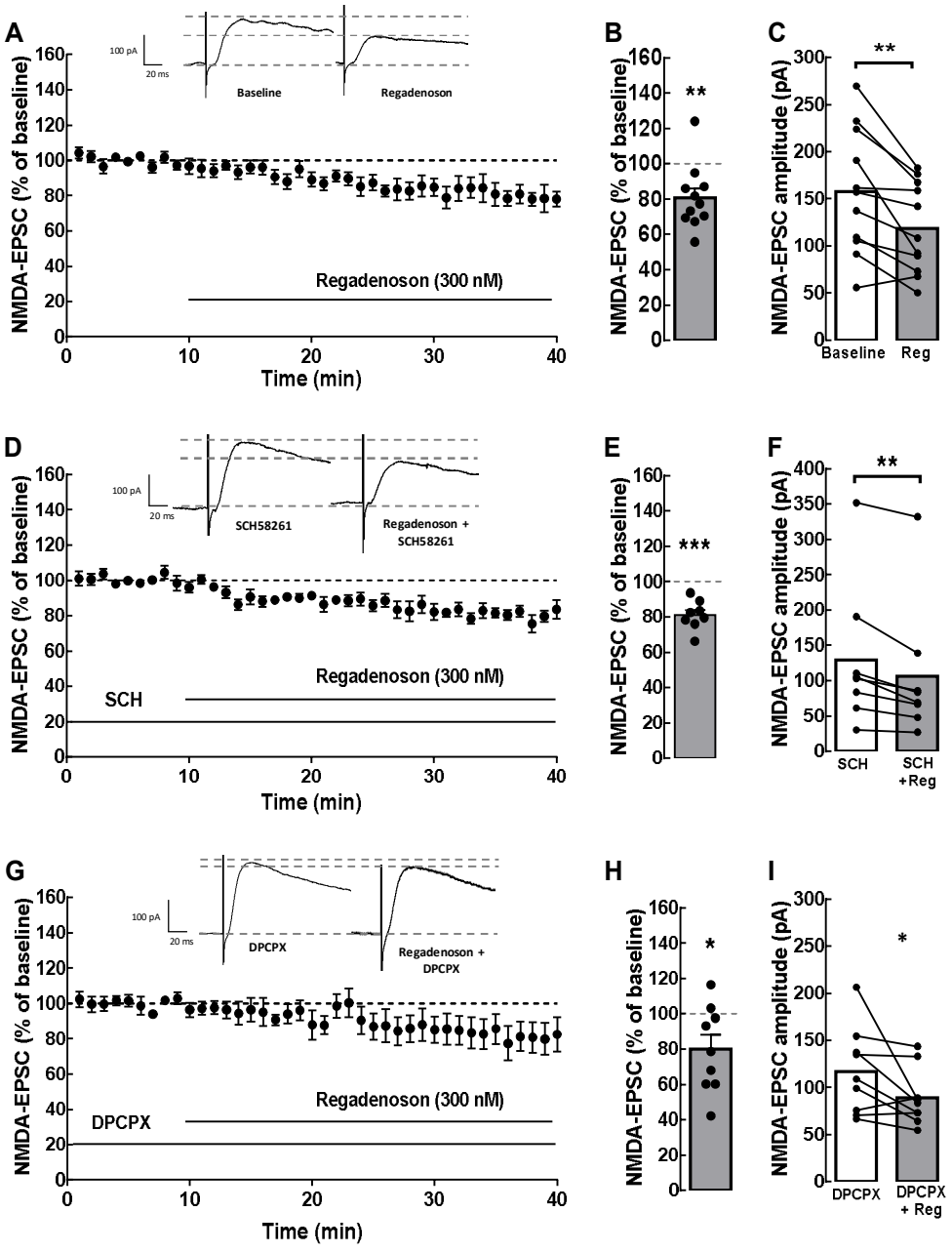


Figure 14. Regadenoson decreases the amplitude of evoked NMDA-EPSCs in BLA pyramidal neurons upon stimulation of the LA A. Time course of the decrease mediated by the $A_{2A}R$ agonist, regadenoson (300 nM), on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC illustrating the decrease of the amplitude of the currents with the application of the $A_{2A}R$ agonist regadenoson (300 nM) are shown in the inset. **B.** Dispersion of the effect of regadenoson (300 nM) during the last 10 minutes of the application of DPCPX (100 nM). **C.** Amplitude (pA) of the evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of regadenoson (300 nM). **D.** Time course of the decrease mediated by regadenoson (300 nM)

in the presence of the selective $A_{2A}R$ antagonist, SCH58261 (50 nM), on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC illustrating the decrease mediated by regadenoson (300 nM) in the presence of SCH58261 (50 nM) are shown in the inset. **E.** Dispersion of the effect of the simultaneous application of SCH58261 (50 nM) and regadenoson (300 nM) during the last 10 minutes of the application of DPCPX (100 nM). **F.** Amplitude (pA) of the evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of regadenoson (300 nM) in the presence of SCH58261 (50 nM). **G.** Time course of the decrease mediated by regadenoson (300 nM) by the selective A_1R antagonist, DPCPX (100 nM), on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC illustrating the decrease mediated by regadenoson (300 nM), in the presence of DPCPX (100 nM), are shown in the inset **H.** Dispersion of the effect of the simultaneous application of DPCPX (100 nM) and regadenoson (300 nM) during the last 10 minutes of the application of DPCPX (100 nM). **I.** Amplitude (pA) of the evoked NMDA-EPSC during baseline and during the last 10 minutes of the application of regadenoson (300 nM), in the presence of DPCPX (100 nM). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, using an one-sample *t*-test with a hypothetical value of 100 for comparison (**B**, **E**, **H**) and ** $p < 0.01$ using paired Student's *t*-test (**C**, **F**).

4.1.3. $A_{2A}R$ are not involved in the A_1R-mediated decrease of evoked NMDA-EPSC in BLA pyramidal neurons

Since A_1R and $A_{2A}R$ are known to interact with each other to control synaptic transmission (Lopes *et al*., 2002; Ferré and Ciruela, 2019), we evaluated if the A_1R-mediated decrease in NMDA-EPSC could be controlled by the $A_{2A}R$. The blockade of $A_{2A}R$ with the selective antagonist, SCH58261 (50 nM), did not change the decrease in NMDA-EPSC mediated by the selective A_1R agonist, CPA (30 nM) (41.02 ± 3.39 % of SCH-baseline; n=5; $p<0.0002$; one-sample *t*-test, when compared to a hypothetical value of 100; n=number of recorded cells; Figure 15A-C)

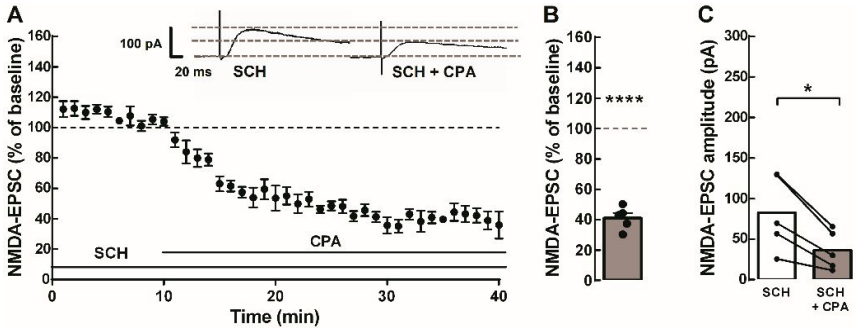


Figure 15. $A_{2A}R$ do not interfere with A_1R-mediated decrease of evoked NMDA-EPSCs in BLA pyramidal neurons. A. Time course of the effect mediated by the selective A_1R agonist CPA (30 nM) in the presence the selective $A_{2A}R$ antagonist SCH58261 (50 nM) of on evoked NMDA-EPSC; representative traces of evoked NMDA-EPSC representing the effect of the simultaneous application of SCH58261 (50 nM) and CPA (30 nM) are shown in the inset. **B.** Dispersion of the effect of the simultaneous application of SCH58261 (50 nM) and CPA (30 nM) during the last 10 minutes of the application of DPCPX (100 nM). **C.** Amplitude (pA) of the evoked NMDA-EPSC during application of SCH58261 (50 nM) and during the last 10 minutes of the simultaneous application of SCH58261 (50 nM) and CPA (30 nM). **** $p<0.0001$ using one-sample *t*-test with a hypothetical value of 100 for comparison (**B**) and * $p<0.05$ using paired Student's *t*-test (**C**).

4.1.4. Discussion

In this part of the work we explored the role of A_1R and $A_{2A}R$ on NMDA receptor-mediated excitatory synaptic transmission, in LA to BLA synapses, in BLA pyramidal neurons.

We observed that A_1R inhibit evoked NMDA-EPSC, similarly to what has already been shown in hippocampal isolated neurons (de Mendonça *et al*., 1995).

Our data also showed that $A_{2A}R$ do not exert tonic effects and do not control A_1R-mediated decrease in NMDA-EPSC, in contrast to other studies of other brain regions, in which it was demonstrated that exogenous activation of $A_{2A}R$ increases the amplitude of NMDA currents, namely in the hippocampus (Dias *et al*., 2012; Mouro *et al*., 2018; Rebola *et al*., 2008; Rombo *et al*., 2014; Tebano *et al*., 2005; Temido-Ferreira *et al*., 2018). Both $A_{2A}R$ agonists which were tested in this study decreased NMDA-EPSC in BLA pyramidal neurons and this effect was not prevented by the selective $A_{2A}R$ antagonist SCH58261, casting doubt on the selectivity of CGS21680 and regadenoson for $A_{2A}R$ in the amygdala.

Others have claimed that the activation of presynaptic A_1R inhibits basal AMPA-EPSCs upon stimulation of medial or external glutamatergic inputs into the BLA (Masneuf *et al*., 2014b). Also, both tonic and agonist-induced activation of postsynaptic $A_{2A}R$ increased the firing rate of BLA pyramidal neurons via cAMP-PKA pathway (Rau *et al*., 2015). These studies suggest that adenosine modulation in the amygdala excitatory synapses depends upon a balance between the inhibitory A_1R-

mediated effects and facilitatory $A_{2A}R$ effects. Our group described that $A_{2A}R$ in the LA facilitated LTP of population spikes evoked by stimulating cortical afferents, as well as their selective downregulation impaired fear acquisition and fear extinction (Simões *et al*., 2016), highlighting the importance of $A_{2A}R$ in amygdala-mediated processes. Interestingly, astrocytes may also be involved in the adenosine modulation activity: it has been reported that the activation of astrocytes in the central amygdala (CeM) decreased the firing rate of CeM neurons and reduced fear expression in a fear-conditioning paradigm (Martin-Fernandez *et al*., 2017). This astrocyte-mediated inhibition was a result of a selective depression of excitatory synapses from the BLA through A_1R activation, and a selective increase of inhibitory synapses of the CeA via $A_{2A}R$ activation (Martin-Fernandez *et al*., 2017). Overall, the existing studies point to a role of adenosine modulation in the amygdala being operated by inhibitory actions of A_1R and facilitatory actions of $A_{2A}R$.

Surprisingly, our data showed that the alleged selective $A_{2A}R$ agonists decreased NMDA-EPSC responses, an effect prevented by A_1R antagonists, rather than antagonists of $A_{2A}R$. However, there are already studies made in other brain areas which questioned the selectivity of these purported $A_{2A}R$ agonists. For instance, the $A_{2A}R$ agonist CGS21680 has been described to have an atypical binding profile in the cortex and hippocampus (as compared to striatum), where CGS21680 seems to behave as an intermediate between A_1R and $A_{2A}R$ (Kirk and Richardson, 1995; Wan *et al*., 1990) and even bind to A_1R (Halldner *et al*., 2004). In cortical regions, where $A_{2A}R$ are present (Cunha *et al*., 1994; Gonçalves *et al*., 2013; Kerkhofs *et al*., 2018; Rebola *et al*., 2005), but A_1R are far more abundant (Cunha *et al*., 2006; Duarte *et al*., 2006; Rebola *et al*., 2005; Rebola *et al*., 2005), CGS21680 binds to a ligand-site associated to A_1R, thereby requiring the interaction between both receptors (Lopes *et al*., 2004). Yet, in our experiments, we blocked the effect of CGS21680 with the selective A_1R antagonist, while the selective A_1R agonist did not occlude the effect of CGS21680. These results suggest that the observed decrease in NMDA-EPSC is not solely mediated, but partially depends on A_1R activation. It is possible that CGS21680 acts at a target other than A_1R and $A_{2A}R$, uses a different intracellular pathway or binds to receptor subpopulations not affected by the A_1R and $A_{2A}R$ antagonists used in this study. The inhibitory effect of CGS21680 has also been observed in other brain regions besides the amygdala: in the striatum, where, in the absence of external Mg^{2+}, the activation of $A_{2A}R$ with CGS21680 (100 nM) blocked NMDAR-mediated currents at a postsynaptic level, in medium spiny neurons from young Wistar rats, via phospholipase C/IP_3/calmodulin and calmodulin kinase II pathway (Wirkner *et al*., 2004, 2000). $A_{2A}R$ levels are highest in the striatum, and binding of CGS21680 was shown to be absent in the striatum of $A_{2A}R$ knockout mice (Lopes *et al*., 2004). It is worth mentioning that others have demonstrated that CGS21680 increased the firing rate of BLA pyramidal neurons, an effect blocked by the selective $A_{2A}R$ antagonist ZM241285 (Rau *et al*., 2015),

and that SCH58261, ZM241285 or genetic downregulation of BLA $A_{2A}R$ decreased high frequency stimulation-induced LTP in the amygdala (Simões *et al*., 2016), which is in accordance to what has been reported in the hippocampus (de Mendonça *et al*., 1995; Rebola *et al*., 2005; Sotres-Bayon *et al*., 2007; Wirkner *et al*., 2000), and opposite to our present observations in the control of NMDA-EPSC in the amygdala. Nevertheless, in these studies, no distinction between pre and post-synaptic receptors was made. It is known that, for instance, in the striatum, different subpopulations of $A_{2A}R$, located at different synaptic sides, with different interacting partners, exhibit different sensitivity to different antagonists (Orrú *et al*., 2011a,b; Wirkner *et al*., 2004). Hence, it would be interesting to define the synaptic locus of $A_{2A}R$ in BLA pyramidal neurons, in order to better understand the results we obtained. Furthermore, while antagonists in general are likely to antagonize only the subpopulations of receptors which are active, agonists likely act on all receptor subpopulations, adding challenges to our data interpretation. However, the use of another $A_{2A}R$ agonist had similar effects: regadenoson (300 nM) had an even more pronounced effect in the decrease of NMDA-EPSC amplitude which was neither prevented by SCH58261 (50 nM) nor by DPCPX (100 nM).

Overall, our data support the need to design more selective $A_{2A}R$ agonists, as well as to explore the roles and signaling pathways of different populations of $A_{2A}R$, with different sensitivities to selective $A_{2A}R$ antagonists, such as SCH58261. A deep understanding of the pharmacological profile of adenosine receptors in different circuits and cell types of the amygdala is crucial, given their potential role in the etiology of emotional disorders and the possibility to be used as therapeutic targets.

4.2. Changes in BLA modulation by $A_{2A}R$ upon repeated restraint stress

Maladaptive responses to prolonged stress are the main risk factor for the development of several neuropsychiatric disorders, especially mood-related dysfunctions, which often lead to depression (McEwen and Akil, 2020). Although depression is highly prevalent in modern society, the available treatments do not have the expected efficacy and are still linked to several adverse effects (Richter-Levin and Xu, 2018). It is noteworthy that the "Diagnostic and Statistical Manual of mental disorders-5th edition, DSM-V) defines one of the main symptoms of depression, which is anxiety, as pathological when "the anxiety, worry, or physical symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning" and distinguishes between three levels of anxiety-related malfunctioning: anxiety disorders, obsessive-compulsive and related disorders (such as several phobias, panic and social anxiety) and stressor-related disorders (such as post-traumatic stress disorder).

Thus, it is of utmost importance to clarify the underlying neural circuitry in depression, in order to understand its basic mechanisms in the CNS and, more specifically, in the amygdala. This brain region is one of the most vulnerable to the effects of prolonged stress, and the abnormal functioning of its neurons is associated to the pathogenesis of depressive-like states (Roozendaal *et al*., 2009). However, it is important to outline that it is particularly challenging to study mood-related pathologies, since they often involve a state that is *per se* difficult to define, due to the overlap between a neurobiological dysfunction and a psychological state (Millan, 2003). Nevertheless, the related behavioral manifestations result from a dysregulation in the complex processing within the amygdala circuitry and, for that reason, they are used in animal models of this pathological state. When a stressor is chosen to create an animal model, it is essential to consider its main features (Anisman and Matheson, 2005; Bai *et al*., 2014). We selected restraining of free movement as the stressor, which is purely psychological and has been shown to be effective in mimicking a depressive-like phenotype when applied at least for 14 days, for 2-8h daily (Kim and Han, 2006). Chronic restraint stress is one of the most used paradigms to induce purely psychological stress in animal models, since it does not cause any physical pain, but limits the ability to freely move and leads to the expression of depressive-like behaviors, including anhedonia and anxiety. This procedure also guarantees that any of the observed stress effects are due to the stressor itself, rather than to physical consequences of possible injuries (Buynitsky and Mostofsky, 2009; Vyas *et al*., 2002). Chronic immobilization stress has also been proved to be one of the most effective in affecting several brain regions, causing dendritic hypertrophy in BLA neurons, as well as dendritic atrophy in hippocampal and prefrontal cortical neurons (Vyas *et al*., 2002). It is worthy to point out that only chronic immobilization stress, and not acute, is able to

induce morphological changes in BLA neurons, although variations in the intensity of the stress protocol modulate the degree of BLA structural plasticity (Mitra *et al*., 2005). In addition to that, it has been shown that a 7-day sub-chronic restraint stress protocol is itself enough to induce over-expression of $A_{2A}R$, at least in the hippocampus (Cunha *et al*., 2006), which is line with the likelihood of adenosinergic modulation, being one of the main mechanisms involved in the etiology and treatment of mood-related disorders (van Calker *et al*., 2020; Yamada *et al*. 2014). In the following parts of this work we sought to explore animal models exhibiting repeated stress-induced increase in anxiety-like behavior and to evaluate changes in $A_{2A}R$ modulation within the basolateral amygdala.

4.2.1. Intermittent repeated restraint stress in adolescent Wistar rats

Adolescence is a key developmental period which is characterized by significant physical, hormonal and behavioral differences when compared to childhood or adulthood and comprises a variety of different behaviors which define the individual as a whole (Spear, 2000). The period of adolescence can be easily compared in different species, and mammalian adolescents typically exhibit common manifestations at this life stage: they are prone to being more impulsive and risk-taking, have frequent mood alterations, are emotionally vulnerable, tend to have augmented social interaction with age-similar subjects and rats typically exhibit increased play behavior and play fighting (Geier *et al*., 2010; Hare *et al*., 2008). The definition of the adolescence age span in animal models is not consensual among the literature, but the most accepted one in rats comprises the period from 28 to 42 postnatal days (Spear and Brake, 1983). This period is characterized not only by distinctive behavioral features, but also by neurodevelopmental alterations, especially for the prefrontal cortex (PFC) and the amygdala, because of their role in the decision-making process and regulation of the behavioral and emotional outputs (Dahl, 2001; Selleck *et al*., 2018). The amygdala is known to be more active during adolescence, typically leading to diminished regulation of appropriate behavioral responses (Hare *et al*., 2008), thereby increasing the vulnerability to the onset of pathological processes associated to affective disorders. However, although it is known that stress leads to an increased susceptibility to experience mood dysfunction, there is scarce information on peri-adolescent behavior and amygdala physiology following exposure to repeated stress. It is known that adolescents show amygdala overactivation when exposed to aversive stimuli, as a consequence of a lack of control by still immature prefrontal cortical regions, which are inefficient during this developmental stage (Hare *et al*., 2008; Kim *et al*., 2011; Selleck *et al*., 2018).

Thus, we used a protocol established by the lab of J. Amiel Rosenkranz (Rosenkranz *et al*., 2010), exposing the animals to an interrupted pattern of stress, which consisted of a single session of 20

minutes of restraint per day, during five consecutive days, followed by two days of handling, and two additional days with similar restraint sessions (Rosenkranz *et al*., 2010). This interrupted pattern of stress has been demonstrated to be more effective than a daily restraint paradigm, since it diminishes the habituation of the animals to the everyday procedure (Zhang *et al*., 2014). Among the changes they observed, the stressed animals had heightened adrenal glands weight and increased corticosterone levels, decreased weight gain, increased anxiety-like behavior in the EPM, impaired fear extinction and increased firing rate of BLA pyramidal neurons (Rosenkranz *et al*., 2010). Therefore, we decided to evaluate whether the same protocol could also result in robust changes in behavior and BLA physiology in adolescent Wistar rats, which could be used as starting points to explore changes in synaptic modulation by $A_{2A}R$ in the BLA.

4.2.1.1. Experimental design

Two different groups of animals were subjected to intermittent repeated restraint. The experimental design is represented in Figure 16. On the day after the last stress session, group 1 was observed in the EPM and open field. On the next day, the animals were subjected to a fear conditioning paradigm. On the last day, fear memory was evaluated, as well as intrasession extinction. Group 2 animals were only observed in the EPM and then used for patch-clamp recordings in BLA pyramidal neurons.

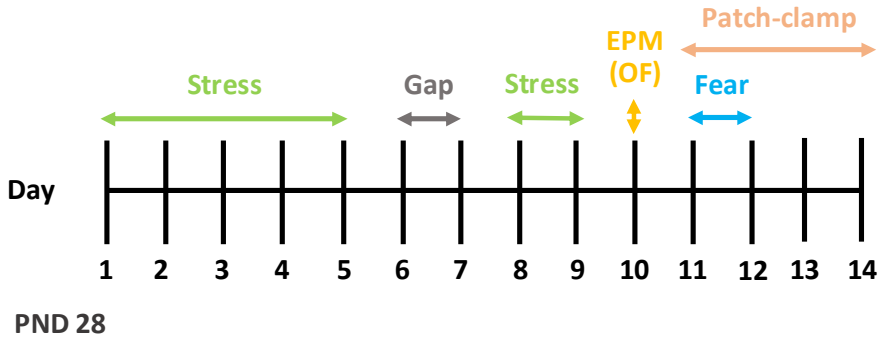


Figure 16. Schematic representation of the experimental design for protocol validation. PND – post-natal days; EPM – elevated plus-maze, OF – open field

4.2.1.2. Stressed adolescent Wistar rats gained similar weight, but had increased weight of adrenal glands

Weight was monitored daily in all the animals, since it is known that stress alters body weight and food intake in animal models (Jeong *et al*., 2013). On the day after the last stress session, the weight gain in relation to the day prior to the beginning of the protocol was 61.56 ± 5.18 g, n=13 in the control group, and 55.82 ± 4.01 g, n=14 in the stressed group (n=number of animals; Figure 17A). The difference between the weight gain in the control and the stressed rats was not significant ($F(1,25)=0.9045$; $p=0.3507$, two-way repeated measures ANOVA).

Stress is known to induce adrenal activation through the immediate response of the HPA axis to stressful stimuli (Goldstein, 2011); thus, the evaluation of the adrenal glands weight can be used as a measure of the effectiveness of a stress protocol. We observed a significant increase of the adrenal glands weight in the stressed group (0.30 ± 0.02 mg/body weight, n=8 *vs.* control 0.23 ± 0.02 mg/body weight, n=6; $p=0.0271$, unpaired Student's *t*-test; n=number of animals) (Figure 17B).

Our data do not fully coincide with the data obtained by Zhang and colleagues, who showed a significant decrease of body weight and a significant increase of adrenal glands weight (Zhang *et al*., 2014).

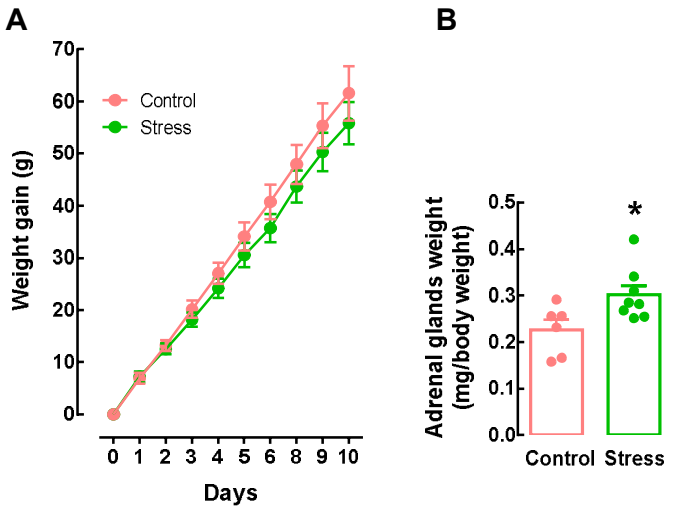


Figure 17. A. Weight gain of the animals during the protocol. **B.** Increased adrenal glands weight in the stressed group of animals. The results are presented as mean ± SEM, n=6-14; $p<0.05$, unpaired Student's *t*-test, two-way repeated measures ANOVA.

4.2.1.3. Stress did not induce anxiety-like behavior in adolescent rats

In order to evaluate the general effectiveness of the stress protocol, the EPM test was performed, since it is known that increased anxiety-like behavior in the EPM is a well-established measure of an effective chronic stress paradigm (Walf and Frye, 2007). No significant differences were observed in the overall anxiety-like behavior (% time spent in the open arms: control 12.14 ± 2.11 % *vs.* stress 19.49 ± 4.05 %, p=0.1507; % entries into the open arms: control 21.33 ± 1.96 % *vs.* stress 24.33 ± 2.09 %, p=0.3257; n=11-14, unpaired Student's *t*-test, n=number of animals; Figure 18A-B) and there was no difference in locomotion (control 11.26 ± 1.02 m *vs.* stress 10.42 ± 0.81 m, n=11-14; p=0.5212, unpaired Student's *t*-test; n=number of animals; Figure 18C), in contrast to the results obtained by Zhang and Rosenkranz, who observed increased anxiety-like behavior in stressed adolescent animals (Zhang and Rosenkranz, 2012).

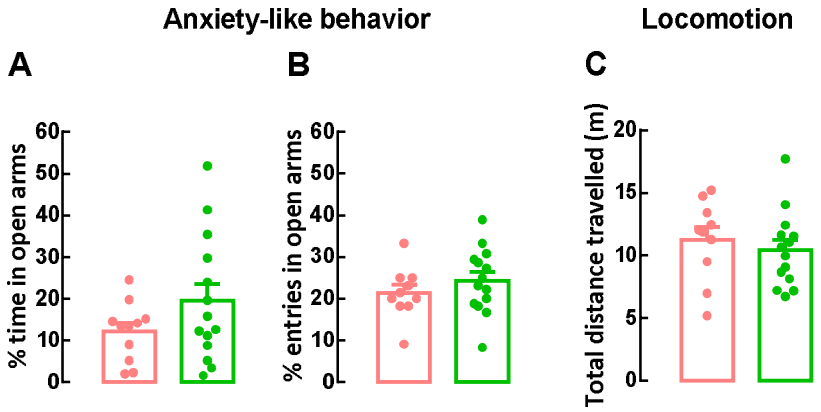


Figure 18. A-B: Evaluation of anxiety-like behavior by measuring the % time (**A**) spent in the open arms, as well as the % entries (**B**) into the open arms. **C:** evaluation of locomotion activity. The results are presented as mean ± SEM, n=11-14, unpaired Student's *t*-test; n=number of animals

4.2.1.4. Stress did not affect locomotion, anxiety-like behavior or habituation in the open field test

In the group of rats which would undergo fear conditioning, an open-field (OF) test was also performed, in order to evaluate whether the control and the stressed groups of animals presented differences in their locomotion activity, measured by the total distance they travelled, as well as to obtain another measurement of anxiety-like behavior, measured by the time spent in the center of the arena (Figure 19A-E). No differences were observed between both groups in locomotion: control 26.42 ± 3.08 m, stress 29.84 ±1.02 m, p=0.2838 (Figure 19A). The anxiety-like behavior was also similar between the groups: % distance in the center - control 18.08 ± 1.41 %, stress 18.38 ± 1.48 %, p=0.8930; % time in the center - control 10.42 ± 1.70 %, stress 9.88 ± 1.46 %, p=0.8153; n=5 control, n=6 stress, unpaired Student's *t*-test, n=number of animals; (Figure 19B). These data are in contrast to the commonly observed results, in which adolescent animals tend to approach the lighted area in the center of the arena more readily than adults (Desikan *et al*., 2008). The OF test was also used to assess the habituation profile of the animals, which represents the animal's learning to disregard irrelevant stimuli (Thompson and Spencer, 1966). More specifically, habituation is frequently used to exclude the effects of the environment which may influence the experiment and typically represents a decrease of locomotion with time (Dubovicky and Jezova, 2004). Several parameters might influence the habituation profile, including strain, sex and age of the animals, as well as exposure to stress (Chapillon and Roullet, 1997; Sestakova *et al*., 2013; Shukitt-Hale *et al*., 2001). In fact, stressed animals usually exhibit a decrease in the habituation profile (Daenen *et al*., 2001; D'Aquila *et al*., 2000; Dubovicky and Jezova, 2004). As expected, we observed a significant decrease in locomotion with time in both groups of animals, but no differences between groups: first 10 min - control 26.42 ± 2.99 m *vs.* stress 29.84 ± 1.49 m; last 10 min – control 7.49 ± 2.99 m *vs.* stress 7.49 ± 2.99 m; n=5-6, $F(1,9)=0.7986$, $p<0.0001$; two-way repeated measures ANOVA; multiple *t*-tests; n=number of animals (Figure 19D-E).

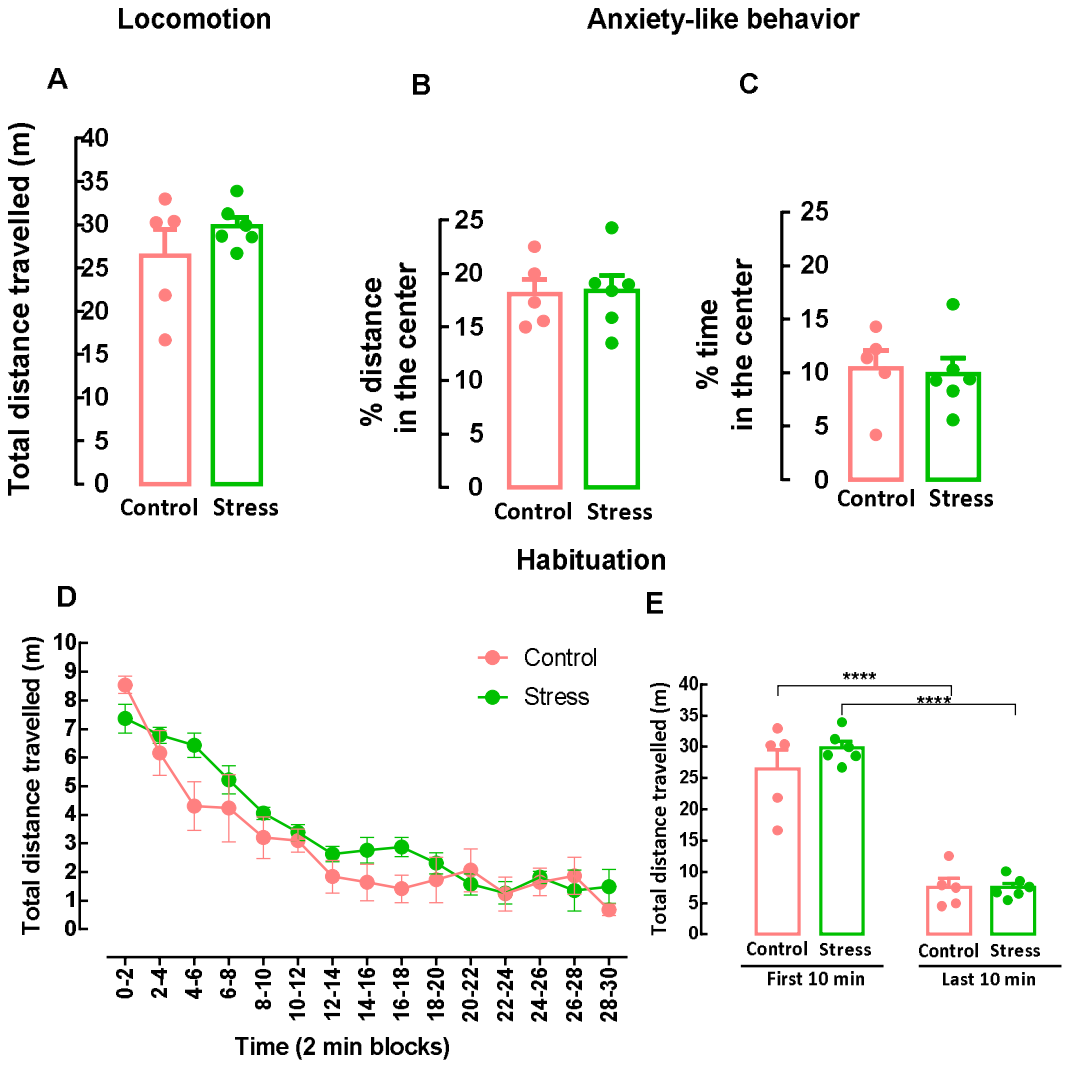


Figure 19. **Open-field test. A.** Evaluation of the locomotor activity: total distance travelled during the first 10 minutes of the test. **B-C.** Evaluation of anxiety-like behavior by measuring the % of the distance travelled (**B**) and the % time (**C**) spent in the center. **D-E.** Habituation profile. **D.** Distance travelled during 20 min, evaluated every 2 min. **E.** Comparison of the distance travelled in the first and last 10 min. The results are presented as mean ± SEM, n=5-6, n=number of animals; A-C: unpaired Student's *t*-test; D-E****<0.0001, two-way repeated measures ANOVA; multiple *t*-tests

4.2.1.5. Stressed animals had decreased acquisition of fear

Fear conditioning was performed by adapting the methodology used by Zhang and his colleagues (Zhang and Rosenkranz, 2013), one day after the EPM test. The percentage of freezing was measured as an indicator of conditioned fear, which is a validated measure of fear in rats, upon re-exposure to

the conditioned stimulus in the absence of the unconditioned stimulus. The footshock intensity used was similar in both groups and induced forepaw withdrawal. A schematic representation of the experimental design is shown in Figure 20A. Surprisingly, the stressed animals exhibited decreased acquisition of fear (34.00 ± 17.36 %, n=5, $p<0.05$, n=number of animals; two-way repeated measures ANOVA, at the fifth trial (T5)), when compared to the control group (72.00 ± 5.51 %, n=5, $p<0.05$, n=number of animals; two-way repeated measures ANOVA, at the fifth trial (T5)) (Figure 20B). On the second day, both groups of animals displayed extinction of fear memory with time. At the fourth trial (T4), the stressed animals displayed significantly diminished % freezing when compared to the control group (control 49.40 ± 19.81 % *vs.* stress - 3.60 ± 3.60 %, n=5, $p<0.01$, two-way repeated measures ANOVA; n=number of animals) and, at the last trial (T15), both group extinguished the fear response (control – 8.00 ± 7.27 % *vs.* stress – 5.60 ± 2.87 %, n=5, two-way repeated measures ANOVA; n=number of animals) (Figure 20C).

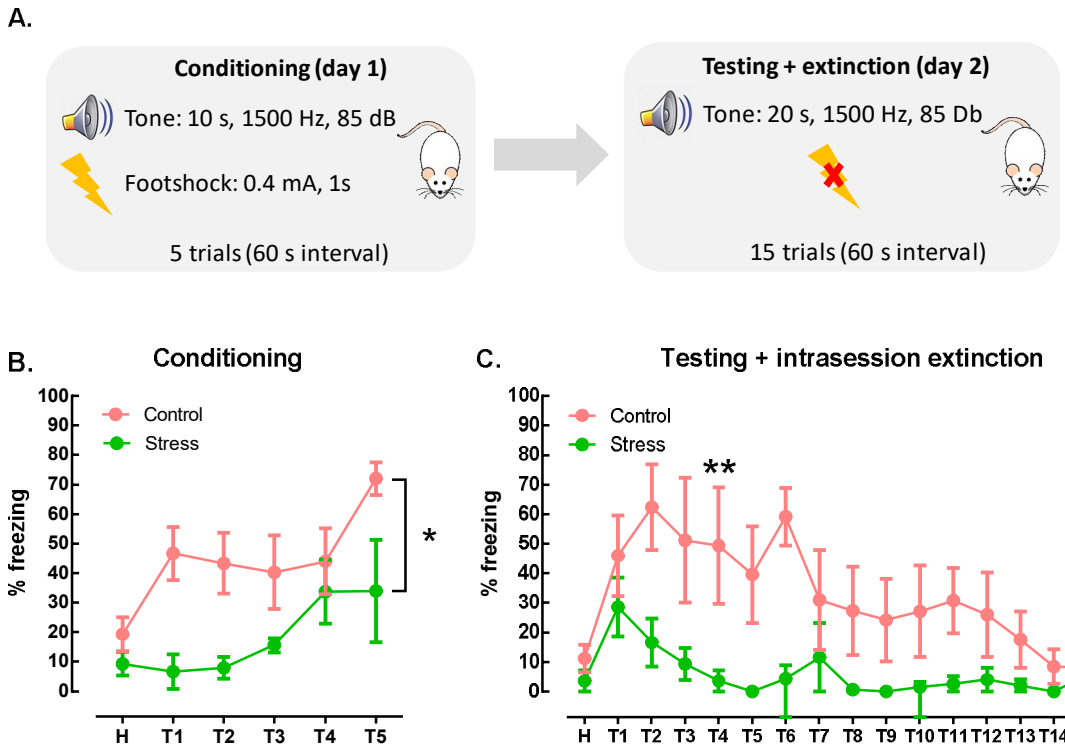


Figure 20. Stressed animals have decreased acquisition of fear. Decreased acquisition of fear by the stressed animals. A. Experimental design. **B.** The Stressed animals have decreased acquisition of fear. **C.** Both groups extinguished the fear when exposed to the tone on the second day. H – habituation; T – trial. The results are presented as mean ± SEM, n=5. * $p<0.05$, ** $p < 0.01$, two-way repeated measures ANOVA; n=number of animals.

4.2.1.6. Electrophysiological changes in BLA pyramidal neurons

Given the robust decrease in fear acquisition observed in stressed animals, we used whole-cell patch-clamp recordings to evaluate whether this could be a result of electrophysiological changes within the basolateral amygdala.

4.2.1.6.1. Pyramidal neurons exhibited similar firing rates in control and stressed animals

Intermittent repeated restraint stress is known to induce a state of hyperexcitability in BLA pyramidal neurons, due to an impairment in GABAergic control mechanisms (Rosenkranz *et al*., 2010; Sharp, 2017) and, for instance, social isolation stress in adolescence has been described to increase the firing rate of pyramidal neurons through a mechanism dependent on calcium-activated potassium channels (Rau *et al.*, 2015). Hence, we evaluated whether our stress paradigm altered the excitability of pyramidal neurons in the BLA by measuring the frequency of action potentials with depolarizing current steps. We observed that the firing rate of the neurons remained similar in both groups (maximum number of fired action potentials – control 14.40 ± 0.60 *vs.* stress 12.43 ± 1.04, n=5-8, multiple *t*-tests; n=number of recorded cells), meaning that BLA pyramidal neurons did not change their intrinsic excitability with the restraint stress protocol (Figure 21A).

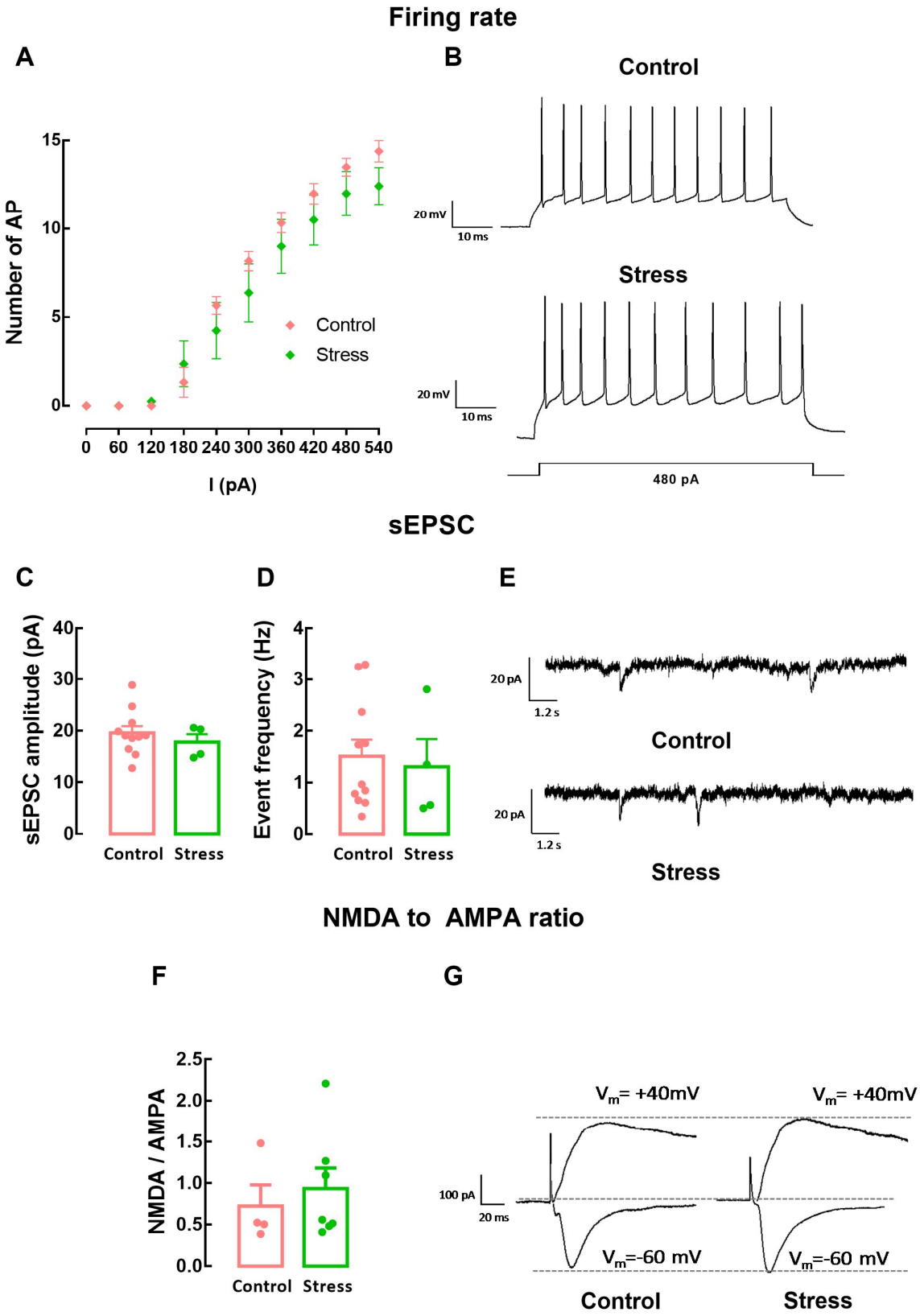
4.2.1.6.2. Spontaneous excitatory currents onto pyramidal neurons were not affected in stressed animals

Stress affects excitatory glutamatergic transmission and malfunction in this system has even been referred as the main mechanism responsible for the onset of mood and anxiety disorders (Sanacora *et al*., 2012). Spontaneous excitatory currents (sEPSC) in the amygdala have been described to increase the frequency of their events, rather than in their amplitude, upon repeated restraint stress in mice (Chauveau *et al*., 2012; Masneuf *et al*., 2014). We tested the existence of changes in spontaneous excitatory transmission in BLA pyramidal neurons, at a holding potential of -60 mV, upon the stress paradigm we used. We did not observe alterations neither in the amplitude (control 19.54 ± 1.32 pA, n=11; stress 17.78 ± 1.52 pA, n=4; p=0.4754, unpaired Student's *t*-test; n=number of recorded cells) nor in the frequency of sEPSC (control 1.51 ± 0.32 Hz, n=11; stress 1.31 ± 0.54 Hz, n=4; p=0.7482, unpaired Student's *t*-test; n=number of recorded cells) (Figure 21C-E).

4.2.1.6.3. Pyramidal neurons exhibited similar NMDA to AMPA ratio

Besides intrinsic electrical activity of BLA pyramidal neurons and sEPSC, evoked EPSC recordings were also performed upon stimulation of the LA (Figure 21F-G).

The NMDA to AMPA ratio is used to assess the predisposition for synaptic potentiation (Kauer and Malenka, 2007), and both synaptic plasticity and the density of NMDA and AMPA receptors are affected upon exposure to chronic stress (Mikasova *et al*., 2017; Pacheco *et al*., 2017; Sanacora *et al*., 2008). Also, the effects of stress on the NMDA to AMPA ratio are not only region-specific, but also synapse-specific (Kallarackal *et al*., 2013). Thus, we used this value to evaluate whether there were changes in the LA-BLA synapses in our stressed group of animals. The NMDA to AMPA ratio value was calculated by comparing the peak amplitude of the evoked NMDA-EPSC obtained at a holding potential of +40 mV in the presence of 20 µM CNQX and the peak amplitude of evoked AMPA-EPSC recorded initially at a holding potential of -60 mV. No differences were observed between the control and the stressed animals (control 0.72 ± 0.25, n=4; stress 0.93 ±0.25, n=7; p=0.5947, unpaired Student's *t*-test; n=number of recorded cells; Figure 21).


Firing rate
A
B
Control
Number of AP
15
10
5
0
0 60 120 180 240 300 360 420 480 540
I (pA)
Control
Stress
20 mV
10 ms
Stress
20 mV
10 ms
480 pA
sEPSC
C
D
E
sEPSC amplitude (pA)
40
30
20
10
0
Control Stress
Event frequency (Hz)
4
3
2
1
0
Control Stress
20 pA
1.2 s
Control
20 pA
1.2 s
Stress
NMDA to AMPA ratio
F
G
NMDA / AMPA
2.5
2.0
1.5
1.0
0.5
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Control Stress
Vm= +40mV
Vm= +40mV
100 pA
20 ms
Vm=-60 mV
Vm=-60 mV
Control
Stress

Figure 21. A-B. BLA pyramidal neurons from control and stressed rats show similar firing rates. A. Average number of action potentials (AP) fired in response to depolarizing current steps. **B.** Representative traces of the action potential fired in response to a 480 pA current, in the control and the stressed rats. I – current amplitude. **C-E. Spontaneous EPSC are similar in the control and the stressed animals.** Amplitude (**C**) and frequency (**D**) of the sEPSC events. **E.** Representative traces show sEPSC recorded from BLA pyramidal neurons in the control and the stressed animals. **F-G. Evoked NMDA to AMPA ratio in BLA pyramidal neurons is similar in the control and the stressed animals. F.** Bar graph **G.** Representative traces. Currents recorded at -60 mV are mainly mediated by AMPA receptors; when the holding potential is increased to +40 mV and 20 μM CNQX is added, the currents are mainly mediated by NMDA receptors. The results are presented as mean ± SEM, n=4-11; unpaired Student's *t*-test; n=number of recorded cells

4.2.1.7. Discussion

The work described in this chapter was done in an attempt to validate an animal model of depression in adolescent male Wistar rats, with robust changes in behavior and BLA physiology, which would allow us to explore the role of BLA $A_{2A}R$ in the expression of those changes. We used a protocol from A.J. Rosenkranz's laboratory and mimicked the stress paradigm which they used in Sprague-Dawley rats in Wistar rats. However, we were not able to obtain an anxiety-like phenotype, when the animals were tested in the EPM. Although the EPM is extensively validated in the literature, it is not uncommon to obtain inconsistent findings, which are generally due to differences in the methodology, strain, the sex of the experimenter or housing conditions (Andrade *et al*., 2003; Walf and Frye, 2007). The age of the animals is also an essential variable to consider, especially during the adolescence period, which is characterized by an extensive variability in anxiety-like behavior (Albani *et al*., 2015; Andrade *et al*., 2003). In fact, decreased anxiety-like behavior in adolescent mice has already been reported (Peleg-Raibstein and Feldon, 2011) and it is known that juvenile animals are more sensitive to environmental stimuli than adults and that they commonly display diminished fear of open spaces (Smith and Morrell, 2007). Indeed, such parameters as illumination and the time of the day when the EPM is performed largely affect the performance of adolescent animals in this test (Albani *et al*., 2015).

We then tested the animals in the OF test, which is typically used to assess locomotor activity and anxiety-related behavior, but often also provides contradictory data (Carola *et al*., 2002). Similarly to the EPM, the OF can be influenced by parameters such as illumination, time of the day and the environment itself (Seibenhener and Wooten, 2015). It is noteworthy that emotional parameters can only be compared between groups if locomotion is similar between them, thereby excluding mobility-related issues (Stanford, 2007). Our animals did not display differences in the total ambulatory distance

travelled, excluding the effect of stress on motor ability. Also, the control and the stressed animals did not differ in anxiety-like behavior, although it would be expected that the stressed animals should spend less time in the center of the arena (Sousa, 2006). Finally, the control and the stressed animals exhibited a similar profile during the habituation period to the maze, while it would be expected that the stressed animals should have it diminished (Daenen *et al*., 2001; D'Aquila *et al*., 2000; Dubovicky and Jezova, 2004).

Although the only behavioral manifestation of the stress effect was the diminished acquisition of fear, we hypothesized that the amygdala could still be altered, since it is the most affected brain region upon fear conditioning, in which the BLA is the main nucleus involved in the integration of the information obtained during the protocol: it is involved in acquisition, expression and extinction of fear memory (Henry *et al* 2008; Maren, 1999; Sah *et al*., 2003). Hence, changes in fear conditioning serve as an index of alterations in the amygdala function and, for that reason, in order to assess whether the amygdala was affected upon the stress paradigm we wanted to validate, we studied the effect of repeated restraint on amygdala-dependent auditory fear conditioning and evaluated if there were differences between the control and the stress groups (Zhang and Rosenkranz, 2013). Fear and anxiety are often mistakenly used as similar concepts, but these terms represent different types of responses in rodents. Fear is described as an active defensive behavior (e.g.: freezing) caused by an imminent threat and both its onset and duration are rapid. In contrast, anxiety is an emotional apprehension state which is not triggered by a specific threat, but rather by a risk which can be circumstantial or just anticipated by the individual (Davis *et al*., 2010; Tsvetkov *et al*., 2015). Although the brain regions and the circuits involved in the processing of fear and anxiety are different, they also overlap, making it possible to study the frequently common and shared behavioral outcome in those two emotional states (Tovote *et al*., 2015). Our data point to a decrease in the acquisition of fear upon stress, which contradicts the results obtained by Zhang and colleagues, who observed no impairment in the acquisition of fear in stressed animals (Zhang and Rosenkranz, 2013). Instead, the authors showed an increased fear memory and decreased fear extinction in the animals subjected to the same stress protocol (Zhang and Rosenkranz, 2013), while we observed a reduced fear response on the testing day, as well as a faster extinction of fear. The majority of studies found in the literature refer to an enhancement of the BLA-dependent fear response in juvenile animals upon repeated immobilization stress (Conrad *et al*., 1999; Wood *et al*., 2008). However, a decrease in conditioned freezing upon stress has already been described by others. For instance, in a study where adolescent male Long-Evans rats were subjected to a protocol of social instability, the authors observed a decrease in acquisition of contextual and auditory fear conditioning (Morrissey *et al*., 2011). Several parameters may account for the above mentioned discrepancies, including variability in the strain of the animals. For example, it has been

known for a long time that Sprague-Dawley rats display higher levels of freezing when compared to other strains, such as Long-Evans, and this divergence was also shown to be dependent on the suppliers which provide the animals (Graham *et al*., 2009; Helmstetter and Fanselow, 1987). Also, Sprague-Dawley and Wistar rats are known to exhibit dissimilar behavior when coping with a stressor, such as a cage intruder, have distinct exploratory behavior, as well as a different resilience to fear conditioning (Walker *et al*., 2009).

Despite the lack of a clear phenotype, the amygdala involvement was suspected, based on the altered behavior of the animals when exposed to fear conditioning. For that reason, we then explored the possibility of the existence of electrophysiological changes in the excitatory synaptic transmission of BLA pyramidal neurons. As a matter of fact, one of the phenomena which has been described to occur upon stressful conditions is the enhancement of glutamate release in several brain structures, including the BLA and CeA nuclei of the amygdala (Reznikov *et al*., 2007). The increase in glutamate levels is typically associated to excessive neuronal activation, leading to excitotoxicity phenomena, which affect both inter-neuronal communication and neuronal morphology (Gorman and Docherty, 2010). Also, changes of glutamate levels in plasma and cerebrospinal fluid have been reported in the brain of depressed patients (Hashimoto *et al*., 2007; Küçükibrahimoglu *et al*., 2009) and, consequently, some of the drugs used in clinics with antidepressant effect are designed to target the alterations in excitatory synaptic transmission (Musazzi *et al*., 2013; Reznikov *et al*., 2007). We began by assessing whether there were changes in excitatory spontaneous events, since it is known that both their frequency and amplitude may be altered upon stress and this effect is region- and synapse-specific (Chauveau *et al*., 2012; Masneuf *et al*., 2014). We did not observe modifications neither in the sEPSC amplitude nor in the frequency of the events, in contrast to a study in which an increase in the sEPSC frequency was described in BLA pyramidal neurons, in mice subjected to a 10-day restraint stress protocol (Masneuf *et al*., 2014). However, these data were obtained when an antagonist of $GABA_A$-receptors was applied, meaning that changes in excitatory synaptic transmission may be revealed when compensatory changes in inhibitory synaptic transmission is eliminated (Masneuf *et al*., 2014). Increased excitability of BLA pyramidal neurons as a result of stress has been reported in several studies and seems to be due to a suppression of GABAergic inhibition, typically leading to an increase in the firing rate of those neurons (Rau *et al*., 2015; Rodríguez Manzanares *et al*., 2005), though in our experimental conditions we did not observe any changes in the number of action potential in BLA pyramidal neurons of the stressed animals. We also did not find changes in the ratio of NMDA to AMPA receptor-mediated currents, although facilitation of long-term potentiation has been shown upon repeated restraint stress in BLA amygdala neurons upon cortical afferents stimulation, as well as in LA neurons upon stimulation of the internal capsule and thalamic afferents (Rodríguez Manzanares *et al*., 2005;

Suvrathan *et al*., 2013). However, others have also described a lack of effect on the NMDA to AMPA ratio in BLA pyramidal neurons upon repeated restraint stress, indicating that NMDA- and AMPA-mediated currents may not always be affected by stressful stimuli (Masneuf *et al*., 2014).

It is worth mentioning that the amygdala should be considered as part of a complex network of intercommunication between several regions, including the PFC and the hippocampus, in which the extensively intermingled amygdala pyramidal neurons might participate in distinct functional circuits and even process differently the events occurring within the amygdala (Felix-Ortiz *et al*., 2013; Sah *et al*., 2003). In fact not long ago, it was described that acute stress enhanced glutamatergic transmission in BLA-PFC synapses without altering the intrinsic excitability of BLA pyramidal neurons (Song *et al*., 2017). Therefore, the lack of alterations in the excitatory synaptic transmission which we observed within the LA-BLA synapses does not necessarily mean that BLA pyramidal neurons were not affected by stress, just that the recordings were not performed in the "right place" to highlight these putative alterations. Our overall results show that the protocol described by Rosenkranz and his colleagues was not effective in our experimental conditions and, more specifically, in adolescent Wistar rats, highlighting the importance of considering how stress impacts emotion not only at different ages, but also in different strains of animals. Since no changes were observed in a set of parameters aimed to evaluate excitatory synaptic transmission in the LA-BLA circuit, we were not able to assess the role of $A_{2A}R$ modulation in these synapses. Therefore, no further experiments were performed in this animal model.

4.2.2. Repeated restraint stress in adult Wistar rats

As mentioned before, restraining of the free movement of the animals is one of the most effective stress paradigms in rodents, which was shown to be effective in mimicking an anxiety state when applied at least for 14 days, for 2-8h daily (Kim and Han, 2006), in inducing morphological changes in BLA neurons (Vyas *et al*., 2002), as well as in causing an imbalance in the ratio of A_1R and $A_{2A}R$ levels (Cunha *et al*., 2006). In this part of the study we used a stress paradigm previously validated by our group (unpublished data[3]) for adult male Wistar rats, which consists in 4h/day immobilization, during 15 consecutive days. The protocol was proved to be effective and consistent among the behavioral tests that which were performed. More specifically, the exploration of open space was significantly diminished in both the EPM and OF, suggesting a clear anxiety-like phenotype (unpublished data[4]). The performance of the stressed animals in the two-trial Y maze was also reduced (unpublished data[4]), indicating a memory deficit, which is also a common feature in depressive states (Bessa *et al*., 2009). Besides the clear behavioral manifestations, neuropharmacological alterations in stressed animals were also described. Particularly, the amygdala of the stressed rats displayed an increase in several synaptic proteins, including vGluT1 (a marker of excitatory nerve terminals), vGAT and gephyrin (markers oh inhibitory synaptic terminals) (unpublished data[4]), implying that the alterations induced by stress occurred at both glutamatergic and GABAergic neurons. The changes in the density of synaptic proteins were accompanied by a significant increase of the density of $A_{2A}R$ in the amygdala of the stressed rats (unpublished data[4]). Following this study, our group performed another set of experiments, in which it was shown that the administration in the BLA of lentivirus expressing sh-RNA to downregulate $A_{2A}R$ was sufficient to revert mood alterations in rodents subjected to repeated restraint stress (unpublished data). Taking all these preliminary data into account, the main aims of this part of the work were to explore which the exact alterations caused by chronic restraint stress in the amygdala excitatory and inhibitory synaptic transmission are, as well as to assess the role of $A_{2A}R$ in these processes.

[3] unpublished data described in "Role of the adenosinergic system in animal models of chronic stress and depression", PhD dissertation, Nuno Miguel de Jesus Machado, 2016

4.2.2.1. Experimental design

In this part of the work, male adult Wistar rats were immobilized in decapicone plastic bags, for 4 hours a day, for 15 days. This stress paradigm was validated in a previous work from our group, in which it was shown that Wistar male rats subjected to this protocol displayed anxiety-like behavior in the EPM and OF tests, as well as decreased memory performance in the modified Y-maze test (unpublished data[4]). Thus, on the day following the last stress session, behavioral analysis was performed only in the EPM, in order to assess an anxiety-like phenotype, followed by patch-clamp electrophysiological recordings (Figure 22A).

4.2.2.2. Stressed animals exhibited anxiety-like behavior in the EPM

The rats that which were subjected to chronic restraint stress spent less time in the open arms (20.19 ± 2.35 %, n=41, p=0.0075, unpaired Student's *t*-test, n=number of animals; Figure 22B) when compared to the control group (29.31 ± 2.35 %, n=42, p=0.0075, unpaired Student's *t*-test, n=number of animals; Figure 22B); they displayed a decreased number of entries into the open arms (control 31.06 ± 2.03 %, n=42; stress 24.18 ± 1.89 %, n=41; p=0.0152, unpaired Student's *t*-test, n=number of animals; Figure 22C), and there were no differences in locomotion activity (control 10.60 ± 0.3608 m, n=42; stress 9.91 ± 0.5183 m, n=41; p=0.2752, unpaired Student's *t*-test, n=number of animals; Figure 22D).

[4] unpublished data described in "Role of the adenosinergic system in animal models of chronic stress and depression", PhD dissertation, Nuno Miguel de Jesus Machado, 2016

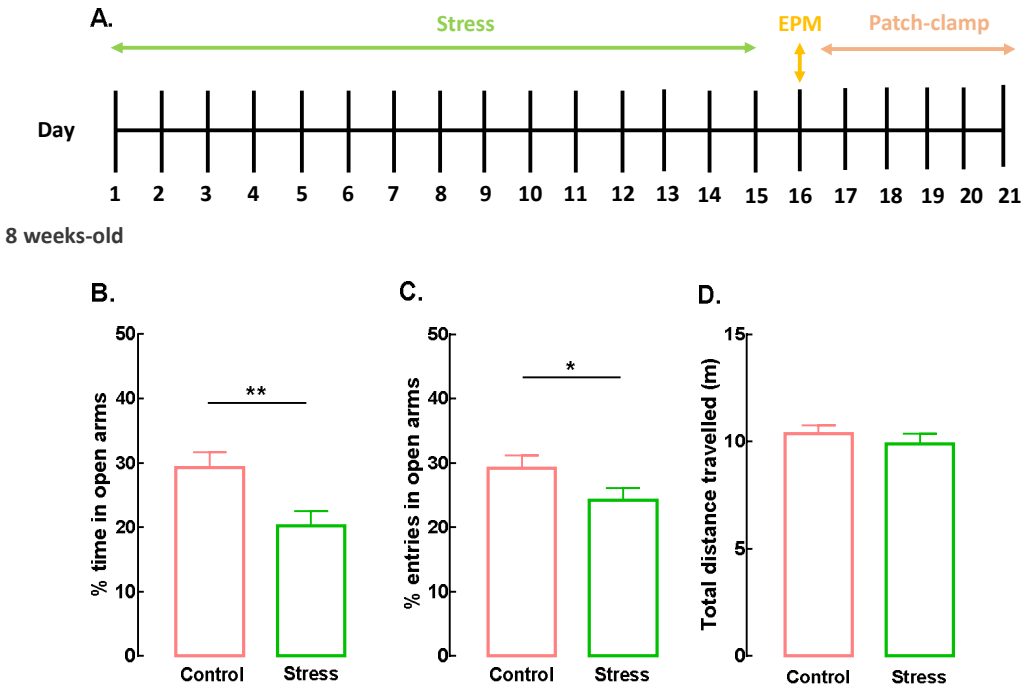

Figure 22. A. Schematic representation of the experimental design to evaluate the behavioral and electrophysiological alterations caused by chronic repeated restraint stress. EPM – elevated plus-maze. **B-D. Stressed animals exhibit anxiety-like behavior. B-C:** Evaluation of anxiety-like behavior by measuring the % time (**B**) spent in the open arms, as well as the % entries (**C**) into the open arms. **D:** evaluation of locomotion activity. The results are presented as mean ± SEM, n=41-42. *p<0.05; **p<0.01, unpaired Student's *t*-test, n=number of animals

4.2.2.3. Electrophysiological changes in BLA pyramidal neurons upon restraint stress

It is well known that chronic stress is associated to changes in the balance between excitatory and inhibitory synaptic transmission (de Kloet *et al*., 2005; Rodríguez Manzanares *et al*., 2005; Sanacora *et al*., 2012; Suvrathan *et al*., 2014). More importantly, previous work from our group showed an increase in the density of glutamatergic (vGLUT) and GABAergic synaptic markers (vGAT), in the BLA of animals subjected to this stress paradigm (unpublished data[5]).

We further assessed the electrophysiological impact of chronic repeated restraint stress, in animals that displayed a clear anxiety-like phenotype and their respective controls, in BLA pyramidal neurons,

[5] unpublished data described in "Role of the adenosinergic system in animal models of chronic stress and depression", PhD dissertation, Nuno Miguel de Jesus Machado, 2016

upon stimulation of the LA, in order to study possible synaptic changes in this circuit upon stress, as well as the role of the $A_{2A}R$.

4.2.2.3.1. Pyramidal BLA neurons of control and stressed rats exhibited similar input-output curves for evoked AMPA-EPSC upon stimulation in the LA

Chronic immobilization stress in rats is well-described as being able to induce a remodeling of neuronal networks, involving a modification of their function and plasticity (Kole *et al*., 2004; Popoli *et al*., 2013). Hence, the integration of synaptic inputs into a given neuron may also vary and we assessed whether there were changes in the strength of basal synaptic transmission. This was accomplished by recording input-output (I/O) curves for evoked AMPA-EPSC, which are obtained by calculating the peak amplitude of the evoked currents (at a holding potential of -60 mV) with increasing steps of electrical stimulation (Figure 23A-B). Although there is a clear tendency for the I/O responses in the stressed group to be higher than in the control one, the divergence is not statistically significant, probably due to the reduced number of recorded cells (n=5-10). For instance, at a stimulation intensity of 300 μA, the difference between the peak amplitudes of both groups is clear, but with a large variability (control 845.64 ± 171.09 pA *vs.* stress 1363.46 ± 454.75 pA, p=0.2129, n=5-10; n=number of recorded cells). The difference was assessed using an unpaired Student's *t*-test analysis, by comparing the peak amplitude values between groups for each stimulation intensity.

4.2.2.3.2. Pyramidal BLA neurons of control and stressed rats exhibited similar NMDA to AMPA ratio

To better understand excitatory synaptic transmission in any neural circuit, it is important to study the function and relative abundance of NMDA and AMPA receptors at glutamatergic synapses, since their number correlates with the efficacy of synaptic transmission, as well as their long-term modification upon a stimulus (Watt *et al*., 2000). Also, restraint stress has been described to alter glutamatergic network functioning in the BLA and one of the commonly observed effects is LTP facilitation (Sarabdjitsingh *et al*., 2012; Suvrathan *et al*., 2013). We, therefore, tested whether there were changes in evoked NMDA to AMPA ratio, to probe for the system's predisposition to undergo synaptic potentiation. We did not observe any significant difference in evoked NMDA to AMPA ratio between the control and the stressed groups (control 0.48 ± 0.06, n=4; stress 0.51 ± 0.44, n=11; p=0.7554, unpaired Student's *t*-test, n=number of recorded cells; Figure 23C-D).

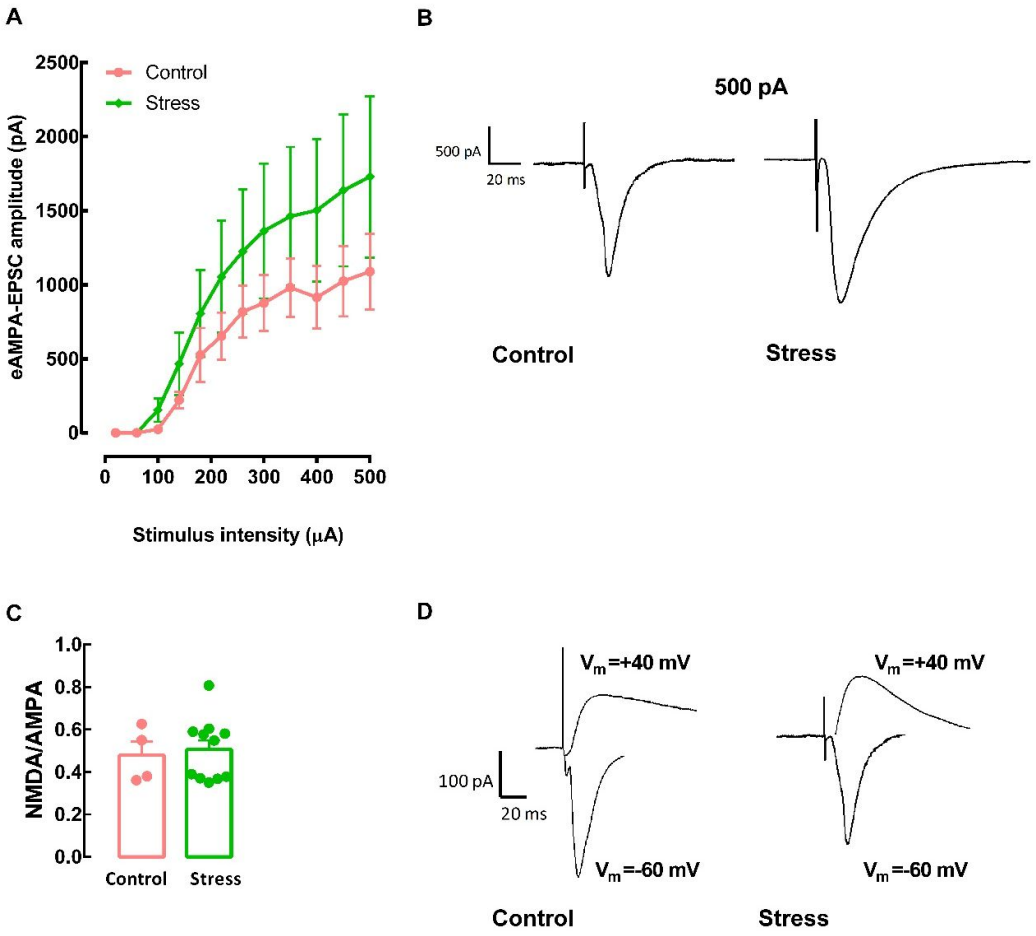

Figure 23. A-B. Input-output curves for evoked AMPA-EPSC in BLA pyramidal neurons upon stimulation in the LA. A. Input-output curves. **B.** Representative traces. The results are presented as mean ± SEM, n=5-9, unpaired Student's *t*-test. **C-D. Evoked AMPA to NMDA ratio in BLA pyramidal neurons is similar in the control and the stressed animals. C.** Bar graph **D.** Representative traces. Currents recorded at -60 mV are mainly mediated by AMPA receptors; when the holding potential is increased to +40 mV and 20 μM CNQX is added, the currents are mainly mediated by NMDA receptors. The results are presented as mean ± SEM, n=4-11; unpaired Student's *t*-test; n=number of recorded cells

4.2.2.3.3. Pyramidal BLA neurons of stressed animals exhibited altered input-output response for evoked IPSC upon stimulation in the LA

Inhibitory neurotransmission in the amygdala prevents the generation of inappropriate emotional and behavioral responses and, under stressful conditions, a reduction in the activity of GABAergic neurons is usually observed (Jie *et al*., 2018). Since the alterations in inhibitory synaptic transmission are well characterized, we investigated whether the number of afferents stimulating an isolated BLA pyramidal neuron varied upon our stress paradigm by recording input-output (I/O) curves for evoked IPSC, which were obtained by calculating the peak amplitude of the evoked currents (at a holding potential of 0 mV), with increasing steps of electrical stimulation. We observed a significant increase in the I/O response in the stressed group when compared to the control group, which was significant at 260 µA intensity stimulation (control 603.79 ± 198.83 pA *vs* stress 1204.22 ± 132.82 pA, p=0.04, n=4-6; n=number of recorded cells). The difference was assessed using unpaired Student's *t*-test analysis by comparing the peak amplitude values for each stimulation intensity between groups (Figure 24A-B). The increase which we observed in the I/O response may be possibly related either to changes in the number of afferent fibers stimulating the neuron or even to an altered sensitivity of the neuron itself.

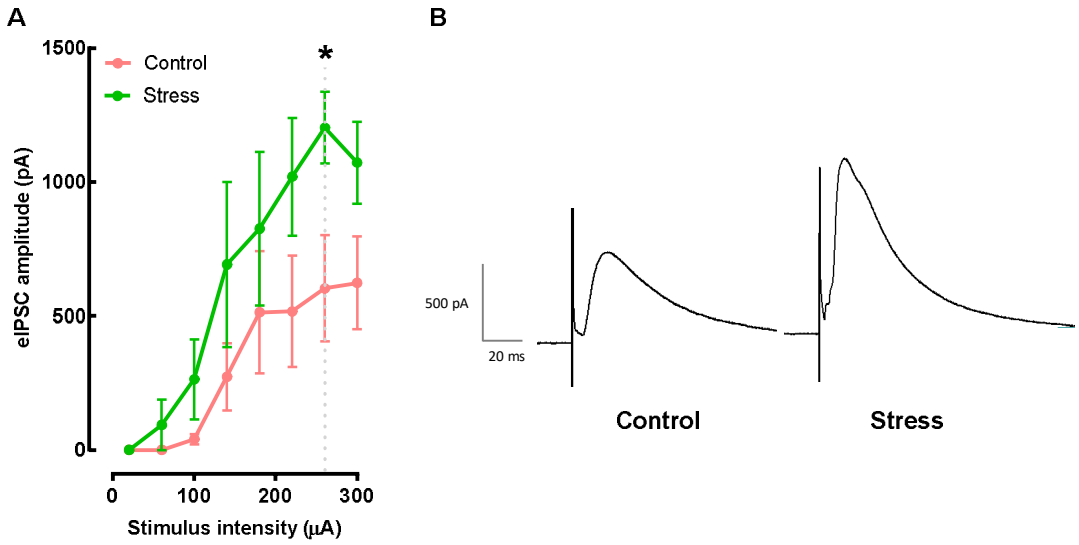

Figure 24. Chronic restraint stress increases the input-output curve for evoked IPSC in BLA pyramidal neurons, upon stimulation in the LA. A. Bar graph of the I/O curve. **B.** Representative traces. The results are presented as mean ± SEM, n=4-6, *p<0.05, unpaired Student's *t*-test, n=number of recorded cells

4.2.2.3.4. Tonic effect of $A_{2A}R$ on eIPSC onto BLA pyramidal neurons is similar in control and stressed animals

Given the robust effect of restraint stress on eIPSC, we asked whether this was dependent on changes in modulation by $A_{2A}R$ within the LA-BLA local circuitry. We assessed the impact of $A_{2A}R$ blockade on eIPSC in BLA pyramidal neurons upon LA stimulation. Upon superfusion of the selective $A_{2A}R$ antagonist SCH58261 (50 nM), all the cells from the control animals responded with an increase in eIPSC amplitude (189.8 ± 33.55% of baseline; n=4; p=0.0753; n=number of recorded cells; Figure 25A-C), suggesting that $A_{2A}R$ exert tonic control over eIPSC onto BLA pyramidal neurons. If this is confirmed with additional experiments, it is, to our knowledge, the first time that $A_{2A}R$ is shown to exert tonic control over basal inhibitory synaptic transmission under physiological conditions in the brain. Similarly, upon superfusion of SCH58261 (50 nM), all the cells from the stressed animals responded with an increase in eIPSC amplitude (171.70 ± 24.57% of baseline, n=3, p=0.1001; n=number of recorded cells; Figure 25A-C); this increase was not significantly different from that observed in the control animals (unpaired Student's *t*-test, p=0.7021; n=number of recorded cells; Figure 25A-C). Taking these data into account, no differences in the tonic actions of adenosine through $A_{2A}R$ between the control and the stressed rats were observed on eIPSC onto BLA pyramidal neurons.

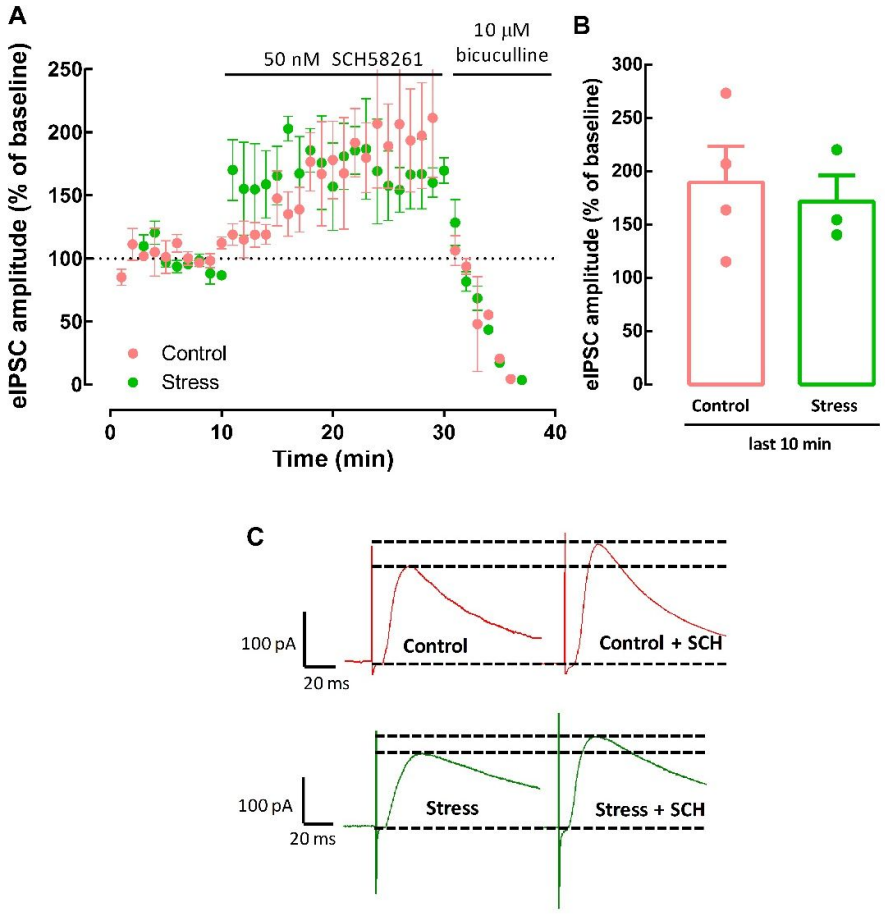

Figure 25. $A_{2A}R$ exert tonic control over eIPSC onto BLA pyramidal neurons, that was not changed in the stressed animals. A. Time course of the effect of the selective $A_{2A}R$ antagonist, SCH58261 (50 nM), on evoked IPSC. **B.** Dispersion of the effect of SCH58261 (50 nM) during the last 10 min of application**. C.** Representative traces of evoked IPSC during the baseline and during the initial 10 minutes of the application of SCH58261 (50 nM); n=3-4; drug effect was compared to a hypothetical value of 100, using a one-sample *t*-test; the control and the stressed groups were compared using unpaired Student's *t*-test; n=number of recorded cells

We also evaluated the effect of the blockade of $A_{2A}R$ on the I/O response by recording the input/output response of the eIPSC before and 20 minutes after the application of the selective $A_{2A}R$ antagonist SCH58261 (50 nM). We did not observe any significant effect of the $A_{2A}R$ blockade in the amplitude of the eIPSC I/O curves in neither the control nor the stressed groups (at 300 μA, control – 518.79 ± 204.43 pA *vs.* control+SCH – 899.86 pA ± 317.39 pA, n=3; stress – 848.80 ± 293.29 pA *vs.* stress+SCH – 915.22 ± 322.09 pA, n=3; two-way repeated-measures ANOVA; n=number of recorded cells; Figure 26). However, it seems that, in the presence of SCH58261 (50 nM), the amplitude of the eIPSC I/O curve in the control group is similar to the I/O curve in the stressed group (at 300 μA, control+SCH – 899.86 pA ± 317.39 pA *vs.* stress – 848.80 ± 293.29 pA, n=3, p=0.3952; n=number of recorded cells; Figure 26). Nevertheless, it is evident that more experiments need to be performed.

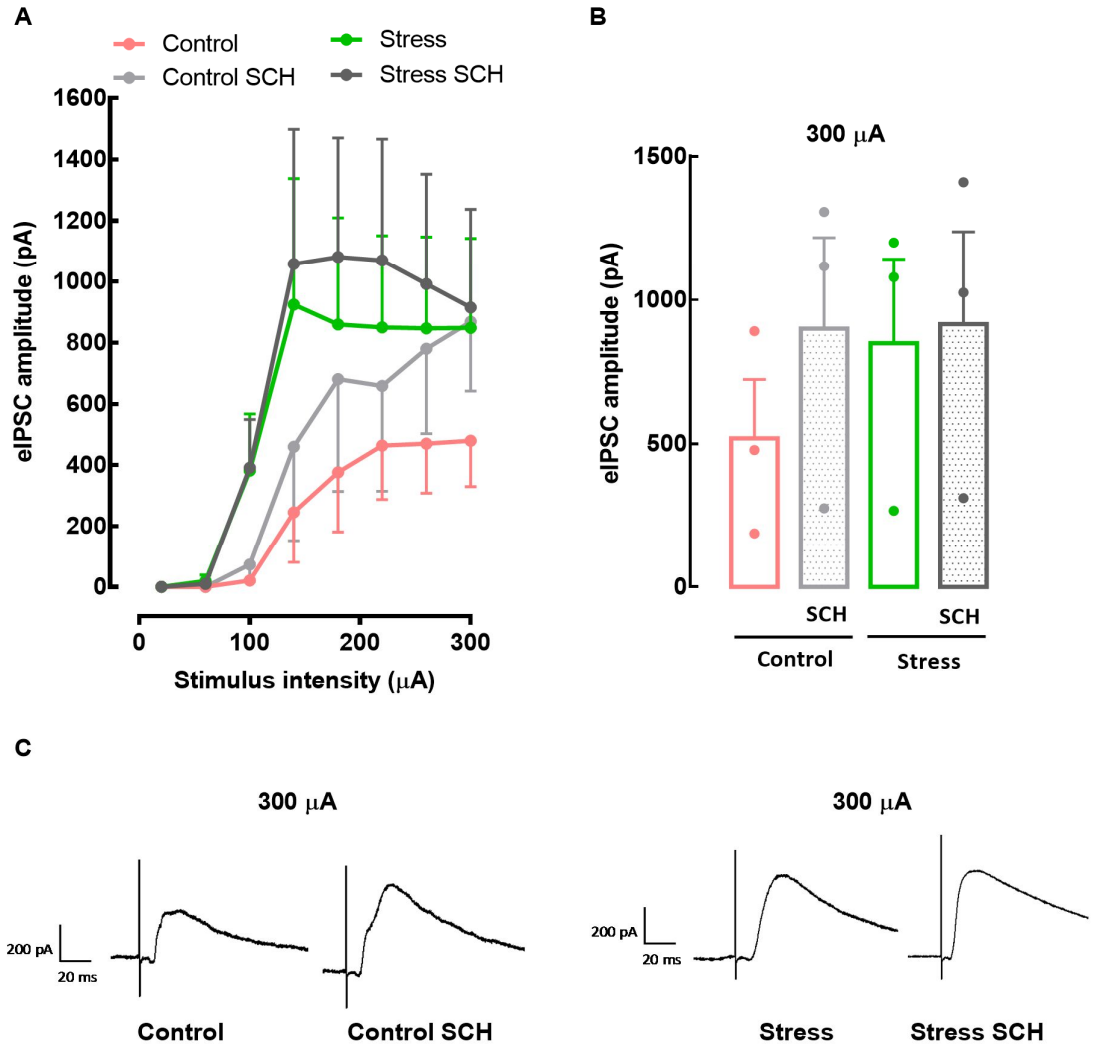

Figure 26. $A_{2A}R$ blockade did not normalize input-output curves for evoked IPSC in BLA pyramidal neurons upon stimulation in the LA. A. I/O curve. **B.** Bar graph of the eIPSC amplitude at 300 μA stimulation. **C.** Representative traces. The results are presented as mean ± SEM, n=3, two-way repeated measures ANOVA; n=number of recorded cells

4.2.2.3.5. Exogenous activation of $A_{2A}R$ produces opposite effect on eIPSC in control and in stressed animals

We tested whether the activation of $A_{2A}R$ would affect the amplitude of eIPSC within the LA-BLA. Upon application of CGS21680 (30 nM), we observed a significant decrease in the amplitude of eIPSC in relation to the baseline during the last 10 minutes of the recording in the control animals (81.84 ± 5.41 %, n=4, p=0.0172, unpaired Student's *t*-test; n=number of recorded cells; Figure 27A-C). Interestingly, we observed the opposite effect in the stressed animals (114.60 ± 8.45 %, n=4, p=0.0172, unpaired Student's *t*-test; n=number of recorded cells; Figure 27A-C). Since the data presented in chapter 4.1 questions the selectivity of CGS21680, it should further be evaluated whether the observed effects are prevented by the selective $A_{2A}R$ antagonist SCH58261.

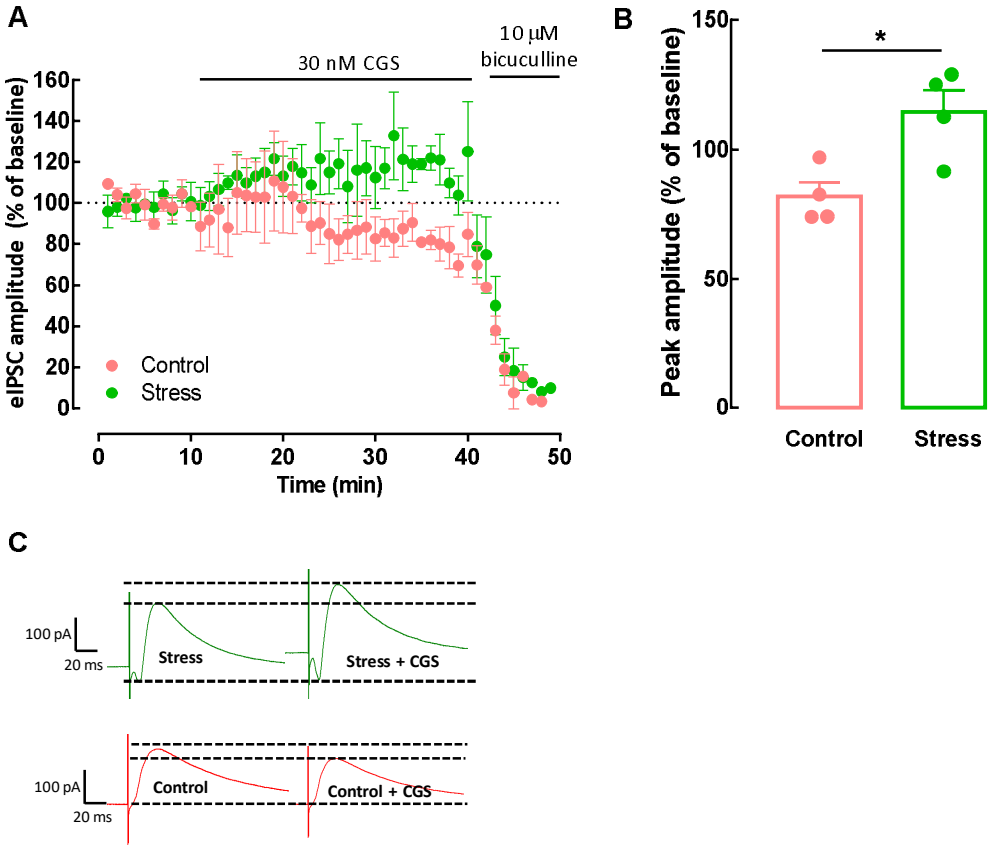

Figure 27. A. Time course of the effect of the selective $A_{2A}R$ agonist, CGS21680 (30 nM), on evoked IPSC. **B.** Dispersion of the effect of CGS21680 (30 nM) **C.** Representative traces of evoked IPSC during baseline and during the last 10 minutes of the application of CGS21680 (30 nM) n=4, * p<0.05, unpaired Student's *t*-test (**B**), one-sample *t*-test with a hypothetical value of 100 (**A**). n=number of recorded cells

4.2.2.4. Discussion

This part of the work provided a new insight on the role of $A_{2A}R$ in the inhibitory synaptic transmission, in LA-BLA synapses, in a well-validated model of chronic restraint stress, in adult Wistar rats. We observed an anxiety-like phenotype upon chronic restraint stress, which is in accordance with other studies, in which 10 days of chronic immobilization caused enhanced anxiety-like behavior (Vyas *et al*., 2004; Wood *et al*., 2008).

We assessed whether there were changes in excitatory synaptic transmission in BLA pyramidal neurons upon chronic restraint stress. It is worth mentioning that altered glutamate transmission is referred as one of the possible causes for the morphological and plasticity changes which pyramidal neurons suffer in the amygdala upon chronic stress exposure, as well as in the hippocampus and the prefrontal cortex (McEwen and Olié, 2005). For instance, it has been shown that chronic restraint stress enhanced excitatory synaptic transmission and increased the number of mature mushroom-like dendritic spines, specifically in the circuit linking BLA pyramidal neurons to the ventral hippocampus, and these circuit-restricted changes were associated with augmented anxiety-like behavior (Magariños *et al*., 1997; Zhang *et al*., 2019). Taking into account the well-described synaptic plasticity changes, we evaluated whether there were modifications in the input-output evoked AMPA-EPSC, but no significant differences were observed. Also, no changes were detected in the NMDA to AMPA ratio in our experimental conditions. The data in the literature are controversial in relation to NMDAR- and AMPAR-mediated currents, according to the brain region studied and the stress paradigm used, since the effects of chronic stress occur both in specific circuits within the amygdala and those which originate from amygdala projections to other output regions, such as the hippocampus (Calhoon and Tye, 2015). For instance, it has been reported, in the lateral amygdala of adult male Wistar rats, that there was an increase in the NMDA to AMPA ratio in pyramidal neurons upon stimulation of thalamic afferents after chronic restraint stress (Suvrathan *et al*., 2014). In hippocampal CA1 pyramidal neurons, a decrease of AMPAR-mediated currents (rather than NMDAR) has been described after chronic unpredictable stress (Kallarackal *et al*., 2013), while, in CA3 neurons, an increase in NMDAR-mediated currents was observed (Kole *et al*., 2002). An enhancement in NMDA to AMPA ratio was also seen in the hippocampus upon *ex vivo* acute corticosterone administration (Tse *et al*., 2011). Some studies even suggest that the administration of NMDAR antagonists is able to reverse depressive symptoms, such as anhedonia and anxiety-like behavior, along with neuronal structural alterations in animal models subjected to chronic stress exposure, further supporting the crucial role of NMDAR (Duman and Li, 2012; Gordillo-Salas *et al*., 2018; Li *et al*., 2011).

Since we did not observe a robust alteration in excitatory synaptic transmission in BLA pyramidal neurons of the stressed animals, we then focused on eventual changes in the inhibitory synaptic transmission.

Several studies have reported a correlation between GABAergic dysfunction (particularly low GABA levels), stress and depression; this became more evident when some antidepressant treatments, including GABA agonists and electroconvulsive therapy, were described to normalize cortical and plasmatic levels of GABA, as well as the expression of GAD67, which is an enzyme involved in the synthesis of GABA, in both human and rodents (Brambilla *et al*., 2003; Sanacora *et al*., 2004; Tunnicliff and Malatynska, 2003).

We observed an enhancement of the inhibitory activity onto BLA pyramidal neurons upon stress, which contradicts some literature describing a reduction in GABAergic activity (Brambilla *et al*., 2003; Fogaça and Duman, 2019). However, it is also known that GABAergic inhibition associated to stress may vary (increase or decrease), according to the affected brain regions and even to the type of stressor. These alterations include changes in the subunit composition of GABA receptors, their Cl^- homeostasis and plasticity in inhibitory synapses (Maguire, 2014). Indeed, unpublished data from our lab show an increase in both vGLUT and vGAT density in animals submitted to the same restraint stress protocol. Additionally, an increase in GABAergic miniature IPSC amplitude has been previously reported, using a similar stress protocol in adult C57BL/6J mice (Masneuf *et al*., 2014). They also found an increase in network-dependent sEPSC frequency only when $GABA_AR$ were blocked, but found no changes in network-independent mEPSC, suggesting a compensatory, and likely protective, increase in inhibitory synaptic transmission to counteract increased excitation onto BLA pyramidal neurons. This could also implicate that our observations from eEPSC amplitude can be further reassessed to evaluate whether under blockade of $GABA_AR$ changes in EPSC become more robust. This, along with increasing the number of recorded cells, recording pyramidal neuron firing rate, as well as evaluating changes in mEPSC and mIPSC frequency and amplitude to assess pre- *versus* postsynaptic changes, will provide us a more complete picture of the synaptic changes within the BLA in our stress model.

Given that hyperexcitability of the amygdala under repeated or chronic stress has been consistently reported, another possible explanation for increased $GABA_AR$ function may come from changes in Cl^- homeostasis following repeated restraint stress. Indeed, dephosphorylation and downregulation of the K^+/Cl^- co-transporter have been described in the paraventricular nucleus after social defeat stress (Miller and Maguire, 2014), pointing to functional deficits as well. In order to better understand the possible effects of stress on inhibitory synaptic transmission, it is important to take into consideration the physiological mechanisms mediated by GABA in mature neurons of the CNS. GABA can act on three receptors ($GABA_AR$, $GABA_BR$, $GABA_CR$) and all of them, when activated, induce

hyperpolarization of the cell, resulting in an increase in the firing rate threshold (a decrease in the probability of action potential initiation), and that is the foundation for the inhibitory actions of GABA, although its receptors are different in the way they function (Tunicliff and Malatynska, 2003). It is noteworthy that, in a developing brain, GABA has been proved to have an excitatory role due to the difference in Cl^- levels inside and outside the cell in relation to an adult brain. More specifically, in immature neurons, the intracellular concentrations of Cl^- are higher than in the extracellular space, while, in mature neurons, the opposite is usually observed. Consequently, in an immature brain, the cell is depolarized upon the actions of GABA and is more likely to fire an action potential (Ben-Ari, 2002; Chen *et al*., 1996; Huang, 2009). In a mature system, although GABA typically acts as an inhibitory neurotransmitter, it has likewise been shown to be able to have excitatory effects (Gutovitz *et al*., 2001) even in normal conditions, but its neurobiological importance remains controversial (Choi *et al*., 2008; Herbison and Moenter, 2011; Wagner *et al*., 2001). This shift in the activity of GABA is also known as its polarity, since, by altering the Cl^- gradient, the net effect of GABA changes from hyper- to depolarization of the postsynaptic cell it targets, and this ability is exclusive to GABAergic currents (Ben-Ari *et al*., 2012). GABA polarity has also been associated to a change in Cl^- normal concentrations under some pathological conditions, including epilepsy and autism disorder (Ben-Ari *et al*., 2012), similarly to what takes place during neuronal development (Ben-Ari, 2002; Haam *et al*., 2012), and this GABA-evoked excitation occurs as part of the mechanisms that lead to the disease. Also, GABA polarity is highly dependent on the levels of neuronal activity and, hence, when there is a malfunction in neuronal circuits, such as their exposure to chronic stress, there also might be enhanced neuronal activity, possibly resulting in a shift from GABA-mediated inhibition to excitation (Ben-Ari *et al*., 2012). Therefore, the increase which we observed in the eIPSC I/O response in the stressed animals might be part of the cellular processes leading to a depression-like phenotype.

The blockade of $A_{2A}R$ with their selective antagonist SCH58261 (50 nM) did not induce a significant effect on the eIPSC I/O response, but the number of recorded cells is too small to infer robust conclusions. More experiments should be performed to clarify the tendency for an increase in I/O response observed in the control animals, and the possible ability of repeated stress to occlude instead of reverting the effect of $A_{2A}R$ blockade on eIPSC. It would be interesting to test whether chronic administration of the $A_{2A}R$ antagonist (SCH58261) *in vivo* would have the same impact or be able to revert the changes in eIPSC which we observed. Interestingly, we determined that $A_{2A}R$ exert tonic inhibition over basal eIPSC onto BLA pyramidal neurons, since their blockade increased eIPSC amplitude. This did not change in the stressed animals. It will be important to clarify whether $A_{2A}R$ act directly in pyramidal neurons to control $GABA_AR$ currents, whether they control excitatory synaptic transmission onto interneurons as in the prefrontal cortex (Kerkhofs *et al*., 2018), or whether

they alternatively increase synaptic communication between feed-forward interneurons, resulting in decreased IPSC onto pyramidal neurons, as is shown in the hippocampus (Rombo *et al*., 2014; Figure 28). Along with Rombo and his colleagues, we also observed that exogenous activation of $A_{2A}R$ decreased eIPSC amplitude onto BLA pyramidal neurons. It should also be mentioned that in the central nucleus of the amygdala, which is mostly constituted by GABAergic cells (Sah *et al*., 2003), ATP derived from astrocytes is converted into adenosine which activates $A_{2A}R$ and increases inhibitory synaptic transmission (Martin-Fernandez *et al*., 2017). If this also applies to BLA GABAergic neurons is, therefore, an important question.

Contrarily to the tonic effect of $A_{2A}R$, which was similar in the control and the stressed animals, exogenous activation of $A_{2A}R$ produced opposite effects in both groups. In the stressed animals, there was a shift towards facilitation of eIPSC onto BLA pyramidal neurons instead of the decrease observed in the control animals. This discrepancy in the findings when using an agonist *versus* an antagonist suggests that these may be targeting different $A_{2A}R$ subpopulations within the local BLA circuitry. It could be receptors located in different synaptic sides (pre- *versus* postsynaptic) or in different cell types and synapses. Another possibility may involve receptors in the same cell type, but which are coupled to different signaling pathways upon repeated stress. These are all questions which will need to be addressed in order to understand the pathophysiological role of our observations.

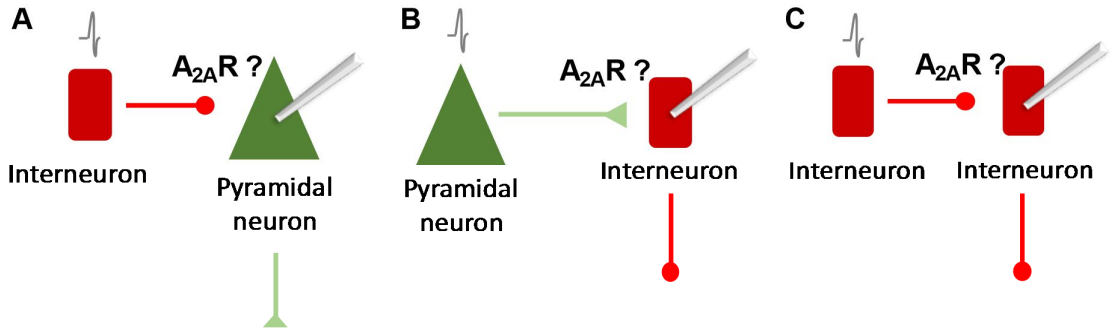

Figure 28. Schematic representation of possible synaptic sites for $A_{2A}R$ to interfere with IPSC onto pyramidal neurons.

Our overall results suggest that, in our model of chronic restraint stress, there are clear alterations in the inhibitory synaptic transmission onto BLA pyramidal neurons synapses. Also, we provided some initial insight concerning a probable role of $A_{2A}R$ in the regulation of these mechanisms, which needs further clarification regarding the exact cell types and/or signaling cascades involved.

5. GENERAL CONCLUSIONS

5. General conclusion

Adenosine, via A_1R and $A_{2A}R$, controls amygdala-dependent behavior, including fear expression (Corodimas *et al*., 2001; Simões *et al*., 2016), which is critically dependent on NMDA receptors. Additionally, general blockade of A_1R and $A_{2A}R$ with caffeine or selective blockade of $A_{2A}R$ prevents mood dysfunction in an animal model of depression (Kaster *et al*., 2015). The amygdala is one of the most affected brain regions in mood disorders. Therefore, this work was focused on the internal circuit of the BLA and was designed to:

1) explore the actions of A_1R and $A_{2A}R$ on NMDA currents, and

2) unravel the possible role of $A_{2A}R$ modulation in changes of both excitatory and inhibitory synaptic transmission in animals submitted to repeated restraint stress which would explain the therapeutic effect of their blockade.

5.1. Main conclusions

✓ In physiological conditions, in LA to BLA synapses, A_1R activation with their selective agonist (CPA) led to a robust inhibition of evoked NMDA-EPSC, an effect which has been described for hippocampal NMDA currents in isolated rat neurons (de Mendonça *et al*., 1995), which is in line with the typically inhibitory function of the A_1R. Surprisingly, $A_{2A}R$ activation with their alleged selective agonist CGS21680 induced a decrease in the amplitude of NMDA-EPSC, an effect that was not prevented by the selective antagonist, SCH58261. This contrasts with the data obtained in the hippocampus, in which $A_{2A}R$ exogenous activation enhanced the amplitude of NMDA-EPSC (Dias *et al*., 2012; Mouro *et al*., 2018; Rebola *et al*., 2008; Rombo *et al*., 2014; Tebano *et al*., 2005; Temido-Ferreira *et al*., 2018). We showed that this curious decrease was prevented by the selective antagonist of A_1R, highlighting the urgent need to design more selective $A_{2A}R$ agonists and explore signaling pathways of potentially different $A_{2A}R$ populations. Moreover, it should not be assumed that $A_{2A}R$ behave in a similar way in different brain regions.

✓ To probe for alterations in pathological conditions, we used two animal models of depression of different ages (adolescent and adult rats), which were subjected to repeated restraint. Unfortunately, the adolescent model of depression did not present a clear anxiety-like phenotype. This obstacle might be explained by the age of the animals, since the adolescence period is characterized by broad variability in anxiety-like behavior (Albani *et al*., 2015; Andrade *et al*., 2003). Also, it is important to take into consideration the fact that distinct animal strains may react differently to a stressor (Walker *et al*., 2009).

✓ In adult Wistar rats, a clear anxiety-like phenotype was obtained upon repeated restraint stress. We focused this part of the work on exploring modulation through $A_{2A}R$, since not only are they generally overexpressed in neurological conditions, but also because there is evidence showing an increased density of these receptors in the BLA of the stressed rats (unpublished data[6]). Furthermore, the selective silencing of BLA $A_{2A}R$ with sh-RNA was sufficient to revert depressive-like symptoms in the stressed animals (unpublished data). This is also in accordance with an increase in the density of glutamatergic and GABAergic synaptic markers (unpublished data). Accordingly, we observed an enhancement of eIPSC I/O response. However, this was not reverted by acute blockade of $A_{2A}R$ in slices. It would be interesting to test whether chronic administration of the $A_{2A}R$ antagonist (SCH58261) *in vivo* would have the same impact. Noteworthy, we showed, for the first time, that $A_{2A}R$ exert tonic control over basal inhibitory synaptic transmission in the BLA. This tonic effect was not altered in the stressed animals. Moreover, we obtained new and curious data when $A_{2A}R$ were exogenously activated with CGS21680: we observed a shift towards an enhancement of inhibitory activity in the stressed rats when compared to the controls. Our current working hypothesis is that different subpopulations of $A_{2A}R$ may be accounting for tonic control of eIPSC *versus* those responding to exogenous agonist treatment which are changed in the BLA stressed animals. Therefore, a detailed analysis of the role of $A_{2A}R$ in different cell types and synapses within the local BLA circuitry, as well as the signaling mechanisms involved, is critical for us to understand the role of $A_{2A}R$ in the expression of mood dysfunction associated with chronic stress and depression.

This study suggests that $A_{2A}R$ may act as central players in the regulation of excitatory/inhibitory balance of neuronal circuits. Further studies of the above mentioned mechanisms may eventually lead to a better knowledge of how the adenosinergic system could be helpful in the prevention and treatment of complex depressive-like conditions. Although the clinical use of both agonists and antagonists of adenosine receptors presents a series of challenges, there are already some drugs approved by the Food and Drug Administration (FDA). For instance, the FDA has recently endorsed an antagonist of $A_{2A}R$, istradefylline, which is marketed as Nourianz, as an adjunctive treatment to traditional medicine used in Parkinson's disease, and is the first non-dopaminergic drug approved in the last 20 years for this pathology (Chen and Cunha, 2020; Torti *et al*., 2018). Another example of a FDA-approved $A_{2A}R$ ligand used in clinics is regadenoson, marketed as Lexican, which is an $A_{2A}R$ agonist used in myocardial imaging, due to its ability to induce coronary vasodilation (Reyes, 2016). In addition to the

[6]unpublished data described in "Role of the adenosinergic system in animal models of chronic stress and depression", PhD dissertation, Nuno Miguel de Jesus Machado, 2016

drugs which are already being used in clinical practice, there is a wide range of drugs undergoing preclinical and clinical trials, which can be accessed on the official clinical trials website: ClinicalTrials.gov (Jacobson *et al*., 2019).

Overall, this work shows that $A_{2A}R$ are promising targets for the development of novel strategies for the treatment of such a complex and challenging disease as depression.

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