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RESUMO

O stresse modula a função cerebral e a cognição. Situações de stresse excessivo podem induzir alterações morfológicas e funcionais no cérebro com consequências cognitivas. Estudos clínicos têm vindo a demonstrar que o stresse pode estar relacionado com uma vulnerabilidade aumentada para sofrer de desordens psiquiátricas como a depressão e a ansiedade. Os modelos de stresse que melhor parecem reproduzir o que se observa clinicamente são aqueles em que este é induzido durante a infância, num período de grande desenvolvimento neuronal. Os estudos publicados indicam que eventos traumáticos ocorridos no período pós-natal têm um efeito permanente no cérebro. Um dos modelos mais aceites e utilizados pela comunidade científica como paradigma de stresse crónico no adulto consiste na perturbação da relação mãe-filho, em animais de laboratório, denominado de separação maternal. A aplicação deste protocolo resulta em níveis plasmáticos de corticosterona permanentemente elevados bem como em alterações no sistema nervoso central. Uma das estruturas cerebrais mais sensíveis ao stresse é o hipocampo, área cerebral crucial na aprendizagem e memória.

A adenosina é um importante neuromodulador da excitabilidade neuronal do hipocampo tendo acções importantes, não só na transmissão basal, mas também na plasticidade sináptica. Os seus efeitos são mediados principalmente por dois receptores, os receptores $A_{1,}$ com acções principalmente inibitórias e presentes em grande densidade; e os receptores $A_{2A,}$ com acções maioritariamente excitatórias e que apresentam baixos níveis de expressão nesta área cerebral. Alterações na densidade destes receptores foram observadas em diversas condições, como actividade convulsiva, em modelos de doenças neurodegenerativas ou mesmo como consequência do envelhecimento. A alteração nos níveis dos receptores da adenosina tem consequências na sua acção neuromoduladora interferindo com a normal regulação da excitabilidade neuronal.

O objectivo do presente trabalho foi o de avaliar, na idade adulta, o efeito de um stresse crónico, induzido logo após o nascimento, nos receptores de adenosina. Foram também avaliadas diversas proteínas intervenientes em processos de plasticidade e memória que podem estar envolvidas no aumento da susceptibilidade do hipocampo como consequência do stress.

Ratos machos da estirpe Wistar foram divididos em dois grupos, controlo (CTR) e separação maternal (MS-*Maternal Separation*). O grupo MS foi separado da mãe diariamente, do dia 2

ao dia 14 de idade, durante 3 horas, enquanto o grupo controlo permaneceu não manipulado. Entre as 6 e as 8 semanas de idade, os animais foram sacrificados e várias áreas cerebrais (hipocampo, córtex e estriado) utilizadas para ensaios de ligação e W*estern Blotting*.

Os níveis dos receptores A_1 e A_{2A} de adenosina foram quantificados por ensaios de ligação utilizando antagonistas selectivos para os receptores de adenosina do subtipo A_1 (DPCPX, 0-10 nM) e A_{2A} (ZM 241385, 0-10nM) ou por W*estern Blotting* nas várias áreas cerebrais. A separação maternal induziu, no hipocampo, um aumento de 56,2±9,4 % (n=3; p<0,05) nos níveis de receptores do subtipo A_{2A} , obtido por *Western Blotting* e uma diminuição de 1202±68 fmol/mg (n=4) para 1073±48 fmol/mg (n=4) dos receptores do subtipo A_1. Nas restantes áreas cerebrais não foram observadas diferenças nos níveis destes receptores.

As alterações induzidas pelo stress nos níveis dos receptores de adenosina seguem a mesma tendência daquelas que ocorrem com o envelhecimento, onde foi observada uma alteração na interacção entre estes receptores. Com o objectivo de estudar a interacção A_1/A_{2A} em condições de stress realizaram-se curvas de deslocamento, na presença e ausência de um agonista selectivo para os receptores do subtipo A_{2A} (CGS 21680 – 30nM). Estas curvas foram realizadas promovendo o deslocamento da DPCPX (2 nM) um antagonista selectivo dos receptores do subtipo A_1 pela CPA (0-6 μM) um agonista selectivo dos mesmos receptores. Não foram observadas alterações a este perfil de deslocamento entre animais CTR e MS, na presença ou ausência de CGS 21680.

A localização regional dos efeitos do stresse nos níveis dos receptores de adenosina, com impacto predominante no hipocampo, conduziu à quantificação dos níveis dos receptores de glucocorticoides e mineralocorticoides nas várias áreas cerebrais por W*estern Blotting*. Estes são os receptores citoplasmáticos para a corticosterona e são fundamentais na mediação da resposta ao stress. Têm ambos uma expressão elevada no hipocampo e alterações na sua razão (MR/GR) implicam alterações nas propriedades neuronais e em fenómenos de plasticidade e memória. A separação maternal induziu, em todas as áreas cerebrais estudadas, uma diminuição nos níveis dos receptores de glucocorticoides sem modificações nos níveis dos receptores de mineralocorticoides. Estas alterações tiveram maior impacto no hipocampo (26,1±4,5% ;P<0,05; n=3) comparando com o córtex (16,3 ±3,4%; P<0,05; n=4) ou o estriado (9,6 ± 2,6%; P<0,05; n=4).

Resumo

Com o objectivo de explorar o potencial impacto destas alterações na função do hipocampo, foram avaliadas várias proteínas envolvidas na transmissão sináptica ou em processos de plasticidade. Foram investigadas alterações nos níveis da subunidade 1 do receptor AMPA para o glutamato ($GluR_1$), da subunidade β_3 do receptor $GABA_A$ (β_3-$GABA_A$), do receptor Trk_B, do factor neurotrófico derivado do cérebro (BDNF), e do receptor 1 da hormona de libertação da corticotrofina (CRH-R_1). A separação maternal induziu alterações nos níveis de todas estas proteínas no hipocampo. Observa-se uma diminuição dos níveis de $GluR_1$ (25%±5%; $P<0,05$;n=3), de β_3-$GABA_A$ (16±2%; $P<0,05$; n=3) e dos receptores Trk_B (17±2,3%; n=3;$P<0,05$) e um aumento de DBNF (67±13%; n=4; $P<0,05$) e do CRH-R_1 (55±7%; $P<0,05$; n=3).

Estas alterações mostram que o stress crónico numa fase precoce da vida, induz modificações permanentes no cérebro, principalmente no hipocampo que envolvem tanto a transmissão inibitória como excitatória. Os receptores $GABA_A$ são os receptores ionótropicos do principal neurotransmissor inibitório do sistema nervoso central e a diminuição dos níveis da subunidade β_3 deste receptor pode traduzir-se em alterações permanentes na neurotransmissão inibitória. Por outro lado, tanto a inserção de receptores AMPA contendo a subunidade $GluR_1$ como a expressão dos receptores Trk_B são importantes na indução da potenciação de longa duração (LTP). As alterações observadas nos níveis destes receptores poderão justificar a diminuição da LTP já observada em animais submetidos a separação maternal.

Com o objectivo de avaliar se todas estas alterações ocorriam em paralelo com modificações na densidade sináptica utilizou-se a sinaptofisina como marcador sináptico e avaliaram-se, por W*estern Blotting*, alterações nos níveis desta proteína entre animais CTR e MS. Não se observaram alterações na densidade sináptica, detectáveis pela técnica utilizada.

Assim, este trabalho vem mostrar que a indução de stresse num período de grande desenvolvimento neuronal se traduz em alterações permanentes no cérebro, detectáveis na idade adulta, com especial impacto no hipocampo. Estas alterações envolvem várias proteínas intervenientes não só na transmissão sináptica basal mas também em fenómenos de plasticidade, tendo consequentemente implicações funcionais na memória. Mostra-se ainda, pela primeira vez, que num modelo de stress crónico usado no estudo de psicopatologias, os receptores da adenosina estão alterados. O facto de estas alterações

seguirem um padrão semelhante ao que aconece no envelhecimento sugere que o stress pode aumentar a susceptibilidade do hipocampo não apenas para futuros insultos, mas pode também exacerbar os défices cognitivos que acompanham o próprio envelhecimento Estes resultados suportam ainda a utilização de antagonistas selectivos dos receptores $A_{2A,}$ que têm sido propostos como antidepressivos em outros modelos.

ABSTRACT

Stress induced early in life interferes with the establishment, maintenance and development of neuronal networks. Some of these changes share similarities with those observed in aging. An imbalance in the density of the adenosine A_1 and A_{2A} receptors in the hippocampus and striatum of aged animals, was previously reported. We now investigated if the induction of chronic stress, through maternal separation, could have a similar impact on adenosine receptors and related proteins in the hippocampus, striatum and cortex.

Male Wistar rats were assigned to either control (CTR) or maternal separated (MS) group. The MS were separated from their mothers for 3h/day (2-14 postnatal days) while CTR were left undisturbed. At 7-8 weeks of age they were sacrificed, the hippocampi and striata dissected and processed. Saturation binding curves with the selective antagonist for A_1 receptors, [^{3}H]DPCPX (0-7 nM) or the selective antagonist for A_{2A} receptors, [^{3}H]ZM 241385 (0-7 nM) were performed. The displacement of [^{3}H]DPCPX (2 nM) by the selective A_1 receptor agonist, CPA (0-6 μM) in the presence of the selective A_{2A} receptor agonist, CGS 21680 (30 nM) was assessed. The levels of A_1 and A_{2A} receptors, GR and MR, $GABA_A$-β_3, $GluR_1$, CRH-R_1, Trk_B and synaptophysin were quantified by immunoblot analysis.

The hippocampus was the only area with changes in the levels of adenosine receptors. A_{2A} receptors were found to be increased by 56.2±9.4 % (n=3; $P<0.05$) and A_1 slightly decreased (1202±68 fmol/mg (n=4) to 1073±48 fmol/mg (n=4)) in MS compared to CTR animals. These changes were not accompanied by alterations in the affinity pattern of A_1 receptors induced by activation of A_{2A} receptors. A region specific effect was further confirmed by observing that the MR/GR ratio was decreased more markedly in the hippocampus of MS animals than in other brain areas. A decrease of 26.1±4.5 % (n=3, $P<0.05$) in the levels of GR was observed in the hippocampus compared to a decrease of 16.3±3.4 % (n=4, $P<0.05$) in cortex and of 9.6±2.6 % (n=4, $P<0.05$) in striatum. MS had also an impact in hippocampal markers of synaptic plasticity. $GluR_1$ subunit, $GABA_A$-β_3 and Trk_B receptors were decreased (n=3, $P<0.05$) while BDNF and CRH-R_1 were increased (n=3-4, $P<0.05$).

Overall these data show that stress induces long-term changes in the hippocampus, impacting on the levels of adenosine receptors and related synaptic plasticity markers, in an "early-ageing" phenomenon. Moreover, these also suggest long-term consequences for hippocampal function that may imply increased susceptibility of this brain area for further insults. Finally, the data obtained further support the involvement of adenosine receptors in

psycopathologies and the blockade of A_{2A} receptors as therapeutical approach against stress-induced cognitive impairments.

KEYWORDS:

Adenosine, A_{2A} receptors, stress, maternal separation, hippocampus

INDEX

ABREVIATIONS LIST

A_1R - Adenosine A_1 Receptor

$A_{2A}R$ - Adenosine A_{2A} Receptor

ACTH - Adrenocorticotrophin

ADA - Adenosine Deaminase

AMPA - L-α-amino-3-hydroxy-5-methylisoxazole-4-propionate

BDNF – Brain-Derived Neurotrophic Factor

CRH - Corticotrophin-Releasing-Hormone

CRH-R_1 – Corticotrophin-Releasing-Hormone Receptor 1

CTR - Control

DAPI - 4',6-diamidino-2-phenylindole

DG - Dentate Gyrus

GABA - γ-Aminobutyric acid

$GABA_A$ - γ-Aminobutyric acid receptor A

GFAP - Glial Fibrillary Acidic Protein

$GluR_1$ - Glutamate Receptor 1 subunit of AMPA Receptor

GR - Glucorticoid Receptor

HPA-axis - Hypothalamic-Pituitary-Adrenal axis system

LTD- Long-Term Depression

LTP - Long-Term Potentiation

MAP_2 – Microtubule-Associated Protein 2.

MR - Mineralocorticoid Receptor

MS – Maternal Separation

NMDAR - N-methyl-D-aspartic acid Receptor

PFA - Paraformaldahyde

RIPA - Radio-immunoprecipitation-assay

SNAP25 - Synaptosomal-associated protein 25

Trk_B – Tropomiosine related kinase B receptor

$\beta_{2/3}GABA_A$ - β_2/β_3 subunit of $GABA_A$ receptor

1| INTRODUCTION

1.1| Stress and HPA-axis system

Stress has been studied for a long time due to its importance in the adaptative response of the organism to the environment. Any type of threat that compromises homeostasis, and by that requires compensatory responses to return to equilibrium can be defined as stress. Therefore, stress plays an important role in all physiological systems through neuronal and endocrine mechanisms (McEwen, 2007). It is now recognized that stressful events may have a role in the development and/or susceptibility for psychiatric disorders (McKinney, 1984; Willner *et al.*, 1997) such as anxiety, depression or posttraumatic stress disorders.

Stressful events are present throughout life, triggering physiological responses that involve changes at peripheral and central levels coordinated by the central nervous system, mostly through the Hypothalamic-Pituitary-Adrenal axis system (HPA-axis) (Herman and Cullinan, 1997). In a stressful situation the paraventricular nucleus of the hypothalamus is activated and releases Corticotrophin-Releasing-Hormone (CRH), which in turn will trigger pituitary neurons to secrete Adrenocorticotrophin (ACTH) to the bloodstream. Circulating ACTH will induce the secretion of glucocorticoids by the adrenal cortex (cortisol in humans and corticosterone in rodents) being those the key mediators of stress response (Tsigos and Chrousos, 2002) (Figure 1.1).

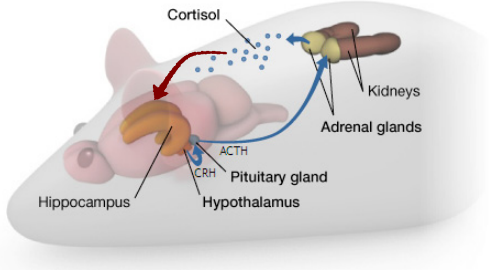

Figure 1.1: Schematic representation of the HPA-axis. The hypothalamus releases CRH that will activate pituitary neurons to release ACTH. This hormone will enter the bloodstream and induce the release of cortisol by the adrenal glands. (Adapted from learn.genetics.utah.edu /content/epigenetics/rats)

Glucocorticoids are the major stress hormones and play a vital role in stress response, mobilizing energy stores, suppressing non-essential physiological systems, modulating behavioral responses to the stressful stimuli, and regulating the stress response system through negative feedback inhibition (Johnson *et al.*, 1992). In addition to the peripheral effects in the immune response or metabolism, glucocorticoids have important effects on the brain, particularly in the hippocampus (reviewed by Joels, 2008).

Physiological actions of glucocorticoids are mediated by two different types of corticosteroid receptors: the Type I, high-affinity, mineralocorticoid receptor (MR) and the Type II, low-affinity, glucorticoid receptor (GR). These are transcription factors and present a distinctive distribution pattern among brain areas. Whereas GRs are ubiquitously distributed in neurons and glial cells, being present in higher levels in the hippocampus, MRs are mostly expressed in hippocampal and septal neurons (Figure 1.2) and have 10 times more affinity for corticosterone than GRs (Reul and de Kloet, 1985). This different affinities will lead to a particular pattern of activation of GR and MR: while MR are tonically activated by circulating glucocorticoids (70% occupancy of MR versus 10% occupancy of GR), GR activation only occurs when the cytoplasmatic levels of these hormones increase (both receptors can reach an occupancy of 90%), as in stressful situations or during the circadian peak (Sandi, 1998; Tsigos and Chrousos, 2002; Joels, 2006). This differential activation of corticosteroid receptors will lead to biphasic effects of stress hormones. In the hippocampus, for example, low levels of corticosterone are associated to small amplitude calcium currents, whereas higher levels of this hormone increase calcium currents (Joels *et al.*, 1994; Karst *et al.*, 1994). Overall, the individual properties, distribution and density of both MR and GR (and particularly their ratio) will lead to distinct effects of stress hormones in different neuronal populations, depending on the activation status of the HPA-axis (Joels, 2006).

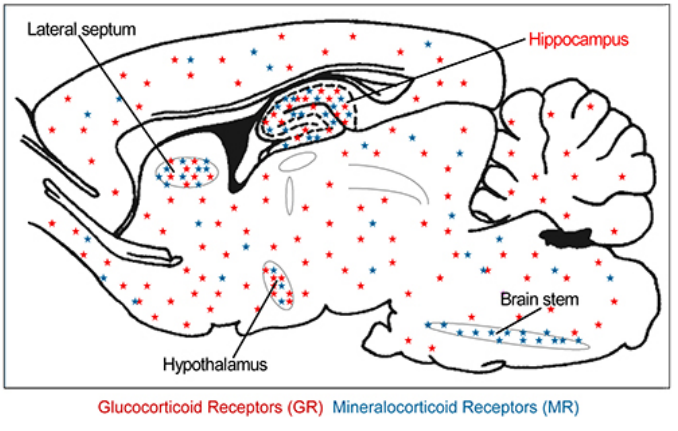

Figure 1.2: Schematic representation of the expression profiles of glucocorticoid (GR) and mineralocorticoid (MR) receptors in the rat brain. The hippocampus is the brain area that presents higer expression of both receptors. From Max Plank Institute of psychiatry web page: www.mpipsykl.mpg.de/en/research/groups/almeida/projects

The control of the HPA-axis activity is essential to maintain a functional and adaptative response to stress and multiple inhibitory pathways are present to achieve that. Stress response can be limited, either by a direct feedback inhibition mediated by the GR present in hypothalamic and pituitary neurons that will decrease the release of ACTH and CRH, or by neuronal pathways projected from other brain areas (Herman and Cullinan, 1997). The most studied neuronal pathway-mediated inhibition of the HPA-axis is the one that arises from the hippocampus, the brain structure that presents higher levels of both corticosteroid receptors and the one more susceptible to stress (Kim *et al.*, 2006). The control of the HPA-axis can however be compromised in situations that are characterized by persistently higher levels of glucocorticoids, such as chronic stress, aging or in psychopathologies (Pardon and Rattray, 2008). Interestingly all of these three situations induce morphological and functional changes in the hippocampus (McEwen, 2007).

1.2| Stress and the hippocampus

The hippocampus is one of the main areas involved in cognition and crucial in memory storage and retrieval (Lopes da Silva *et al.*, 1990). Hippocampal neuroanatomy is highly organized providing a unidirectional circuit divided in 3 areas: Dentate gyrus (DG), CA3 and CA1 (Figure 1.3). The information arises from the entorhinal cortex to the DG which axons (mossy fibers) innervate CA3 synapse. CA3 projects through Schaffer collaterals axons to CA1 and this back to the entorhinal cortex closing the unidirectional circuit of input integration (Lopes da Silva *et al.*, 1990).

The effects of stress on hippocampal plasticity were first explored by Foy and co-workers when studying stress–induced impairments in Long-Term Potentiation (LTP) (Foy *et al.*, 1987). Since then, stress and corticosterone have been shown to have multiple effects, being involved in hippocampal dependent learning and memory (Diamond *et al.*, 1999), in the regulation of neuroexcitability (Prager and Johnson, 2009), LTP and long term depression (LTD) (Kim *et al.*, 1996; Xu *et al.*, 1997), the most characterized molecular models of memory.

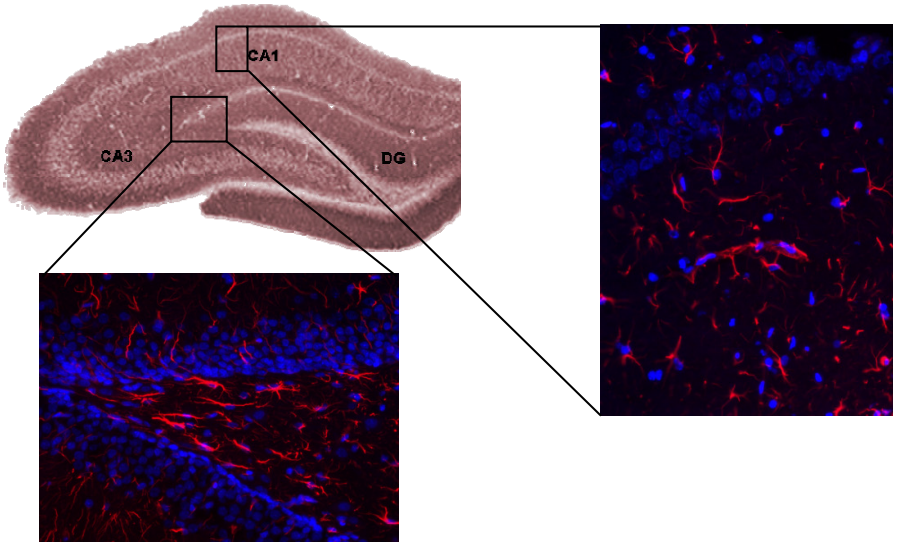

Figure 1.3: Image of one hippocampal slice with the three main areas illustrated: the dentate gyrus (DG), CA3 and CA1. Amplified images of CA1 (A) and DG (B) are fluorescence immunohistochemistry images obtained by the author (40x amplification) from a rat hippocampal slice section with 12µm. Neuronal nuclei are stained with DAPI (blue), astrocytes are identified by red fluorescence using anti Glial Fibrillary Acidic Protein (GFAP) immunoreactivity.(For a detailed discription of the protocol see Annex I)

The permanent activation of hippocampal GR can have profound deleterious effects on this brain structure at both cellular and molecular levels (reviewed by Kim *et al.*, 2006). Different studies have described neuronal death (Sapolsky, 1985), morphological changes, as atrophy of apical dendrites in CA3 pyramidal neurons (Magarinos *et al.*, 1997) and even impaired adult neurogenesis (Dagyte *et al.*, 2009) in the hippocampus of chronic stressed animals. Changes in the levels of neurotransmitters receptors were also reported. The levels of $GABA_A$ and NMDA receptors are decreased (Caldji *et al.*, 2000b; Roceri *et al.*, 2002) and the subunits content of AMPA receptor is also changed (Pickering *et al.*, 2006). Changes in neurotrophins were observed in stressed animal such as lower levels of Brain-Derived Neurotrophic Factor (BDNF) (Smith *et al.*, 1995b). All these profound changes, together with the acute effects mediated by GR activation, lead to an altered excitability and synaptic plasticity, such as impairments of LTP and LTD, with implications in learning and memory. This is confirmed by the observed learning deficits (Sousa *et al.*, 2000) and anxious behavior (Caldji *et al.*, 2000a) characteristic of stressed animals. Figure 1.4 presents a summarized representation of the stress effects in the hippocampus.

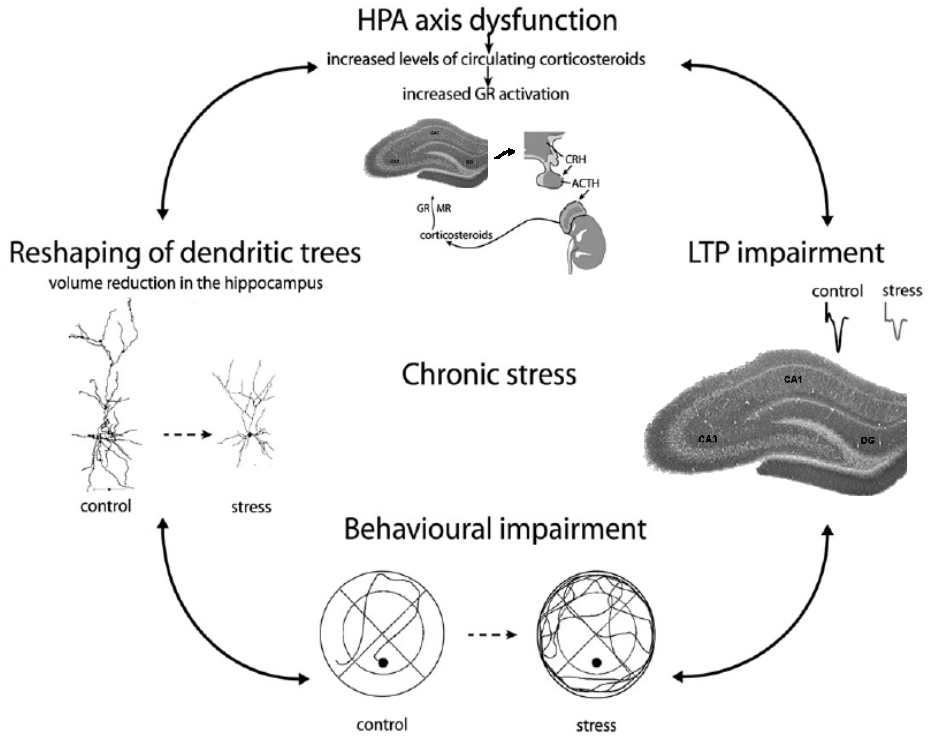

Figure 1.4: Schematic representation of the stress effects in the hippocampus. Changes in the balance between MR and GR activation will trigger cellular and molecular changes in the hippocampus, ultimately leading to behavioral impairment, adapted from Sousa (2008).

1.3| Adenosine: possible role of A_{2A} receptors in stress response

Adenosine is present in the cell as a metabolite (Stone *et al.*, 1985) where, in situations of compromised energy status, acts with an homeostatic role in the control of cellular metabolism (Arch and Newsholme, 1978). Moreover, this nucleotide is a well known neuromodulator with important actions, not only in the regulation of neurotransmitter release and post-synaptic excitability, but also modulating the response of other receptors in several brain structures (reviewed by Sebastião and Ribeiro, 2009).The neuromodulatory role of adenosine in the hippocampus is mediated by the balance between inhibitory and excitatory actions of adenosine A_1 and A_{2A} receptors (A_1R and A_{2A}R) respectively (for a review see Cunha, 2001). Adenosine receptors are metabotropic G protein coupled receptors. A_1 receptors are usually coupled to adenylate cyclase inhibitory G proteins (Gi/Go) and A_{2A} receptors to adenylate cyclase excitatory G proteins (Gs) (Linden, 2001). The expression of adenosine receptors in the central nervous system is different among brain areas (Figure 1.5). A_1 receptors are widely distributed, being more abundant in the cortex, cerebellum and hippocampus (Reppert *et al.*, 1991), while A_{2A} receptors display a more restricted pattern. High expression levels of A_{2A} receptors are observed at the olfactory bulb and striatum (Jarvis and Williams, 1989), whereas in the neocortex and hippocampus they are present at very low levels (Cunha *et al.*, 1994a; Kirk and Richardson, 1995). By differential activation of A_1 and A_{2A} receptors, adenosine is able to modulate neuronal excitability and synaptic plasticity in the hippocampus, mainly regulating glutamate release (Sebastião and Ribeiro, 1996). In spite of the low expression and density of A_{2A} receptors in the hippocampus, they play an important role in the modulation of synaptic transmission. Different effects resulting from A_{2A} receptor activation were

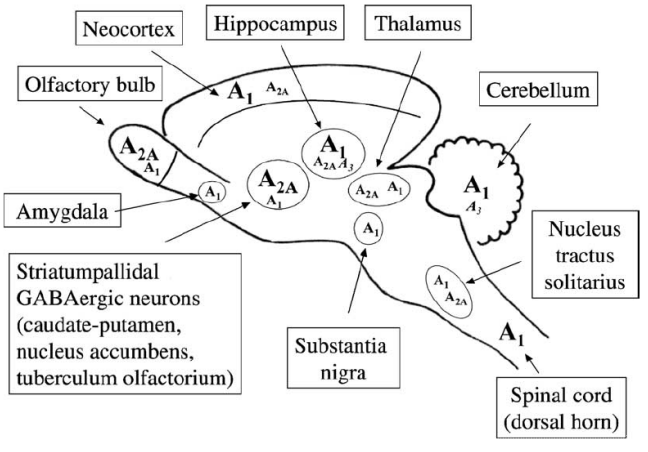

Figure 1.5: Schematic representation of the distribution of adenosine receptors in the brain. The hippocampus presents high levels of adenosine A_1 receptors and low levels of adenosine A_{2A} receptors (Adapted from Ribeiro *et al.*, 2003)

observed, not only in neurons but also in astrocytes (reviewed by Sebastião and Ribeiro, 2009). Presynaptic A_{2A} receptors were shown to modulate A_1 receptor inhibitory actions, resulting in a facilitatory effect on synaptic transmission (Cunha *et al.*, 1994b; O'Kane and Stone, 1998). In neurons, other pre-synaptic effects were observed in the modulation of release or uptake of different neurotransmitters, such as glutamate (Lopes *et al.*, 2002) GABA (Cunha and Ribeiro, 2000; Cristóvão-Ferreira *et al.*, 2009) or acetylcholine (Cunha *et al.*, 1994b). Post-sinaptically, A_{2A} receptors are being implicated in the modulation of AMPA mediated currents (Dias *et al.*, 2008). Evidences also point towards a crosstalk between A_{2A}R and Trk_B receptor (neurotrophin receptor) with implications for BDNF effects (Diogenes *et al.*, 2004; Assaife-Lopes *et al.*, 2007; Diogenes *et al.*, 2007). In the astrocytes, A_{2A} receptors are involved in the modulation of glutamate release (Nishizaki *et al.*, 2002) and GABA uptake (Critóvão-Ferreira *et al.*, 2008).

Extracellular levels of adenosine affect adenosine signaling. If increased, as in noxious brain conditions, such as hypoxia or ischaemia, A_{2A} receptors are activated (Fredholm, 1997) and A_1 receptors desensitized (Fernandez *et al.*, 1996). If this is prolonged, the levels of adenosine receptors will change. It was observed that in different chronic noxious brain conditions the levels of A_{2A} receptors in the hippocampus are usually increased whereas those of A_1 receptors are decreased (reviewed by Cunha, 2005). Aged animals, which also present an imbalance in adenosine receptors (Cunha *et al.*, 1995), display changes in the transduction mechanisms associated to these receptors (Lopes *et al.*, 1999a), suggesting that the balance between A_1 and A_{2A} receptors is crucial to adenosine response.

The first observation that adenosine and stress where linked came from the work of Scaccianoce and collaborators (1989) showing that adenosine could modulate ACTH production, probably in the anterior pituitary, as was later confirmed (Chau *et al.*, 1999). However, the evidence that stress could change adenosine neuromodulatory system comes only in 2006. Cunha and co-workers observed that one episode of sub-chronic stress could lead to an imbalance of adenosine receptors in a similar pattern to what happens in noxious brain conditions (Cunha *et al.*, 2006). Moreover, the use of a selective A_{2A} antagonist was able to counteract not only the changes in adenosine receptors but also the synaptic loss present in stressed animals (Cunha *et al.*, 2006).

Both stress and adenosine are being implicated in psychiatric disorders. Adenosine receptors are being studied as possible therapeutic targets for its treatment (Cunha *et al.*, 2008); and several animal models are being validated for the study of these disorders (Kalueff and

Tuohimaa, 2004). The stress models that are more accepted for the study of psychiatric disorders are those where stress is induced early in life. The induction of stress in a period of massive brain development will lead to permanent changes in the central nervous system (Heim *et al.*, 1997) and interfere in brain and behavioral development. As a consequence, brain circuits are more susceptible to further challenges and consequently predisposed to pathology (Caldji *et al.*, 2000a).

1.4| Maternal Separation Model

Maternal separation protocol (MS) is a neonatal chronic stress model that has been proposed for the study of mood disorders. Daily separation of the litter from their mother for 180 minutes each day during postnatal days 2–14 induces repeated neonatal stress (Ladd *et al.*, 2000). The parent offspring interaction is altered, not only the pups are deprived from maternal care, maternal behavior also remains aberrant after the reunion (Newport *et al.*, 2002).

Among different stress models, MS is the one thought to be more physiological, it induces a psychological stress by altering maternal care, that can be transposed to humans (Newport *et al.*, 2002). In fact, different clinical observations have been linking many adulthood psychiatric disorders with stressful childhood events. A significant coincidence was found between the occurance of an early trauma as parental loss, sexual abuse or physical assault in childhood, and the chance of developing affective disorders (Heim and Nemeroff, 2001; Sullivan *et al.*, 2006). Chronic neonatal stress will culminate in an over-activation of the HPA-axis that persists in the adult life leading to permanently higher levels of corticosterone and to cognitive deficits (Figure 1.4). The Increased corticosterone levels in this period of brain development lead to permanent changes at the levels of gene expression, neurochemistry, electrophysiology, and morphology (Bakshi and Kalin, 2000; Kaufman *et al.*, 2000).

2| AIM

Chronic stress is known to induce a dysfunction in the HPA-axis leading to higher circulating levels of stress hormones which have deleterious effects on brain function. The hippocampus is one of the main brain areas affected by stress hormones with implications for synaptic plasticity and behavior. Interestingly other situations in which an impairment of hippocampal function occurs, as aging or in psychopathological conditions, also present a dysfunction in the HPA-axis.

Different observations have been linking adenosine signaling with stress effects and pointing adenosine receptors as potential targets for anti-depressive drugs and even recovery from damage. Nevertheless, it is not known whether a chronic stress situation induces sustained changes in the neuromodulation by adenosine.

This study aimed **to evaluate the effect of chronic stress in the levels of adenosine A_1 and A_{2A} receptors in the hippocampus.** An imbalance of these receptors could modify adenosine signaling and by that contribute for the hippocampal associated deficits observed in chronic stressed animals. Moreover, several other markers linked to hippocampal function or related to adenosine were quantified **to assess if the induction of stress early in life could lead to permanent effects observed at adult age.**

Clarifying the involvement of adenosine receptors in the chronic stress effects will be helpful to highlight new strategies to treat psychopathologies. An overview of hypothesis underlying the present thesis is illustrated in Figure 2.1.

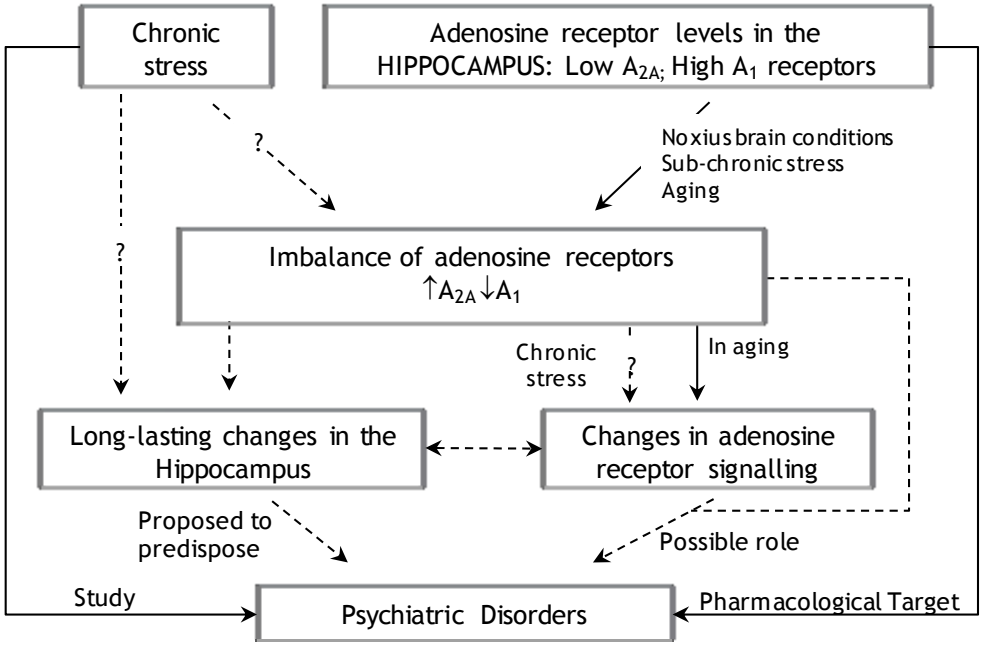

Figure 2.1: Adenosine neuromodulation in the hippocampus under stress conditions. The levels of adenosine receptors are fundamental for adenosine signaling, and may change, as occur in noxious brain conditions, or during the aging process, with consequences for hippocampal function. Broken arrows indicate connections that are not fully characterized. Interrogation points indicate the questions to be addressed in the present work.

3| METHODS

3.1| Maternal separation protocol

The protocol used has been validated and described before (Daniels *et al.*, 2004). Wistar dams (Harlan, Barcelona) and their litters were assigned to the control (CTR) (non-separated) protocol or to the maternal separation (MS) protocol as described before (Lopes *et al.*, 2008b). To exclude artifacts from genetic background, at PND 2 all the litters were collected together and the pups were randomly distributed to foster dams. Separated pups were removed from their cages and dams at postnatal days 2-14 for 180 min, daily around 10 a.m. They were removed as a group from the nest and placed in an isolation cage in an adjacent room kept at 32.0±0.5ºC. At the end of the separation period pups were returned to their home-cage and rolled in the soiled home cage bedding before reuniting them with the mother. Control pups were not handled and were maintained in their home-cages until weaning. At day 21 the pup's sex was accessed, they were weaned and housed in individual cages until further use.

3.2| Brain extraction and dissection

MS and CTR male Wistar rats (6-8 week old) were anesthetized under isoflorane atmosphere before being decapitated. The brain was rapidly removed from the brain cavity and the striata, hippocampi and neocortices were dissected free in ice-cold Krebs/HEPES solution of the following composition (mM): NaCl 124, Glucose 10, HEPES 25, KCl 3, $MgCl_2$ 1, $CaCl_2$ 2, pH 7.4. The different brain areas were either homogenized, fixed or separately frozen at -80ºC until further use, as described below.

3.3| Tissue processing

a) Whole tissue lysates

Frozen tissue (striatum, cortex or hippocampus) was placed in 400 µL of Radio-Immunoprecipitation-Assay (RIPA) buffer (50 mM Tris, 1 mM EDTA,150 mM NaCl 0,1% SDS, 1% NP 40, pH 8) supplemented with protease inhibitors (ROCHE) and homogenized in a Potter-Elvehjem homogenizer with a Teflon piston (6 up and down strokes), according to (Palacios *et al.*, 2004). The volume of the suspension was completed to 600 µL with RIPA and left shaking for 1 hour. The samples were then centrifuged at 14000g during 15 minutes at 4ºC,

the supernatant was collected and corresponds to the whole tissue lysate. Protein was quantified using the BioRad Dc Protein assay Kit based on Lowry (1951) due to the high levels of detergents in the sample. This type of sample was used for relative quantification of cytoplasmatic proteins.

b) Whole tissue homogenates

Frozen tissue (striatum, cortex or hippocampus) was placed in 300 µL to 1 mL of a chilled 0.32 M sucrose solution with 50 mM Tris at pH 7.6, plus protease inhibitors (ROCHE) and homogenized in a Potter-Elvehjem homogenizer with a Teflon piston (6 up and down strokes), as before (Cunha *et al.*, 1996). The volume of the suspension was completed to 500 µL to 1.5 mL with the sucrose solution and centrifuged at 1000g during 10 minutes at 4ºC. The supernatant was collected and corresponds to the whole tissue homogenate. Protein was quantified according to Bradford (1976) using the BioRad Protein assay kit. This sample was used for relative quantification of all membrane proteins except for adenosine A_{2A} receptors.

c) Membrane fractions

The protocol used was as previously described (Cunha *et al.*, 1996). Frozen tissue (striatum, cortex or hippocampus) was placed in 5 mL of a chilled 0.32 M sucrose solution with 50 mM Tris, 2 mM EGTA and 1 M DTT pH 7.6 and homogenized in a Potter-Elvehjem homogenizer with a Teflon piston (2 up and down strokes). The volume of the suspension was completed to 10 mL with sucrose solution and centrifuged at 3000g during 10 minutes at 4ºC, the supernatant collected and centrifuged at 14000g for 12 minutes at 4ºC. The pellet is the membrane fraction. Membrane samples were used for radioligand binding assays and relative quantification of adenosine A_{2A} receptors.

d) Synaptosomal fractions

The synaptosomes were prepared as before (Lopes *et al.*, 1999a). Freshly dissected tissue was added to 8 mL of a chilled 0.32 M sucrose solution containing 1 mM EDTA, 1 mg/mL bovine serum albumin and 5 mM HEPES, pH 7.4, and homogenized in a Potter-Elvehjem homogeneizer with a Teflon piston (2 up and down stroks). The volume of the suspension was completed to 10 mL with sucrose solution and centrifuged at 3000g for 10 minutes at 4ºC, the supernatant collected and centrifuged at 14000g for 12 minutes at 4ºC. The pellet

was resuspended in 2 mL of 45% v/v Percoll solution made in Krebs-Ringer solution (composition in mM: NaCl 140, KCl 5,HEPES 25, EDTA 1, Glucose 10, pH 7.4). After centrifugation at 14000g for 2 minutes the top layer was collected (synaptosomal fraction) and washed two times in Krebs-Ringer solution by ressuspension and subsequent centrifugation at 14000g for 2 minutes. Synaptossomes were used for displacement binding assays and relative quantification of adenosine A_{2A} receptors.

3.4| Saturation binding assays

The radioligand binding experiments were performed as described (Lopes *et al.*, 1999a). The membrane fraction was resuspended in pre-incubation solution (50 mM Tris, 1 mM EDTA, 2 mM EGTA, pH 7.4) and incubated for 30 minutes at 37ºC with 2 U/mL of adenosine deaminase (ADA; EC 3.5.4.4), which removes endogenous adenosine catalyzing the conversion of adenosine into its inactive metabolite inosine. The suspension was centrifuged at 14000g for 15 minutes at 4ºC and the pellet resuspended in Tris/Mg^{2+} solution (50 mM Tris-HCl, 2 mM MgCl2, pH 7.4) containing 4 U/mL of ADA. [^{3}H]ZM 241385 binding (0-10 nM) was for 1 hour with 20-35 µg of protein/well for striatum membranes; [^{3}H]DPCPX (0-10 nM) binding was for 2 hours with 40-60 µg protein/well of hippocampal, 60-100 µg protein/well of cortex and 20-40 µg protein/well of striatum membranes. The incubation was made in Tris/Mg^{2+} solution with 4 U/mL of ADA at room temperature in a final volume of 300 µL. Specific binding was determined subtracting non specific binding, measured in the presence of 2 µM of XAC and normalizing for protein concentration. Binding reactions were stopped by vacuum filtration with a Skatron semi-automatic cell harvester using chilled incubation solution. Filtermats 1.5 µm (Molecular Devices) were used and placed in scintillation vials, 3 mL scintillation cocktail (OptiPhase 'HiSafe' 2, PerkinHelmer) were added. Radioactivity bound to the filters was determined after 12 hours with an efficiency of 55-60% for 2 minutes. All binding assays were performed in triplicate. The membranes left from the assay were frozen for further use.

3.5| Displacement binding assays

Competition binding curves of A_1 receptor antagonist [^{3}H]DPCPX by the A_1 receptor agonist CPA were performed as before (Lopes *et al.*, 1999a), in the absence and in the presence of the A_{2A} receptor selective agonist CGS 21680 for CTR and MS animals. Synaptossomes (22-35 µg/well) ressuspended in Krebs/HEPES solution (124 nM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ 10 mM glucose buffered with 25 mM HEPES, pH 7.4) plus ADA (8 U/mL) were incubated with [^{3}H]DPCPX (2 nM) and increasing concentrations of CPA (0 to 6 µM) in a final volume of 300 µL. All samples were assayed in triplicate. For each experiment four assays were performed simultaneously, for CTR and MS animals and for each, one in the presence and other in the absence of CGS 21680 (30 nM). Non-specific binding was determined for each assay in the presence of the non selective antagonist XAC at 2 µM. Microplates were incubated for 2 hours at room temperature and the reaction was stopped by vacuum filtration with a Skatron semi-automatic cell harvester with chilled incubation solution. Filtermats 1.5 µm (Molecular Devices) were used and placed in scintillation vials, 3 mL of scintillation cocktail (OptiPhase 'HiSafe' 2, PerkinHelmer) were added. Radioactivity bound to the filters was determined after 12 hours with an efficiency of 55-60% for 2 minutes. Synaptosomes left from the assay were frozen at -20ºC for further use.

3.6| Western Blotting

After protein quantification the appropriate volume of each sample was diluted in four volumes of water and one volume of sample buffer (350 mM Tris pH 6.8, 30% glycerol, 10% SDS, 600 mM DTT and 0,012% Bromophenol blue). For membrane samples, the appropriate volume was first centrifuged at 14000g at 4ºC for 20 minutes and the pellet ressuspended in four volumes of water and one of sample buffer. Prior to loading, the samples were denatured either at 60-70ºC for 15-20 minutes for membrane proteins, or at 95º for 5 minutes for cytosolic proteins (these conditions were tested and membrane samples had a stronger signal when not boiled). The samples and the molecular weight marker were separated by SDS-PAGE (8%, 10%, or 12% according to the protein molecular weight and a 5% stacking) in denaturing conditions and electro-transferred to nitrocellulose membranes (GE Healthcare) or PVDF membranes (Millipore) when probing A_{2A} receptors (shown to afford a stronger signal). The percentage of resolving gels and protein loading amounts are summarized in table 3.1. Membranes were blocked with 5% non-fat dry milk for 1 hour and a half, washed with TBS-T 0.1% (Tris buffer saline solution, 200 nM Tris, 1.5 M NaCl with 0.1%

Tween-20) and incubated with primary antibody overnight at 4ºC. After washing again for 30 minutes, the membranes were incubated with secondary antibody for 1 hour at room temperature (primary and secondary antibody dilution are in table 3.1). After 40 minutes of washing with TBS-T, chemoluminescent detection was performed with ECL-PLUS western blot detection reagent (GE Healthcare) using X-Ray films (Fujifilm). Optical density was determined with Image-J software and normalized to the respective β-actin band density.

3.7| Drugs

4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzene propanoic acid (**CGS 21680**); N_6-cyclopentyladenosine (**CPA**) and 8-{4-[(2-aminoethyl)amino]carbonylmethyloxyphenyl}xanthine (**XAC**) were purchased from Tocris Cookson, UK). These solutions were diluted in the assay solution from 5 mM sock aliquots made in DMSO stored at -20ºC.

Adenosine Deaminase (ADA, from calf intestine 10 mg/2 mL, EC 3.5.4.4) was from ROCHE; [*propyl*- ^{3}H]8-Cyclopentyl-1,3-dipropylxanthine **([^{3}H]DPCPX**, specific activity 100 Ci/mmol) was from Amersham, Buckinghamshire UK, and (**[^{3}H]ZM 241385,** specific activity 27.4 Ci/mmol) was from ArC Inc, St. Louis, USA. All these drugs were prepared directly into the incubation solution each day.

All other reagents used were of the highest purity available either from Merck, Germany or Sigma Aldrich, Spain.

3.8| Statistics

The values presented are mean ±SEM of *n* experiments, except the K_i values that are presented as mean ±95% confidence interval (95%Cl). To test the significance of the differences between CTR and MS groups, a paired Student's *t* test was used. Values of $P<0.05$ were considered to be statistically significant. The IC_{50} values obtained from displacement binding curves were converted into K_i values upon non-linear fitting of the semi-logaritmic curves derived from the competition curves. The K_D value used was determined experimentally from the saturation binding curves (K_D=0.65 nM). An F-test ($P<0.05$) was used to determine whether the competition curves were best fitted by one or two independent binding site equation and if the parameters obtained from the CTR and MS saturation curves (B_{max} and K_D) were different.

Table 3.1: Primary and secondary antibodies and related conditions used in the Western blot experiments for individual proteins. All primary antibodies were diluted in 3% Bovine Serum Albumin with 0,1% NaN_3 and secondary antibodies in 5% non-fat dry milk.

Protein	Protein loading (μg)	Resolving gel %	Primary antibody	Animal	Dilution	Secondary antibody	Dilution
$A_{2A}R$	130-150	12	Upstate (05-717)	Mouse	1:2000	Sta. Cruz Biotechnology (goat anti-mouse; goat anti-rabbit; donkey anti-goat)	1:5000
GR	45-80	8	Sta. Cruz Biotechnology (sc-1004)	Rabbit	1:1000		1:7500
MR	45-80	8	Sta. Cruz Biotechnology (sc-11412)	Rabbit	1:250		1:10000
$GluR_1$	20-40	8	Millipore (05-855)	Rabbit	1:6000		1:10000
$\beta_{2/3}GABA_A$	40-60	10/8	Upstate (05-474)	Mouse	1:2000		1:10000
BDNF	80	15	Abcam (ab46176)	Rabbit	1:1000		1:10000
Trk_B	20-40	8	BD Biosciences (610101)	Mouse	1:1500		1:10000
CRH-R_1	40-80	10/8	Sta. Cruz Biotechnology (sc-12381)	Goat	1:100		1:10000
Synaptophysin	30	12	SIGMA (S 5768)	Mouse	1:1500		1:7500
β-Actin	----	----	Abcam (ab8227)	Rabbit	1:1000		1:20000

Abreviations: $A_{2A}R$ – Adenosine A_{2A} Receptor; GR – Glucocorticoid Receptor; MR –Mmineralocorticoid Receptor; $GluR_1$ - Glutamate receptor 1 subunit of AMPA receptors; $\beta_{2/3}GABA_A$ - β_2 or β_3 subunit of $GABA_A$ receptor; BDNF – Brain-Derived-Neurotrofic-Factor; Trk_B – Tropomyosine related Kinase B; CRH-R_1 – Receptor 1 for Corticotrophin Releasing Hormone.

4| RESULTS

Maternal separation is a well characterized paradigm that induces stress in the early phases of development (Ladd *et al.*, 2000). It is widely used for the study of different psychopathologies, and is known to induce permanent changes in stress response and brain function, with repercussions for hippocampal-dependent memory and plasticity (Aisa *et al.*, 2009). Some of these changes share similarities with those observed in aging (Miller and O'Callaghan, 2005). It was previously observed an imbalance in the density of the adenosine A_1 and A_{2A} receptors in the hippocampus and striatum of aged animals (Cunha *et al.*, 1995; Lopes *et al.*, 1999a). We now investigated if the induction of chronic stress, through maternal separation, could have a similar impact on adenosine receptors.

4.1| Levels of Adenosine A_1 and A_{2A} receptors

Adenosine A_1 and A_{2A} receptor levels were quantified in the hippocampus, striatum and cortex from control (CTR) and maternal separated (MS) animals at 8 weeks of life. Whenever technically possible, saturation binding assays were used since they allow for absolute quantification and assess the affinity state of the receptors. Being the levels of A_{2A} receptors in the hippocampus and cortex very low, saturation binding techniques were not possible in these brain areas due to the high amounts of protein required. Instead, Western blotting was used. To further increase the signal-to-noise ratio one used preparations of nerve terminals (synaptosomes) which are particularly enriched in A_{2A} receptors (Figure 4.1).

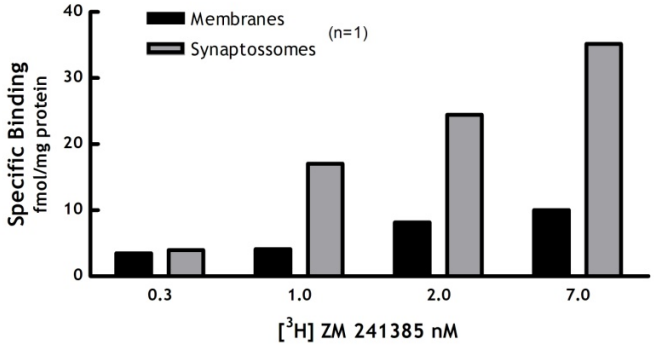

Figure 4.1: Levels of adenosine A_{2A} receptor in membranes and synaptossomes of the hippocampus determined by the binding of the selective A_{2A} receptor antagonist [^{3}H]ZM 241358 (0.3-7 nM). It is evident from the image that in the same amount of total protein synaptossomes have at least twice the levels of A_{2A} receptors than membranes (as in Rebola *et al.*, 2005). The bars present only one assay performed with membranes (black bar) obtained from 16 hippocampi (300 μg/well) and synaptossomes (grey bar) from 8 hippocampi (100μg/well).

In the hippocampus, MS animals presented an increase of 56.2 ± 9.4% (n=3; p<0.05) in A_{2A} receptors in synaptic terminals compared to CTR animals (Figure 4.2 A). A positive control using striatum tissue was always used to confirm that the observed band corresponded to specific A_{2A} receptors immunoreactivity. The A_1 receptor levels, analyzed by binding studies, were decreased in MS compared to CTR animals. The B_{max} values obtained from the saturation binding curve were of 1202 ± 68 fmol/mg protein (n=4) for CTR animals and 1073 ± 48 fmol/mg protein for MS animals (n=4; P<0.05) (Figure 4.2 B). The dissociation constants (K_d) presented no significant changes (Table 4.1).

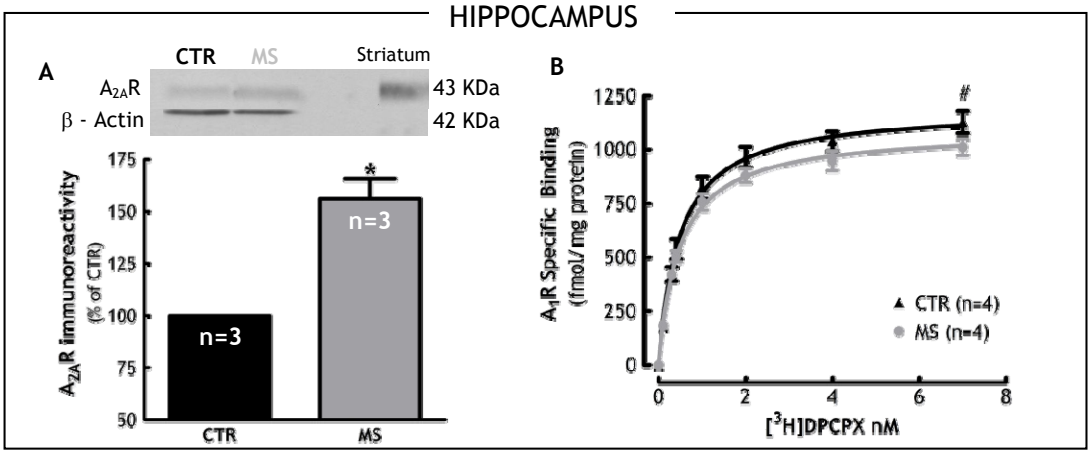

Figure 4.2: Adenosine receptor levels in the rat hippocampus. **(A)** Adenosine A_{2A} receptor immunoreactivity was measured in synaptic terminals of CTR (black) and MS (grey) animals and normalized by β-Actin immunoreactivity. Results are the mean ±SEM of three experiments. At the top a representative image of the western blot is presented. **(B)** Saturation binding curves of the A_1 receptor selective antagonist [^{3}H]DPCPX were performed for CTR (black) and MS (grey) animals. [^{3}H]DPCPX (0-7 nM) was incubated with 40-60 μg of hippocampal membranes in a final volume of 300 μL for 2h at room temperature. The ordinates represent the specific binding of [^{3}H]DPCPX obtained upon subtraction of the non-specific binding, determined in the presence of 2 μM of XAC, from total binding. Each point is the mean ± SEM of four experiments performed in triplicate. (*): P<0.05 calculated using a paired Student t-test compared to control; ($^{\#}$):p<0.05 calculated using an F-test compared to control.

In the cortex, the A_{2A} receptor immunoreactivity was unchanged in MS compared to CTR animals (Figure 4.3 A). When looking at synaptic terminals a tendency for a decrease was observed but it needs further confirmation (n=2), whereas in membranes no differences were found (n=3). Regarding A_1 receptors, the saturation binding curves indicated no differences between CTR and MS animals: the B_{max} values were of 912.3 ± 131 fmol/mg protein (n=3) in CTR animals and of 1009 ± 78 fmol/mg protein (n=3) in MS animals (Figure 4.3 B). The dissociation constants showed no significant changes (Table 4.1).

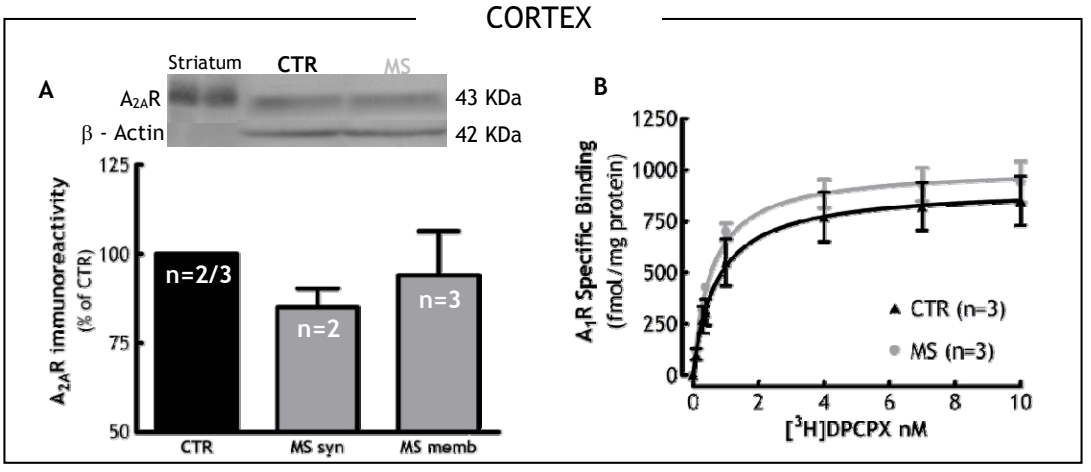

Figure 4.3: Adenosine receptor levels in the rat cortex. **(A)** Adenosine A_{2A} receptor immunoreactivity was measured in synaptic terminals (MS syn) and membranes (MS memb) of CTR (black) and MS (grey) animals and normalized by β-Actin immunoreactivity. Results are the mean ±SEM of two experiments for synaptossomes and three experiments for membranes. At the top a representative image of the western blot in synaptossomes is presented. **(B)** Saturation binding curves of the A_1 receptor selective antagonist [^{3}H]DPCPX were performed for CTR (black) and MS (grey) animals. [^{3}H]DPCPX (0-10 nM) was incubated with 60-100 μg of hippocampal membranes in a final volume of 300 μL for 2h at room temperature. The ordinates represent the specific binding of [^{3}H]DPCPX obtained upon subtraction of the non-specific binding, determined in the presence of 2 μM of XAC, from total binding. Each point is the mean ± SEM of three experiments performed in triplicate.

In the striatum, the levels of both A_1 and A_{2A} receptors were evaluated using saturation binding curves. The B_{max} for the selective A_{2A} receptor antagonist, [^{3}H]ZM 241385 (0-10 nM), was similar between CTR (3044 ± 436 fmol/mg protein; n=5) and MS animals (3169 ± 400 fmol/mg protein; n=5) (Figure 4.4 A). The respective dissociation constant was also unaltered (Table 5.1). When performing saturation binding curves for A_1 receptor the B_{max} obtained for the selective A_1 receptor antagonist, [^{3}H]DPCPX (0-8 nM), presented no significant changes between CTR (819 ± 46 fmol/mg protein; n=5) and MS animals (3169 ± 400 fmol/mg protein; n=5) (Figure 4.4 B). Similar values for the dissociation constants were also obtained (Table 4.1).

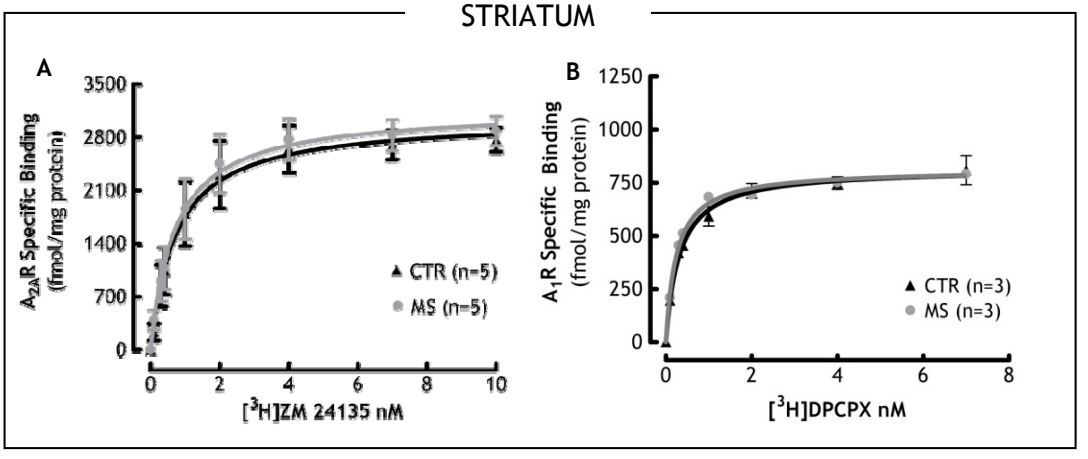

Figure 4.4: Saturation binding curves of the A_{2A} receptor selective antagonist [^{3}H]ZM 241385 **(A)** and of the A_1 receptor selective antagonist [^{3}H]DPCPX **(B)** were performed for CTR (black) and MS (grey) animals in the striatum. [^{3}H]ZM 241385 (0-10 nM) was incubated with 20-35 μg of hippocampal membranes in a final volume of 300 μL for 1h at room temperature. [^{3}H]DPCPX (0-7 nM) was incubated with 20-40 μg of hippocampal membranes in a final volume of 300 μL for 2h at room temperature. The ordinates represent the specific binding of [^{3}H]ZM 241385 (A) or [^{3}H]DPCPX (B) obtained upon subtraction of the non-specific binding, determined in the presence of 2 μM of XAC, from total binding. Each point is the mean ± SEM of five (A) or three (B) experiments performed in triplicate.

Table 4.1: Average B_{max} and K_D values for A_1 or A_{2A} receptors obtained with saturation binding curves performed with [^{3}H] DPCPX or [^{3}H] ZM 241385 (0-10 nM) for CTR and MS animals with 7-8 weeks. Values are mean ± SEM of n experiments. ($^{\#}$): P<0.05 calculated using an F-test compared to control.

Brain Area	Receptor	Group	B_{max} (fmol/mg protein)	K_D (nM)	*n*
Hippocampus	A_1R	CTR	1202 ± 68	0.51 ±0.11	4
		MS	1073 ± 48$^{\#}$	0.45 ±0.06	4
Cortex	A_1R	CTR	912 ± 131	0.72 ±0.41	3
		MS	1009 ± 78	0.56 ±0.17	3
Striatum	A_1R	CTR	819 ± 46	0.32 ±0.07	3
		MS	812 ± 77	0.24 ±0.09	3
	A_{2A}R	CTR	3044 ± 436	0.74 ± 0.41	5
		MS	3169 ± 400	0.71 ± 0.36	5

4.2| Interaction between A_1 and A_{2A} receptors

In young animals, A_{2A} receptors attenuate the affinity of A_1 receptors and thereby alter synaptic transmission in the hippocampus (O'Kane and Stone, 1998; Lopes *et al.*, 1999b). Upon ageing, changes in the levels of adenosine receptors were shown to impair this crosstalk between A_1 and A_{2A} receptors (Lopes *et al.*, 1999a). Given that maternal separation induced changes in the levels of the receptors, one went to investigate possible consequences to the A_1/A_{2A} receptor interaction.

The effect of A_{2A} receptor activation on the affinity of A_1 receptor was evaluated by displacement of the A_1 selective antagonist, [^{3}H] DPCPX (2 nM), by the A_1 receptor selective agonist CPA (0-6 μM) in the absence and in the presence of the A_{2A} receptor selective agonist CGS 21680 (30 nM). This assay was performed for CTR and MS animals with 7 weeks. The curves obtained (figure 4.5) for both CTR (n=5) and MS (n=5) animals in the absence of CGS 21680 fitted best with a two binding site model, a low and high affinity binding site. In CTR animals the K_{i1} was 0.81 nM (95%Cl: 0.30-1.24 nM; n=5) whereas for MS animals the K_{i1} was 0.61 nM (95%Cl: 0.40-0.94 nM; n=5). Activation of A_{2A} receptors by CGS 21680 induced no changes in the displacement parameters in any of the groups (see Table 4.2 for dissociation constants). The K_{i1} with CGS for CTR animals was 0.67 nM (95%Cl: 0.44-1.02 nM; n=5) whereas for MS animals the K_{i1} was 0.53 nM (95%Cl: 0.34-0.84 nM; n=5).

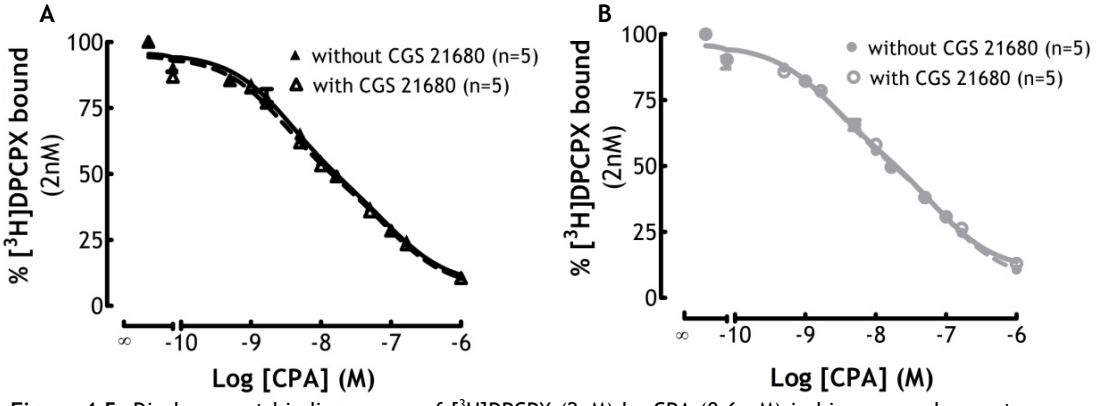

Figure 4.5: Displacement binding curves of [^{3}H]DPCPX (2nM) by CPA (0-6 μM) in hippocampal synaptossomes of youg adult (7 weeks old) rats in the absence (closed symbol) and in the presence (open symbol) of the A_{2A} receptor agonist CGS 21680 (30 nM) for either CTR **(A)** or MS **(B)** animals. The ordinates represent the percentage of [^{3}H]DPCPX obtained upon subtraction of the non specific binding, determined in the presence of 2 μM of XAC, from the total binding. Each point is the mean ± SEM of five experiments performed in triplicate.

Table 4.2: Ability of A_{2A} receptor agonist, CGS 21680 (30 nM), to attenuate the displacement by the A_1 receptor agonist, CPA (0-6 μM), of the A_1 receptor antagonist, [^{3}H] DPCPX (2 nM), binding to rat hippocampal synaptossomes prepared from 7 weeks old CTR or MS animals. The values of K_{i1}, K_{i2} and 95% confidence values (95%Cl) are presented in nM and were derived from five experiments.

Group	Condition	K_{i1} (nM)	95%Cl	K_{i2} (nM)	95%Cl
CTR (n=5)	-CGS 21680	0.81	0.30-1.24	26.60	14.11-47.947
	+CGS 21680 (30 nM)	0.67	0.44-1.02	24.69	13.92-43.81
MS (n=5)	-CGS 21680	0.61	0.40-0.94	25.26	15.22-41.94
	+CGS 21680 (30 nM)	0.53	0.34-0.84	18.62	11.45-30.27

4.3| Levels of GR and MR receptors

The ratio between mineralocorticoid and glucocorticoid receptors (MR and GR respectively) can interfere on the impact of stress in different brain areas (reviewed by Joels, 2006). Therefore, it was investigated whether the observed changes in adenosine receptor levels in the different brain areas, were correlated with regional MR/GR changes. Due to their cytoplasmatic and nuclear location (Htun *et al.*, 1996), the levels of these receptors were quantified in whole tissue lysates that include both fractions.

In the hippocampus, the GR immunoreactivity decreased by 26.1 ± 4.5% ($P<0.05$) in MS (n=3) compared to CTR (n=3) animals, whereas the levels of MR (n=4) were not affected by the stress paradigm (Figure 4.6).

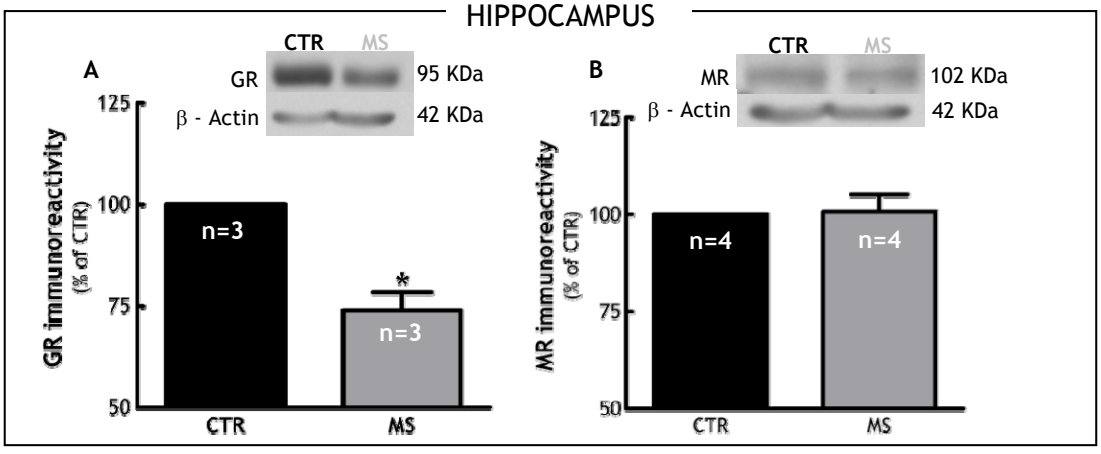

Figure 4.6: GR **(A)** and MR **(B)** levels in the rat Hippocampus. Both proteins were quantified in whole tissue lysates either for CTR (black) or MS (grey) animals. Specific immunoreactivity was normalized to that of β - Actin. Results are the mean ±SEM of three (A) or four (B) experiments. At the top a representative image of the western blot is presented. (*):$P<0.05$ calculated using a paired Student t-test.

In the cortex, a similar pattern was obtained. The GR levels were lower in MS animals (16.3 ± 3.4%; n=4; P<0.05) than in CTR animals (Figure 4.7 A), while MR levels seemed to be unchanged (n=2) (Figure 4.7 B).

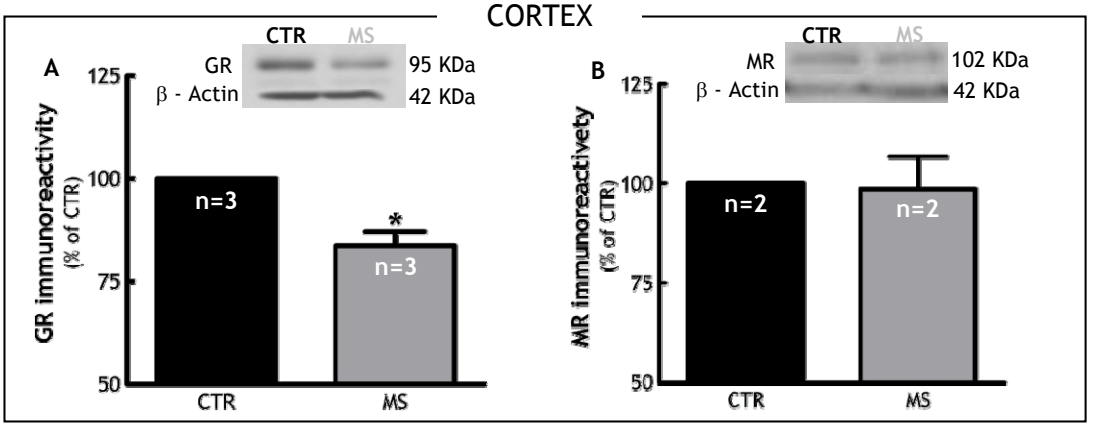

Figure 4.7: GR **(A)** and MR **(B)** levels in the rat cortex. Both proteins were quantified in whole tissue lysate either for CTR (black) or MS (grey) animals. Specific immunoreactivity was normalized to that of β - Actin. Results are the mean ±SEM of three (A) or two (B) experiments. At the top a representative image of the western blot is presented. (*):P<0.05 calculated using a paired Student t-test.

For the striatum, a decrease in the GR immunoreactiviy was also observed, although in a lower magnitude. MS animals had 9.6 ± 2.6% (n=4; P<0.05) less GR than CTR (n=4) animals (Figure 4.8 A). For MR, no apparent changes were obtained (n=2) (Figure 4.8 B).

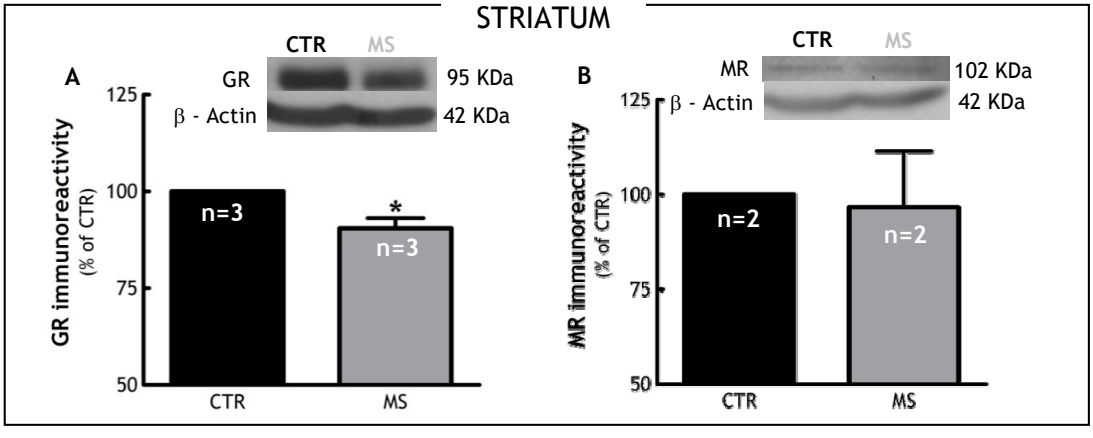

Figure 4.8: GR **(A)** and MR **(B)** levels in the rat striatum. Both proteins were quantified in whole tissue lysate either for CTR (black) or MS (grey) animals. Specific immunoreactivity was normalized to that of β - Actin. Results are the mean ±SEM of three (A) or two (B) experiments. At the top a representative image of the western blot is presented. (*):P<0.05 calculated using a paired Student t-test.

4.4| Levels of different markers of synaptic plasticity

Stress induced an increase in the MR/GR ratio, which is more pronounced in the hippocampus than in cortex or striatum. To evaluate how profound the effects of maternal deprivation were for the hippocampus, one went to evaluate further changes in different mediators of synaptic transmission and plasticity.

The GluR1 subunit of the AMPA receptors is required for several forms of hippocampal LTP. Changes in the pool of AMPA receptors containing $GluR_1$ subunit influence the magnitude of LTP (Andrasfalvy *et al.*, 2003). Analysis of the GluR1 immunoreactivity (Figure 4.9) revealed a decrease of 25 ± 5% ($P<0.05$) in the levels of this subunit in the hippocampus of MS (n=3) when comparing to CTR animals (n=3).

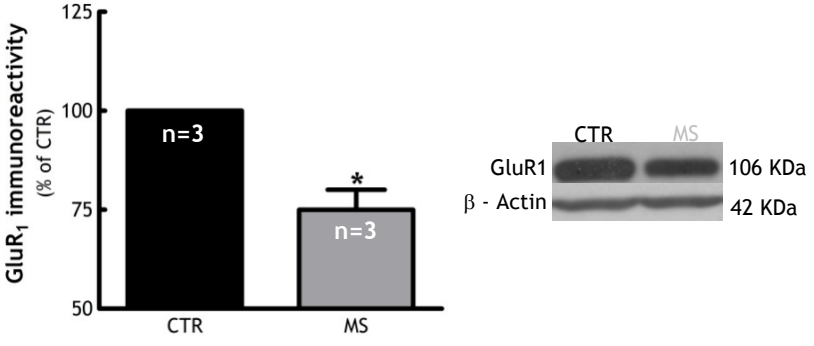

Figure 4.9: $GluR_1$ levels in the rat Hippocampus. Immunoreactivity of $GluR_1$ was measured in whole tissue homogenates either for CTR (black) or MS (grey) animals. Specific immunoreactivity was normalized to that of β - Actin. Results are the mean ±SEM of three experiments. At the top a representative image of the western blot is presented. (*):$P<0.05$ calculated using a paired Student t-test.

The $GABA_A$ receptors are the major receptors involved in GABA-mediated inhibitory signaling in the hippocampus. Changes in this receptor are associated with changes in the inhibitory tonus of the hippocampus (Steiger and Russek, 2004). $GABA_A$ receptor levels were evaluated specifically through the β_3 subunit of these receptors, one of the most expressed $GABA_A$ subunit in the hippocampus (Sperk *et al.*, 1997). Analysis of $GABA_A$-β_3 immunoreactivity (figure 4.10) revealed a decrease of 16 ± 2% ($P<0.05$) of $GABA_A$ receptors in MS (n=3) when comparing to CTR animals (n=3).

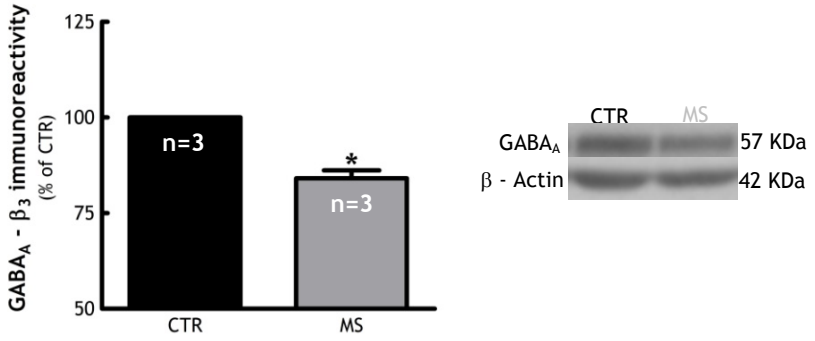

Figure 4.10: $GABA_A$-β_3 levels in the rat Hippocampus. Immunoreactivity of $GABA_A$-β_3 receptor subunit was measured in whole tissue homogenates either for CTR (black) or MS (grey) animals. Specific Immunoreactivity was normalized to that of β - Actin. Results are the mean ±SEM of three experiments. At the top a representative image of the western blot is presented. (*):P<0.05 calculated using a paired Student t-test.

The Brain-Derived-Neurotrophic-Factor (BDNF) is a neurotrophin highly expressed in the rat hippocampus that, by activation of the receptor B from the family of the Trk_B receptor, is able to modulate hippocampal synaptic plasticity (Kang and Schuman, 1995). The levels of BDNF and Trk_B receptor were evaluated in CTR and MS animals (Figure 4.11). The stress paradigm induced an increase in BDNF levels accompanied by a decrease in the Trk_B levels. MS animals had 67±13% (n=4; P<0.05) higher BDNF content and 17±2.3% (n=3;P<0.05) lower Trk_B levels than CTR animals.

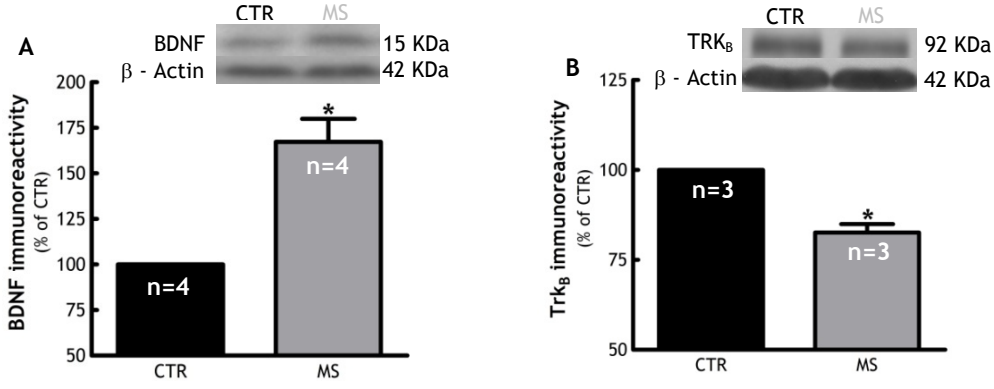

Figure 4.11: BDNF **(A)** and Trk_B receptor **(B)** levels in the rat hippocampus. Both proteins were quantified in whole tissue homogenates either for CTR (black) or MS (grey) animals. Specific Immunoreactivity was normalized to that of β - Actin. Results are the mean ±SEM of four (A) or three (B) experiments. At the top a representative image of the western blot is presented. (*):P<0.05 calculated using a paired Student t-test.

Corticotrophin-Releasing-Hormone (CRH) is an important neuro-endocrine mediator of HPA-axis activation that has been described to have extra hypothalamic effects (Aldenhoff *et al.*, 1983). In the hippocampus CRH has been described to induce or potentiate LTP (Wang *et al.*, 1998; Pollandt *et al.*, 2006). To evaluate the effects of maternal deprivation on CRH system one went to investigate the levels of CRH-R_1, the most expressed CRH receptor in the hippocampus (Chalmers *et al.*, 1995).

Immunoblot analysis revealed a significant increase in the levels of CRH-R_1 (Figure 4.12). CRH-R_1 protein levels increased by 55±7% ($P<0.05$) in MS (n=3) when comparing to CTR animals (n=3).

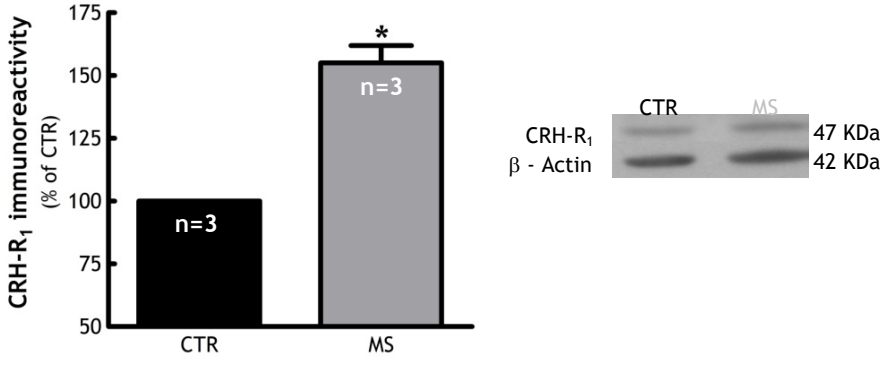

Figure 4.12: CRH-R_1 levels in the rat Hippocampus. Immunoreactivity of CRH-R_1 was measured in whole tissue homogenates either for CTR (black) or MS (grey) animals. Specific mmunoreactivity was normalized to that of β - Actin. Results are the mean ±SEM of three experiments. At the top a representative image of the western blot is presented. (*):$P<0.05$ calculated using a paired Student t-test

Several stress paradigms were shown to lead to hippocampal shrinkage and to synaptic loss (Sapolsky, 1985; Watanabe *et al.*, 1992). To evaluate changes in synaptic density in this stress model the levels of synaptophysin, a protein expressed in synaptic terminals, were quantified (Wiedenmann and Franke, 1985) (Figure 4.13). Results revealed no changes in total synaptic density in the hippocampus of MS (n=4) compared to CTR animals.

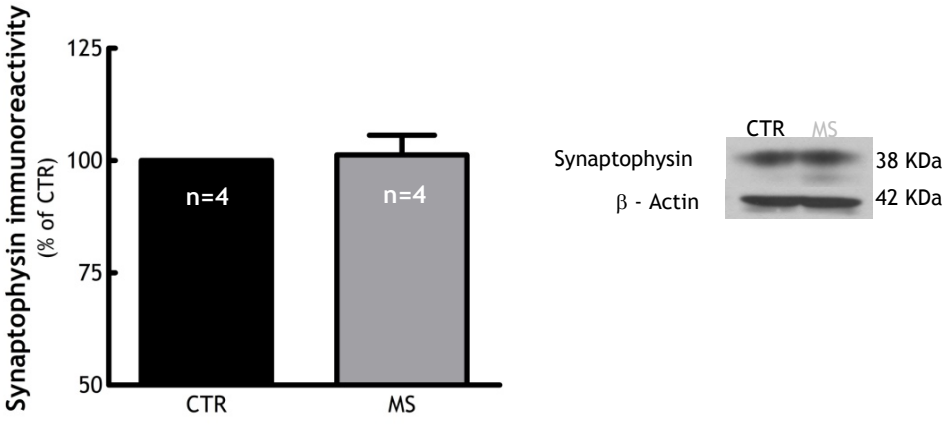

Figure 4.13: Synaptophysin levels in the rat Hippocampus. Immunoreactivity of synaptophysin was measured in whole tissue lisates either for CTR (black) or MS (grey) animals. Specific immunoreactivity was normalized to that of β - Actin. Results are the mean ±SEM of four experiments. At the top a representative image of the western blot is presented.

5| DISCUSSION

Stress in the neonatal period can impair neuronal development and lead to long-lasting effects in physiology and behavior (Ladd *et al.*, 2000) that share similarities with those observed in aging (Miller and O'Callaghan, 2005). Although many pharmacological and behavioural aspects of neonatal stress are already clarified, nothing is known regarding its consequences on adenosine neuromodulation. We have previously observed that aging leads to an imbalance in the density of the adenosine A_1 and A_{2A} receptors in the hippocampus and striatum (Cunha *et al.*, 1995; Lopes *et al.*, 1999a). Being the hippocampus particularly susceptible to stress (Smith *et al.*, 1995a), our hypothesis is that, in part by changing the balance between adenosine receptors, stress increases hippocampal vulnerability and by that induces an early ageing in the hippocampus. To test this hypothesis we used a model of chronic stress, known as maternal separation (MS), a well characterized paradigm used in the study of anxiety and depression (Kalueff and Tuohimaa, 2004). Previous work from our group has shown that MS in neonatal period reduced the magnitude of long-term potentiation (LTP) in the adult hippocampus accompanied by impairments of the spatial memory (Lopes *et al.*, 2008a). Now it was evaluated the impact of MS on the levels and affinity state of adenosine receptors in the adult brain, as well as changes in stress-related receptors and synaptic plasticity markers known to be altered upon ageing.

The main finding of the present work is that neonatal stress induced a long-lasting increase in the levels of A_{2A} receptors and a decrease of A_1 receptors in the hippocampus. In contrast, in striatum and cortex, neither A_1 nor A_{2A} receptor levels were modified. In accordance with a differential effect of chronic stress in the hippocampus, we found regional differences in the mineralocorticoid/glucocorticoid receptors ratio (MR/GR) which may result from the dominant expression of corticosteroid receptors in this brain area over others. Moreover, the $GluR_1$ subunit of AMPA receptors, the β_3 subunit of $GABA_A$ receptors and Trk_B receptors were also found to be decreased in this brain area, together with increased levels of BDNF and CRH-R_1, whereas synaptophysin was found unchanged.

5.1|Changes in adenosine receptors

The first observation linking adenosine to stress was made by Geiger and Galvin (1985) showing that administration of an adenosine A_1 receptor agonist, was able to reduce the plasmatic levels of rat corticosterone. More recently, an episode of restraint stress was shown to induce an acute and reversible increase in the levels of hippocampal adenosine A_{2A}

receptors (Cunha *et al.*, 2006). The changes now observed result from an impact of neonatal stress in the adenosine receptors that remains until adulthood.

The increased levels of A_{2A} receptors found in synaptic terminals may not reflect changes in the expression or total levels of the receptor, rather they may only result from an enrichment of the receptors in the synapses as a consequence of stress. In the same way, the low magnitude of the alterations seen for A_1 receptors might be consequence of using whole hippocampal membranes rather than synaptosomes. A decrease in A_1 receptors may become more significant if only synapses were analyzed. The use of real-time PCR would be important to evaluate if the observed changes in the protein levels are accompanied by changes in the expression of the mRNA encoding for the receptors. Moreover, the increased levels in adenosine A_{2A} receptors now reported might explain the higher efficiency of an A_{2A} receptor antagonist, SCH 58261 (50 nM) in enhancing LTP of MS compared to CTR animals (Lopes *et al.*, 2008a).

Lopes and co-workers showed that the imbalance of adenosine receptors have repercussions for adenosine signaling in synaptic transmission (Lopes *et al.*, 1999a). In aging, when A_{2A} receptors are increased and A_1 receptors decreased, A_{2A} receptors are able to directly increase glutamate release by a protein kinase A - dependent mechanism. In contrast, in younger animals, A_{2A} receptors do not affect glutamate release on their own; instead they decrease the affinity of A_1 receptors, via protein kinase C activation (Lopes *et al.*, 1999b; Rebola *et al.*, 2003). Given that the changes in adenosine receptor levels induced by MS follow a similar pattern, we anticipated a similar shift in the A_1/A_{2A} receptors crosstalk. However, the displacement binding curves revealed no decrease in the affinity of A_1 receptors by A_{2A} receptor activation in MS, not even in CTR animals. The dissociation constants (K_d) now obtained for A_1 receptors were higher than obtained previously (Lopes *et al.*, 1999b), meaning that the receptors were in a lower affinity state than expected which may mask any further change induced by MS. This could be attributed to several causes, from compromised integrity of the synaptosomes, which need to be “functional” in order to have G protein-coupled receptors and preserve the downstream mechanisms (Lopes *et al.*, 1999b), to unexpected higher levels of endogenous adenosine that compete for the receptor binding site. Different protocols were tested in CTR animals, such as milder re-suspension techniques or increasing the amount of adenosine deaminase to 8 U/ml to improve the removal of endogenous adenosine. None of these procedures was able to increase the affinity state of A_1 receptors. Therefore, in spite of the increased levels of A_{2A} receptors in

MS animals, their activation loss the ability of influencing A_1 receptor affinity. Whether this is a true consequence from stress and thus similar to ageing, or just an experimental artifact remains to be clarified.

5.2|Region specific effects on the ratio MR/GR

The levels of adenosine A_1 and A_{2A} receptor were quantified in other brain areas, namely in cortex and striatum, however no changes were observed in any of those areas. This indicates that stress induces region-specific effects regarding adenosine. Region-specific effects of stress were already obtained using different stress models (Gruen *et al.*, 1995; Meller *et al.*, 2003), as a consequence of a differential GR and MR expression (the main mediators of stress response) among brain areas (Reul and de Kloet, 1985). The observation that the translocation of GR to the nucleus is enhanced in stress and lasts longer in the hippocampus comparing to cortex (Kitchener *et al.*, 2004), further supports a region specific effect of stress. To investigate possible regional correlations, the levels of GR and MR in the three brain areas were studied. The results obtained show that neonatal stress induced a decrease in GR, but no changes in MR levels. Moreover, the changes observed were more marked in the hippocampus than in cortex and striatum. As a response to increasing circulating levels of corticosteroids and as a way to limit their action usually occurs a down-regulation in GR levels (Sapolsky *et al.*, 1984; 1985; 1986) which is reversible, when the plasma levels of corticosterone return to normal values (Sapolsky *et al.*, 1984). However, in MS animals the increase in the levels of plasma corticosterone is sustained throughout life (Lopes, 2008) and may result in the now observed decrease in the levels of GR, with the consequent increase in MR/GR ratio. The ratio MR/GR is crucial in stress response being involved in the regulation of processes such as memory and synaptic plasticity (for review see Joels, 2006; Sousa *et al.*, 2008). Aged animals, who also display increased circulating levels of corticosteroids and lower basal levels of GR in the hippocampus, have an impaired negative regulation of the HPA-axis (Mizoguchi *et al.*, 2009). On the other hand, increased levels of GR in the hippocampus confer a "resistance" to future stress stimuli by increasing the inhibition of the HPA-axis (Meaney *et al.*, 1988). All these observations support the role this imbalance of MR/GR ratio in the preferential deleterious effect of MS paradigm in the hippocampus when compared to other brain areas.

5.2|Changes in synaptic plasticity

Corticotrophin releasing hormone is an important stress mediator. Activation of the corticotrophin releasing hormone receptor one (CRH-R_1) was shown to have neuroprotective effects against β-amyloid-induced neuronal death (Pedersen *et al.*, 2002; Contoreggi *et al.*, 2003). Maternal separated animals display increased levels of CRH-R_1 in the hippocampus. This increase may result of an adaptative change against the noxious conditions induced by the chronic high levels of glucocorticoids. However more recent observations seem to indicate that CRH-R_1 effects are responsible for the spine retraction and dendritic atrophy observed after chronic stress induction (Chen *et al.*, 2008). These data are not mutually exclusive. By inducing dendritic retraction, CRH may decrease neuronal vulnerability and protect from cell death. In situations of acute stress this is reversible (Sousa *et al.*, 2000) however if the corticosterone levels in the bloodstream increase chronically, as in MS animals (Lopes, 2008), a permanent remodeling of the dendrites may occur, ultimately leading to the observed cognitive impairment (Lopes *et al.*, 2008a). This issue was further evaluated by measuring the density of synaptophysin, a protein present in synaptic terminals and involved in activity-dependent synapse formation (Tarsa and Goda, 2002). No changes were observed in synaptic density which may be related to: the sensibility of the technique applied; the subtleness of the changes or a region specific effect, since stress induced changes in hippocampal density are usually more dramatic in CA3 area (Watanabe *et al.*, 1992; Magarinos and McEwen, 1995) or even a possible effect in neurite length without impairing the total number of synapses. As an alternative a more selective marker for dendritic atrophy could be used, as SNAP 25, involved in axonal terminal remodeling (Osen-Sand *et al.*, 1993) or immunohistochemistry techniques could be employed.

To further explore the extension of these long lasting effects the levels of different important proteins for synaptic plasticity were evaluated. The insertion of AMPA receptors containing glutamate receptor one ($GluR_1$) subunit is a constitutive part of LTP induction (Andrasfalvy *et al.*, 2003) and is modulated by GR (Martin *et al.*, 2009). $GABA_A$ receptors are fundamental for inhibitory neurotransmission in the hippocampus controlling neuronal firing (McCormick *et al.*, 1993) and are also regulated by glucocorticoids (Majewska *et al.*, 1985). TRK_B receptors, besides their neurotrophic action (Soppet *et al.*, 1991), have important roles on basal synaptic transmission (Levine *et al.*, 1996; Diogenes *et al.*, 2004) and in LTP induction (Fontinha *et al.*, 2009) when activated by BDNF. Maternal separation induced a long-lasting decrease in these three proteins. The decrease observed in TRK_B receptors and

particularly in $GluR_1$ subunit of AMPA receptors may underlie the impairments in LTP observed in MS animals (Lopes *et al.*, 2008a). Moreover the observed increase in the levels of BDNF together with a decrease in TRK_B receptor may enhance the negative impact of stress in the hippocampus, if BDNF activates its low affinity receptor – the P75 receptor. P75 is known as a death receptor, increasing intracellular calcium and consequently inducing apoptosis (reviewed by Lu *et al.*, 2005). The evaluation of the levels of this receptor in maternal separated animals would help to clarify how the neurotrophin system is modulated by chronic stress. Finally a decrease in the levels of $GABA_A$-β_3 subunit, if accompanied by a decrease in the affinity of the receptor and the consequent reduced inhibitory tonus, may explain the anxious behaviour observed in MS animals (Lopes *et al.*, 2008a).

More importantly, the animals were not tested right after the stress induction period, from PND2 until PND 14, where the effects could still be attributed to acute effects in the HPA-axis and hence reversible. Rather, the changes are shown to be present at 6-8 weeks of age, long after the application of the stress paradigm and at the same age at which the impairments in synaptic plasticity were observed (Lopes *et al.*, 2008a). Taken together, this data strongly suggests that the impact of MS on adenosine modulatory function is chronic. Interestingly, many of the proteins shown to be altered are somehow related to adenosine signaling or their function is modulated by adenosine receptors. GABA and glutamate release and uptake are modulated by adenosine (reviewed by Sebastião and Ribeiro, 2009), GluR1 insertion in the membranes is potentiated by A_{2A} receptor activation (Dias *et al.*, 2008), BDNF effects on synaptic transmission are dependent on A_{2A} receptor activation (Diogenes *et al.*, 2004; Diogenes *et al.*, 2007). Furthermore, the effects of chronic stress on LTP (mediated by chronic activation of GR) were reverted by blocking adenosine A_{2A} receptors *in vitro* (Lopes *et al.*, 2008a). These observations strongly suggest an altered adenosine neuromodulation and an involvement of adenosine A_{2A} receptors in stress-induced deficits in the hippocampus that needs further confirmation. Finally the fact that maternal separation is a model for the study of psychopathologies that presents long lasting changes in adenosine receptors further supports the study of A_{2A} receptor antagonism as a potential therapeutic tool for these pathologies. However, the functional consequences of the observed changes in the levels of adenosine receptors should be further evaluated.

In conclusion, our results suggest that early-life stress leads to chronic changes in the levels of adenosine receptors and synaptic plasticity markers, which can have an important long-term impact in hippocampal structure and function. These molecular changes may account for cognitive disturbances observed in stress-related neuropsychiatric disorders. Moreover, many of the changes observed in the hippocampus, the increased levels of A_{2A} receptors, the decreased levels of A_1, GR and also TRK_B receptors are common features to what is observed in the aging hippocampus (summarized in Figure 5.1). This suggests that the observed stress-induced changes in the hippocampal formation may increase the susceptibility, not only for further noxious stimuli but also for the aging associated cognitive deficits.

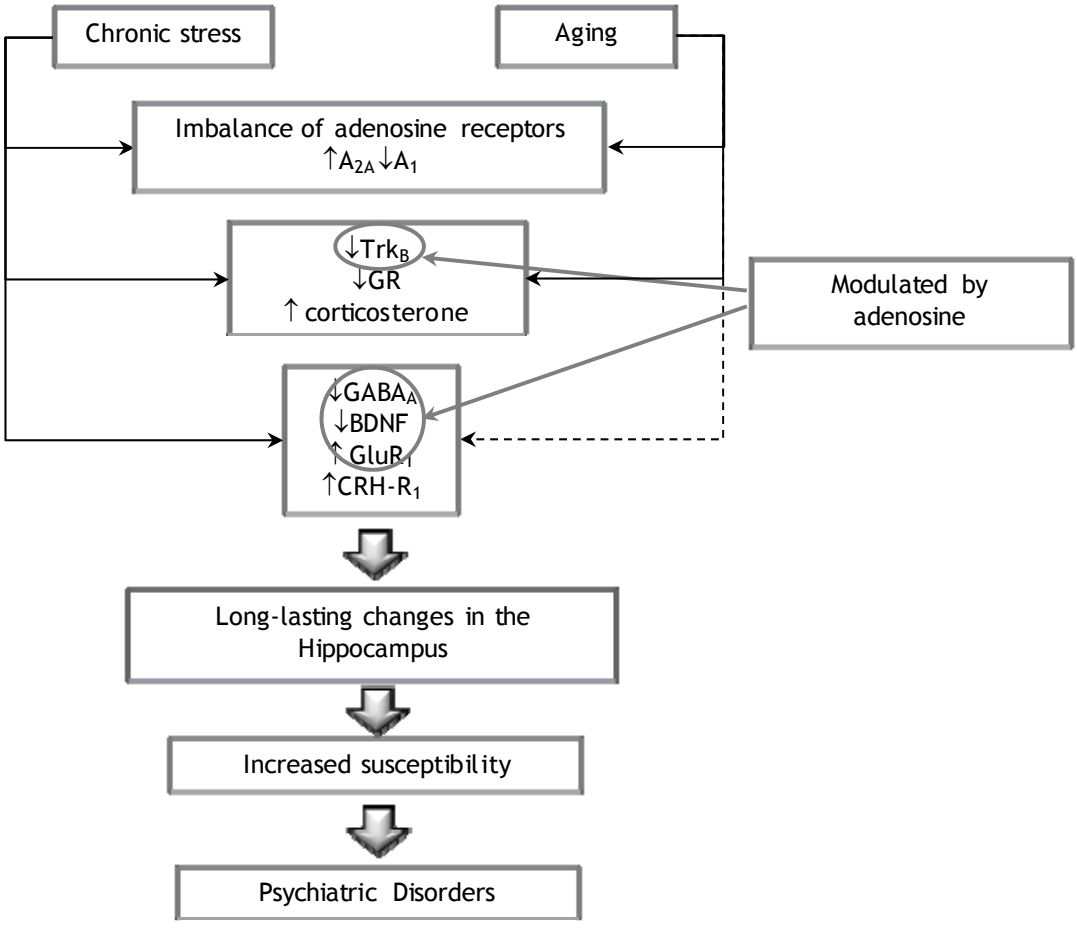

Figure 5.1: Involvement of adenosine receptors in the similarities between chronic stress and aging in the hippocampus. Broken arrows indicate connections that are not fully characterized.

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7| REFERENCES

AISA B, ELIZALDE N, TORDERA R, LASHERAS B, DEL RIO J, and RAMIREZ MJ. Effects of neonatal stress on markers of synaptic plasticity in the hippocampus: Implications for spatial memory. *Hippocampus*, 2009.

ALDENHOFF JB, GRUOL DL, RIVIER J, VALE W, and SIGGINS GR. Corticotropin releasing factor decreases postburst hyperpolarizations and excites hippocampal neurons. *Science,* 221: 875-7, 1983.

ANDRASFALVY BK, SMITH MA, BORCHARDT T, SPRENGEL R, and MAGEE JC. Impaired regulation of synaptic strength in hippocampal neurons from glur1-deficient mice. *J Physiol,* 552: 35-45, 2003.

ARCH JR and NEWSHOLME EA. The control of the metabolism and the hormonal role of adenosine. *Essays Biochem,* 14: 82-123, 1978.

ASSAIFE-LOPES N, PEREIRA DB, RIBEIRO JA, CHAO MV, and SEBASTIÃO AM. *Activation of adenosine a_{2a} receptors increases trkb receptors translocation to lipid rafts. Secondary Titl.*, 2007.

BAKSHI VP and KALIN NH. Corticotropin-releasing hormone and animal models of anxiety: Gene-environment interactions. *Biol Psychiatry,* 48: 1175-98, 2000.

CALDJI C, DIORIO J, and MEANEY MJ. Variations in maternal care in infancy regulate the development of stress reactivity. *Biol Psychiatry,* 48: 1164-74, 2000a.

CALDJI C, FRANCIS D, SHARMA S, PLOTSKY PM, and MEANEY MJ. The effects of early rearing environment on the development of gabaa and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. *Neuropsychopharmacology,* 22: 219-29, 2000b.

CHALMERS DT, LOVENBERG TW, and DE SOUZA EB. Localization of novel corticotropin-releasing factor receptor (crf2) mrna expression to specific subcortical nuclei in rat brain: Comparison with crf1 receptor mrna expression. *J Neurosci,* 15: 6340-50, 1995.

CHAU A, ROSE JC, and KOOS BJ. Adenosine modulates corticotropin and cortisol release during hypoxia in fetal sheep. *Am J Obstet Gynecol,* 180: 1272-7, 1999.

CHEN Y, DUBE CM, RICE CJ, and BARAM TZ. Rapid loss of dendritic spines after stress involves derangement of spine dynamics by corticotropin-releasing hormone. *J Neurosci,* 28: 2903-11, 2008.

CONTOREGGI C, HERNING RI, NA P, GOLD PW, CHROUSOS G, NEGRO PJ, BETTER W, and CADET JL. Stress hormone responses to corticotropin-releasing hormone in substance abusers without severe comorbid psychiatric disease. *Biol Psychiatry,* 54: 873-8, 2003.

CRISTÓVÃO-FERREIRA S, VAZ SH, RIBEIRO JA, and SEBASTIAO AM. Adenosine a2a receptors enhance gaba transport into nerve terminals by restraining pkc inhibition of gat-1. *J Neurochem,* 109: 336-47, 2009.

CRITÓVÃO-FERREIRA S, VAZ SH, RIBEIRO AJ, and SEBASTIÃO AM. *Adenosine modulates astrocytic gaba uptake in a biphasic way. Secondary Titl.*, 2008.

CUNHA GM, CANAS PM, OLIVEIRA CR, and CUNHA RA. Increased density and synapto-protective effect of adenosine a2a receptors upon sub-chronic restraint stress. *Neuroscience,* 141: 1775-81, 2006.

CUNHA RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: Different roles, different sources and different receptors. *Neurochem Int,* 38: 107-25, 2001.

CUNHA RA. Neuroprotection by adenosine in the brain: From a(1) receptor activation to a (2a) receptor blockade. *Purinergic Signal,* 1: 111-34, 2005.

CUNHA RA, CONSTANTINO MC, SEBASTIAO AM, and RIBEIRO JA. Modification of a1 and a2a adenosine receptor binding in aged striatum, hippocampus and cortex of the rat. *Neuroreport,* 6: 1583-8, 1995.

CUNHA RA, FERRE S, VAUGEOIS JM, and CHEN JF. Potential therapeutic interest of adenosine a2a receptors in psychiatric disorders. *Curr Pharm Des,* 14: 1512-24, 2008.

CUNHA RA, JOHANSSON B, CONSTANTINO MD, SEBASTIAO AM, and FREDHOLM BB. Evidence for high-affinity binding sites for the adenosine a2a receptor agonist [3h] cgs 21680 in the rat hippocampus and cerebral cortex that are different from striatal a2a receptors. *Naunyn Schmiedebergs Arch Pharmacol,* 353: 261-71, 1996.

CUNHA RA, JOHANSSON B, VAN DER PLOEG I, SEBASTIAO AM, RIBEIRO JA, and FREDHOLM BB. Evidence for functionally important adenosine a2a receptors in the rat hippocampus. *Brain Res,* 649: 208-16, 1994a.

CUNHA RA, MILUSHEVA E, VIZI ES, RIBEIRO JA, and SEBASTIAO AM. Excitatory and inhibitory effects of a1 and a2a adenosine receptor activation on the electrically evoked [3h]acetylcholine release from different areas of the rat hippocampus. *J Neurochem,* 63: 207-14, 1994b.

CUNHA RA and RIBEIRO JA. Purinergic modulation of [(3)h]gaba release from rat hippocampal nerve terminals. *Neuropharmacology,* 39: 1156-67, 2000.

DAGYTE G, VAN DER ZEE EA, POSTEMA F, LUITEN PG, DEN BOER JA, TRENTANI A, and MEERLO P. Chronic but not acute foot-shock stress leads to temporary suppression of cell proliferation in rat hippocampus. *Neuroscience,* 162: 904-13, 2009.

DANIELS WM, PIETERSEN CY, CARSTENS ME, and STEIN DJ. Maternal separation in rats leads to anxiety-like behavior and a blunted acth response and altered neurotransmitter levels in response to a subsequent stressor. *Metab Brain Dis,* 19: 3-14, 2004.

DIAMOND DM, PARK CR, HEMAN KL, and ROSE GM. Exposing rats to a predator impairs spatial working memory in the radial arm water maze. *Hippocampus,* 9: 542-52, 1999.

DIAS RA, FERNANDES CC, RIBEIRO JA, and SEBASTIÃO AM. *Enhancement of ampa currents and glur1 membrane expression through pka-coupled adenosine a_{2a} receptors. Secondary Titl.*, 2008.

DIOGENES MJ, ASSAIFE-LOPES N, PINTO-DUARTE A, RIBEIRO JA, and SEBASTIAO AM. Influence of age on bdnf modulation of hippocampal synaptic transmission: Interplay with adenosine a2a receptors. *Hippocampus,* 17: 577-85, 2007.

DIOGENES MJ, FERNANDES CC, SEBASTIAO AM, and RIBEIRO JA. Activation of adenosine a2a receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices. *J Neurosci,* 24: 2905-13, 2004.

FERNANDEZ M, SVENNINGSSON P, and FREDHOLM BB. Adaptive changes in adenosine receptors following long-term treatment with the adenosine receptor agonist r-phenylisopropyl adenosine. *Life Sci,* 58: 769-76, 1996.

FONTINHA BM, DELGADO-GARCIA JM, MADRONAL N, RIBEIRO JA, SEBASTIAO AM, and GRUART A. Adenosine a(2a) receptor modulation of hippocampal ca3-ca1 synapse plasticity during associative learning in behaving mice. *Neuropsychopharmacology,* 34: 1865-74, 2009.

FOY MR, STANTON ME, LEVINE S, and THOMPSON RF. Behavioral stress impairs long-term potentiation in rodent hippocampus. *Behav Neural Biol,* 48: 138-49, 1987.

FREDHOLM BB. Adenosine and neuroprotection. *Int Rev Neurobiol,* 40: 259-80, 1997.

GEIGER JD and GLAVIN GB. Adenosine receptor activation in brain reduces stress-induced ulcer formation. *European Journal of Pharmacology,* 115: 185-90, 1985.

GRUEN RJ, WENBERG K, ELAHI R, and FRIEDHOFF AJ. Alterations in gabaa receptor binding in the prefrontal cortex following exposure to chronic stress. *Brain Res,* 684: 112-4, 1995.

HEIM C and NEMEROFF CB. The role of childhood trauma in the neurobiology of mood and anxiety disorders: Preclinical and clinical studies. *Biol Psychiatry,* 49: 1023-39, 2001.

HEIM C, OWENS MJ, PLOTSKY PM, and NEMEROFF CB. Persistent changes in corticotropin-releasing factor systems due to early life stress: Relationship to the pathophysiology of major depression and post-traumatic stress disorder. *Psychopharmacol Bull,* 33: 185-92, 1997.

HERMAN JP and CULLINAN WE. Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci,* 20: 78-84, 1997.

HTUN H, BARSONY J, RENYI I, GOULD DL, and HAGER GL. Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. *Proc Natl Acad Sci U S A,* 93: 4845-50, 1996.

JARVIS MF and WILLIAMS M. Direct autoradiographic localization of adenosine a2 receptors in the rat brain using the a2-selective agonist, [3h]cgs 21680. *European Journal of Pharmacology,* 168: 243-6, 1989.

JOELS M. Corticosteroid effects in the brain: U-shape it. *Trends Pharmacol Sci,* 27: 244-50, 2006.

JOELS M. Functional actions of corticosteroids in the hippocampus. *European Journal of Pharmacology,* 583: 312-21, 2008.

JOELS M, HESEN W, KARST H, and DE KLOET ER. Steroids and electrical activity in the brain. *J Steroid Biochem Mol Biol,* 49: 391-8, 1994.

JOHNSON EO, KAMILARIS TC, CHROUSOS GP, and GOLD PW. Mechanisms of stress: A dynamic overview of hormonal and behavioral homeostasis. *Neurosci Biobehav Rev,* 16: 115-30, 1992.

KALUEFF AV and TUOHIMAA P. Experimental modeling of anxiety and depression. *Acta Neurobiol Exp (Wars),* 64: 439-48, 2004.

KANG HJ and SCHUMAN EM. Neurotrophin-induced modulation of synaptic transmission in the adult hippocampus. *J Physiol Paris,* 89: 11-22, 1995.

KARST H, WADMAN WJ, and JOELS M. Corticosteroid receptor-dependent modulation of calcium currents in rat hippocampal ca1 neurons. *Brain Res,* 649: 234-42, 1994.

KAUFMAN J, PLOTSKY PM, NEMEROFF CB, and CHARNEY DS. Effects of early adverse experiences on brain structure and function: Clinical implications. *Biol Psychiatry,* 48: 778-90, 2000.

KIM JJ, FOY MR, and THOMPSON RF. Behavioral stress modifies hippocampal plasticity through n-methyl-d-aspartate receptor activation. *Proc Natl Acad Sci U S A,* 93: 4750-3, 1996.

KIM JJ, SONG EY, and KOSTEN TA. Stress effects in the hippocampus: Synaptic plasticity and memory. *Stress,* 9: 1-11, 2006.

KIRK IP and RICHARDSON PJ. Further characterization of [3h]-cgs 21680 binding sites in the rat striatum and cortex. *Br J Pharmacol,* 114: 537-43, 1995.

KITCHENER P, DI BLASI F, BORRELLI E, and PIAZZA PV. Differences between brain structures in nuclear translocation and DNA binding of the glucocorticoid receptor during stress and the circadian cycle. *Eur J Neurosci,* 19: 1837-46, 2004.

LADD CO, HUOT RL, THRIVIKRAMAN KV, NEMEROFF CB, MEANEY MJ, and PLOTSKY PM. Long-term behavioral and neuroendocrine adaptations to adverse early experience. *Prog Brain Res,* 122: 81-103, 2000.

LEVINE ES, DREYFUS CF, BLACK IB, and PLUMMER MR. Selective role for trkb neurotrophin receptors in rapid modulation of hippocampal synaptic transmission. *Brain Res Mol Brain Res,* 38: 300-3, 1996.

LINDEN J. Molecular approach to adenosine receptors: Receptor-mediated mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol,* 41: 775-87, 2001.

LOPES DA SILVA FH, WITTER MP, BOEIJINGA PH, and LOHMAN AH. Anatomic organization and physiology of the limbic cortex. *Physiol Rev,* 70: 453-511, 1990.

LOPES L. Adenosine a2a receptors and chronic stress in the hippocampus of the rat. *Purinergic Signalling,* 4, 2008.

LOPES LV, CUNHA RA, KULL B, FREDHOLM BB, and RIBEIRO JA. Adenosine a(2a) receptor facilitation of hippocampal synaptic transmission is dependent on tonic a(1) receptor inhibition. *Neuroscience,* 112: 319-29, 2002.

LOPES LV, CUNHA RA, and RIBEIRO JA. Cross talk between a(1) and a(2a) adenosine receptors in the hippocampus and cortex of young adult and old rats. *J Neurophysiol,* 82: 3196-203, 1999a.

LOPES LV, CUNHA RA, and RIBEIRO JA. Zm 241385, an adenosine a(2a) receptor antagonist, inhibits hippocampal a(1) receptor responses. *European Journal of Pharmacology,* 383: 395-8, 1999b.

LOPES LV, FONTINHA BM, COSTENLA AR, SOUSA V, and SEBASTIÃO AM. *Behavioural and electrophysiological assessment of the impact of early life stress in the rat hippocampus. Secondary Titl.*, 2008a.

LOPES LV, MARVIN-GUY LF, FUERHOLZ A, AFFOLTER M, RAMADAN Z, KUSSMANN M, FAY LB, and BERGONZELLI GE. Maternal deprivation affects the neuromuscular protein profile of

the rat colon in response to an acute stressor later in life. *J Proteomics,* 71: 80-8, 2008b.

LU B, PANG PT, and WOO NH. The yin and yang of neurotrophin action. *Nat Rev Neurosci,* 6: 603-14, 2005.

MAGARINOS AM and MCEWEN BS. Stress-induced atrophy of apical dendrites of hippocampal ca3c neurons: Comparison of stressors. *Neuroscience,* 69: 83-8, 1995.

MAGARINOS AM, VERDUGO JM, and MCEWEN BS. Chronic stress alters synaptic terminal structure in hippocampus. *Proc Natl Acad Sci U S A,* 94: 14002-8, 1997.

MAJEWSKA MD, BISSERBE JC, and ESKAY RL. Glucocorticoids are modulators of gabaa receptors in brain. *Brain Res,* 339: 178-82, 1985.

MARTIN S, HENLEY JM, HOLMAN D, ZHOU M, WIEGERT O, VAN SPRONSEN M, JOELS M, HOOGENRAAD CC, and KRUGERS HJ. Corticosterone alters ampar mobility and facilitates bidirectional synaptic plasticity. *PLoS One,* 4: e4714, 2009.

MCCORMICK DA, WANG Z, and HUGUENARD J. Neurotransmitter control of neocortical neuronal activity and excitability. *Cereb Cortex,* 3: 387-98, 1993.

MCEWEN BS. Physiology and neurobiology of stress and adaptation: Central role of the brain. *Physiol Rev,* 87: 873-904, 2007.

MCKINNEY WT. Animal models of depression: An overview. *Psychiatr Dev,* 2: 77-96, 1984.

MEANEY MJ, AITKEN DH, VAN BERKEL C, BHATNAGAR S, and SAPOLSKY RM. Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science,* 239: 766-8, 1988.

MELLER E, SHEN C, NIKOLAO TA, JENSEN C, TSIMBERG Y, CHEN J, and GRUEN RJ. Region-specific effects of acute and repeated restraint stress on the phosphorylation of mitogen-activated protein kinases. *Brain Res,* 979: 57-64, 2003.

MILLER DB and O'CALLAGHAN JP. Aging, stress and the hippocampus. *Ageing Res Rev,* 4: 123-40, 2005.

MIZOGUCHI K, IKEDA R, SHOJI H, TANAKA Y, MARUYAMA W, and TABIRA T. Aging attenuates glucocorticoid negative feedback in rat brain. *Neuroscience,* 159: 259-70, 2009.

NEWPORT DJ, STOWE ZN, and NEMEROFF CB. Parental depression: Animal models of an adverse life event. *Am J Psychiatry,* 159: 1265-83, 2002.

NISHIZAKI T, NAGAI K, NOMURA T, TADA H, KANNO T, TOZAKI H, LI XX, KONDOH T, KODAMA N, TAKAHASHI E, SAKAI N, TANAKA K, and SAITO N. A new neuromodulatory pathway with a glial contribution mediated via a(2a) adenosine receptors. *Glia,* 39: 133-47, 2002.

O'KANE EM and STONE TW. Interaction between adenosine a1 and a2 receptor-mediated responses in the rat hippocampus in vitro. *European Journal of Pharmacology,* 362: 17-25, 1998.

OSEN-SAND A, CATSICAS M, STAPLE JK, JONES KA, AYALA G, KNOWLES J, GRENNINGLOH G, and CATSICAS S. Inhibition of axonal growth by snap-25 antisense oligonucleotides in vitro and in vivo. *Nature,* 364: 445-8, 1993.

PALACIOS SD, PAK K, RIVKIN AZ, KAYALI AG, AUSTEN D, ALETSEE C, MELHUS A, WEBSTER NJ, and RYAN AF. Role of p38 mitogen-activated protein kinase in middle ear mucosa hyperplasia during bacterial otitis media. *Infect Immun,* 72: 4662-7, 2004.

PARDON MC and RATTRAY I. What do we know about the long-term consequences of stress on ageing and the progression of age-related neurodegenerative disorders? *Neurosci Biobehav Rev,* 32: 1103-20, 2008.

PEDERSEN WA, WAN R, ZHANG P, and MATTSON MP. Urocortin, but not urocortin ii, protects cultured hippocampal neurons from oxidative and excitotoxic cell death via corticotropin-releasing hormone receptor type i. *J Neurosci,* 22: 404-12, 2002.

PICKERING C, GUSTAFSSON L, CEBERE A, NYLANDER I, and LILJEQUIST S. Repeated maternal separation of male wistar rats alters glutamate receptor expression in the hippocampus but not the prefrontal cortex. *Brain Res,* 1099: 101-8, 2006.

POLLANDT S, LIU J, OROZCO-CABAL L, GRIGORIADIS DE, VALE WW, GALLAGHER JP, and SHINNICK-GALLAGHER P. Cocaine withdrawal enhances long-term potentiation induced by corticotropin-releasing factor at central amygdala glutamatergic synapses via crf, nmda receptors and pka. *Eur J Neurosci,* 24: 1733-43, 2006.

PRAGER EM and JOHNSON LR. Stress at the synapse: Signal transduction mechanisms of adrenal steroids at neuronal membranes. *Sci Signal,* 2: re5, 2009.

REBOLA N, CANAS PM, OLIVEIRA CR, and CUNHA RA. Different synaptic and subsynaptic localization of adenosine a2a receptors in the hippocampus and striatum of the rat. *Neuroscience,* 132: 893-903, 2005.

REBOLA N, COELHO JE, COSTENLA AR, LOPES LV, PARADA A, OLIVEIRA CR, SOARES-DA-SILVA P, DE MENDONCA A, and CUNHA RA. Decrease of adenosine a1 receptor density and of adenosine neuromodulation in the hippocampus of kindled rats. *Eur J Neurosci,* 18: 820-8, 2003.

REPPERT SM, WEAVER DR, STEHLE JH, and RIVKEES SA. Molecular cloning and characterization of a rat a1-adenosine receptor that is widely expressed in brain and spinal cord. *Mol Endocrinol,* 5: 1037-48, 1991.

REUL JM and DE KLOET ER. Two receptor systems for corticosterone in rat brain: Microdistribution and differential occupation. *Endocrinology,* 117: 2505-11, 1985.

RIBEIRO JA, SEBASTIAO AM, and DE MENDONCA A. Participation of adenosine receptors in neuroprotection. *Drug News Perspect,* 16: 80-6, 2003.

ROCERI M, HENDRIKS W, RACAGNI G, ELLENBROEK BA, and RIVA MA. Early maternal deprivation reduces the expression of bdnf and nmda receptor subunits in rat hippocampus. *Mol Psychiatry,* 7: 609-16, 2002.

SANDI C. The role and mechanisms of action of glucocorticoid involvement in memory storage. *Neural Plast,* 6: 41-52, 1998.

SAPOLSKY RM. A mechanism for glucocorticoid toxicity in the hippocampus: Increased neuronal vulnerability to metabolic insults. *J Neurosci,* 5: 1228-32, 1985.

SAPOLSKY RM, KREY LC, and MCEWEN BS. Stress down-regulates corticosterone receptors in a site-specific manner in the brain. *Endocrinology,* 114: 287-92, 1984.

SAPOLSKY RM, KREY LC, and MCEWEN BS. The neuroendocrinology of stress and aging: The glucocorticoid cascade hypothesis. *Endocr Rev,* 7: 284-301, 1986.

SAPOLSKY RM, MEANEY MJ, and MCEWEN BS. The development of the glucocorticoid receptor system in the rat limbic brain. Iii. Negative-feedback regulation. *Brain Res,* 350: 169-73, 1985.

SCACCIANOCE S, NAVARRA D, DI SCIULLO A, ANGELUCCI L, and ENDROCZI E. Adenosine and pituitary-adrenocortical axis activity in the rat. *Neuroendocrinology,* 50: 464-8, 1989.

SEBASTIÃO AM and RIBEIRO JA. Adenosine a2 receptor-mediated excitatory actions on the nervous system. *Prog Neurobiol,* 48: 167-89, 1996.

SEBASTIÃO AM and RIBEIRO JA. Adenosine receptors and the central nervous system. *Handb Exp Pharmacol*: 471-534, 2009.

SMITH MA, MAKINO S, KVETNANSKY R, and POST RM. Effects of stress on neurotrophic factor expression in the rat brain. *Ann N Y Acad Sci,* 771: 234-9, 1995a.

SMITH MA, MAKINO S, KVETNANSKY R, and POST RM. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mrnas in the hippocampus. *J Neurosci,* 15: 1768-77, 1995b.

SOPPET D, ESCANDON E, MARAGOS J, MIDDLEMAS DS, REID SW, BLAIR J, BURTON LE, STANTON BR, KAPLAN DR, HUNTER T, NIKOLICS K, and PARADE LF. The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkb tyrosine kinase receptor. *Cell,* 65: 895-903, 1991.

SOUSA N, CERQUEIRA JJ, and ALMEIDA OF. Corticosteroid receptors and neuroplasticity. *Brain Res Rev,* 57: 561-70, 2008.

SOUSA N, LUKOYANOV NV, MADEIRA MD, ALMEIDA OF, and PAULA-BARBOSA MM. Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. *Neuroscience,* 97: 253-66, 2000.

SPERK G, SCHWARZER C, TSUNASHIMA K, FUCHS K, and SIEGHART W. Gaba(a) receptor subunits in the rat hippocampus i: Immunocytochemical distribution of 13 subunits. *Neuroscience,* 80: 987-1000, 1997.

STEIGER JL and RUSSEK SJ. Gabaa receptors: Building the bridge between subunit mrnas, their promoters, and cognate transcription factors. *Pharmacol Ther,* 101: 259-81, 2004.

STONE EA, SLUCKY AV, PLATT JE, and TRULLAS R. Reduction of the cyclic adenosine 3',5'-monophosphate response to catecholamines in rat brain slices after repeated restraint stress. *J Pharmacol Exp Ther,* 233: 382-8, 1985.

SULLIVAN R, WILSON DA, FELDON J, YEE BK, MEYER U, RICHTER-LEVIN G, AVI A, MICHAEL T, GRUSS M, BOCK J, HELMEKE C, and BRAUN K. The international society for developmental psychobiology annual meeting symposium: Impact of early life experiences on brain and behavioral development. *Dev Psychobiol,* 48: 583-602, 2006.

TARSA L and GODA Y. Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. *Proc Natl Acad Sci U S A,* 99: 1012-6, 2002.

TSIGOS C and CHROUSOS GP. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J Psychosom Res,* 53: 865-71, 2002.

WANG HL, WAYNER MJ, CHAI CY, and LEE EH. Corticotrophin-releasing factor produces a long-lasting enhancement of synaptic efficacy in the hippocampus. *Eur J Neurosci,* 10: 3428-37, 1998.

WATANABE Y, GOULD E, and MCEWEN BS. Stress induces atrophy of apical dendrites of hippocampal ca3 pyramidal neurons. *Brain Res,* 588: 341-5, 1992.

WIEDENMANN B and FRANKE WW. Identification and localization of synaptophysin, an integral membrane glycoprotein of mr 38,000 characteristic of presynaptic vesicles. *Cell,* 41: 1017-28, 1985.

WILLNER P, SANGER D, and OGLESBY M. The behavioural pharmacology of anxiety and depression. *Behav Pharmacol,* 8: 475-6, 1997.

XU L, ANWYL R, and ROWAN MJ. Behavioural stress facilitates the induction of long-term depression in the hippocampus. *Nature,* 387: 497-500, 1997.

ANNEX I

Immunohistochemistry method used to obtain the photos in Figure 1.3

(Based in Manabe *et al.*, 2002).

Hippocampi were fixed in freshly prepared paraformaldehyde 4% (PFA 4%) either overnight at 4ºC or for two hours at room temperature. After rinsing in ice-cold PBS (phosphate buffer saline solution, NaCl 137 mM; KCl 2.7 mM; KH_2PO_4 1.5 mM; Na_2HPO_4 8 mM) the tissue was left sinking overnight at 4ºC in sucrose 30% made in PBS. It was equilibrated with gelatin sucrose for 30/40 minutes at 37ºC in 15 % sucrose/7.5 % gelatin in PBS, included in the same solution and then left to solidify for 1 to 2 hours at 4ºC. The included tissue was cut into blocks and slowly frozen to -70ºC submerging in 2-methylbutane cooled with liquid nitrogen. The blocks were kept at -80ºC until use. Cryostat sections of 12 µm thick were obtained with a cryostat (CM3050 S; Leica, Germany), collected in Superfrost slides and stored at -20ºC.

For immunohistochemistry the slides were left for 30 minutes at 37ºC in PBS to remove the gelatin from the slices, after washing three times with PBS at room temperature, a 10-minute incubation with glycine 0.1 M was performed. At room temperature the slices were them permeabilized with 0.5% Triton X100 for 15-30 minutes and blocked with 10% Fetal Bovine Serum (FBS) prepared in PBS-T (PBS with 0.1% Tween-20) for 1 hour. Incubation with primary antibody was performed overnight at 4ºC and slides were washed in PBS-T before incubation with secondary antibody (anti-mouse Alexa 568, Invitrogen 1:500) for 90 minutes at room temperature. Immunodetection was performed for Glial fibrillary acidic protein (GFAP) with anti-GFAP P11137 from Chemicon (mouse, 1:100). For nuclei identification a 5 min incubation with 4',6-diamidino-2-phenylindole (DAPI, a fluorescent stain that binds strongly to the DNA) was performed after washing with PBS-T. One last wash was performed to remove DAPI, the slides were mounted in Mowiol and let to dry overnight at room temperature protected from light exposure. Fluorescence visualization was done with a fluorescence microscope (Inverted Widefield Fluorescence Microscope, Zeiss Axiovert 200M, Germany). All antibody dilutions were freshly prepared in 10% of fetal bovine serum FBS in PBS-T.

MANABE N, HIRAI S, IMAI F, NAKANISHI H, TAKAI Y, and OHNO S. Association of asip/mpar-3 with adherens junctions of mouse neuroepithelial cells. *Dev Dyn,* 225: 61-9, 2002.