UNIVERSIDADE DE LISBOA

Faculdade de Medicina de Lisboa

Interaction between glucocorticoid and adenosine A_{2A} receptors in the hippocampus – implications for learning and memory

Vânia Luísa Neves Batalha

Doutoramento em Ciências Biomédicas

Especialidade em Neurociências

2014

UNIVERSIDADE DE LISBOA

Faculdade de Medicina de Lisboa

Interaction between glucocorticoid and adenosine A_{2A} receptors in the hippocampus – implications for learning and memory

Vânia Luísa Neves Batalha

Tese orientada por Doutora Luísa Vaqueiro Lopes

Doutoramento em Ciências Biomédicas

Especialidade em Neurociências

A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 20 de Maio de 2014

The experimental work herein described was performed at Instituto de Medicina Molecular - Faculdade de Medicina da Universidade de Lisboa except when otherwise stated, under the supervision of Dr. Luísa V. Lopes.

Abstract

Chronic stress and ageing are closely linked to a dysfunction in the hypothalamic pituitary adrenal axis (HPA-axis) that leads to higher circulating levels of stress hormones. This has deleterious effects on brain function such as neuronal loss, synaptic plasticity impairments and cognitive deficits, all actions related to activation of glucocorticoid receptors (GR). Adenosine A_{2A} Receptors ($A_{2A}R$) are constitutively activated G-protein coupled-receptors, and one of the main brain targets of the homeostatic neuromodulator adenosine. Upon aging and stress an imbalance in the levels of adenosine receptors was observed, and recent results indicate that their blockade could prevent stress effects. However, it was still unknown the extent to which $A_{2A}R$ were involved in neuronal dysfunction and in stress mediated effects. Therefore the goal of this thesis was first, to evaluate if $A_{2A}R$ blockade could revert stress induced deficits, clarifying the role of $A_{2A}R$ in the deleterious effects of stress; second, to assess if an increase in $A_{2A}R$ is sufficient to drive hippocampal deficits and third, if $A_{2A}R$ could directly modulate GR, being this the mechanism by which they are triggering neuronal dysfunction.

By treating maternal separated rats with a selective antagonist of $A_{2A}R$, orally delivered for one month, it was shown that the blockade of these receptors can: overcome memory deficits evaluated in the Morris Water Maze task; reestablish synaptic plasticity impairments, recorded as Schaffer collaterals-CA3 long-term potentiation; and finally recover CA1 pyramidal neuronal branching in stressed animals. This was accompanied by a reestablishment of the HPA-axis function, quantified as circadian oscillation of corticosterone plasma levels. These results revealed the instrumental role of $A_{2A}R$ in stress effects and their effectiveness as therapeutic targets.

To further explore if $A_{2A}R$ were the trigger for hippocampal dysfunction the effects of overexpressing $A_{2A}R$ under the control of calcium calmodulin protein Kinase II

(CaMKII) promoter were evaluated. $A_{2A}R$ overexpression in rats lead to a shift in adenosine neuromodulation, from a protein kinase C- to a protein kinase A- dependent signaling, much similar to what is observed upon aging. This was also present in stressed animals and reverted by blocking $A_{2A}R$, providing the first evidence that an abnormal $A_{2A}R$ signaling can be the trigger to pathology. Additionally it was shown that the neuronal increase in $A_{2A}R$ is sufficient to drive hippocampal dependent memory impairments and to modify synaptic plasticity in an age-like manner. Interestingly, $A_{2A}R$ overexpression also induces a dysfunction in HPA-axis by increasing circulating corticosterone levels, revealing again, an involvement of $A_{2A}R$ in the control of stress response.

Finally it was shown that $A_{2A}R$ blockade decreases GR/GRE (glucocorticoid receptor/glucocorticoid response element) transcriptional activity in a neuroblastoma cell line. This is probably due to their ability to modify GR nuclear translocation induced by the GR analogue, dexamethasone, since we observed a prevention of this translocation by blocking $A_{2A}R$. This has important consequences for synaptic plasticity: the impairments in hippocampal synaptic plasticity induced by GR activation are more profound under $A_{2A}R$ overexpression and are prevented if $A_{2A}R$ are blocked. Additionally, $A_{2A}R$ blockade therapy in vivo increased hippocampal histone H3 acetylation of the *Nr3c1* gene encoding GR and GR mRNA levels.

Globally it is now possible to conclude that $A_{2A}R$ dysfunction, depending on their levels and signaling targets, has an instrumental role in stress and aging, driving memory and synaptic plasticity impairments. Together, these results suggest that $A_{2A}R$ directly modulate GR, unveiling an important therapeutic alternative to GR antagonists for clinical applications. These findings are significant for the treatment of not only psychopathologies but can also be extended to the multiple age-related conditions where glucocorticoid response is impaired.

Resumo

O stresse crónico e o envelhecimento estão ambos associados a um aumento dos níveis plasmáticos de corticosterona e a uma disfunção do eixo hipotalâmico hipofisário (HPA, do inglês *Hypotalamic-Pituitary-Adrenal*). Estas alterações têm efeitos deletérios para a função cerebral com comprometimento da função neuronal, da plasticidade sináptica estando frequentemente associadas a danos cognitivos, tudo isto consequência da activação dos receptores de glucocorticóides (GR, do inglês *glucocorticoid receptors*). Os receptores A_{2A} de adenosina (receptores A_{2A}) são receptores acoplados a proteínas G constitutivamente activados e, no cérebro, um dos principais mediadores da resposta ao neuromodulador homeostático, a adenosina. Ao longo do envelhecimento e com o stresse, ocorre um desequilíbrio dos níveis destes receptores e alguns estudos sugerem que o seu bloqueio pode mesmo prevenir os efeitos do stresse. Contudo, não era ainda conhecido até que ponto os receptors A_{2A} estariam envolvidos na disfunção neuronal e nos efeitos mediados pelo stresse. Assim, os objectivos desta tese foram: em primeiro lugar, avaliar se o bloqueio dos receptores A_{2A} poderia reverter os efeitos deletérios originados pelo stresse, clarificando deste modo a importância destes receptores; segundo, avaliar se um aumento dos receptores A_{2A} seria suficiente para originar défices ao nível do hipocampo e em terceiro lugar, se os receptores A_{2A} poderiam modular os efeitos dos GR, sendo este o mecanismo pelo qual estariam a conduzir ao dano e à disfunção neuronal.

Submetemos ratos Wistar machos a um paradigma de stresse por separação maternal. Neste protocolo, os animais são separados das mães durante 3 horas por dia, do dia 2 ao dia 14 de vida. Quando avaliados na idade adulta às 8 semanas, estes animais apresentam um comprometimento da memória dependente do hipocampo e da plasticidade sináptica. Para estudar o envolvimento dos receptores A_{2A} nestes défices e o seu potencial terapêutico, os animais foram tratados com KW6002, um antagonista selectivo dos receptores A_{2A}, a partir das 6 semanas de vida, durante pelo menos um mês, período após o qual foram avaliados em testes de comportamento animal. A administração de KW6002 reverteu os défices de memória e o comportamento ansioso

apresentado por estes animais. As alterações na plasticidade sináptica, avaliadas na sinapse fibras de Schaffer/dendrites de CA3 do hipocampo, e na arborização dendrítica foram também revertidas. A função do eixo HPA foi também avaliada: os animais sujeitos a separação maternal não apresentaram uma variação circadiária fisiológica dos corticosteróides, com níveis de corticosterona no plasma elevados permanentemente. Após o tratamento, os níveis plasmáticos desta hormona reduziram e a variação circadiária foi reestabelecida. Estes resultados revelam assim um papel instrumental dos receptores A_{2A} nos danos causados pelo stresse e o seu potencial terapêutico para diversas patologias.

Com o objectivo de explorar em maior detalhe o papel dos receptores A_{2A} na origem da disfunção do hipocampo, que ocorre tanto com o envelhecimento como com o stresse, os efeitos que advêm da sobreexpressão deste receptor foram avaliados. Utilizaram-se ratos transgénicos com sobreexpressão do receptor A_{2A} humano controlada pelo promotor da CaMKII, abundante em neurónios do córtex e hipocampo. A sobreexpressão de receptores A_{2A} alterou a neuromodulação adenosinérgica de forma muito semelhante ao envelhecimento. Isto também foi observado em animais sujeitos a separação maternal e revertido com o tratamento com o antagonista dos receptores A_{2A}. Adicionalmente, demonstrou-se que o aumento neuronal dos receptores A_{2A} é suficiente para gerar défices na memória dependente do hipocampo e para modificar a plasticidade sináptica de forma similar à que ocorre no envelhecimento. É interessante notar que todos estes efeitos ocorrem associados a uma disfunção dos eixo HPA, também consequência do aumento destes receptores. Isto revela novamente um papel importante dos receptores A_{2A} no controlo da resposta ao stresse.

Era então fundamental compreender se a acção dos receptores A_{2A} na modulação dos efeitos do stresse se devia:

1) apenas a um efeito indirecto na função do hipocampo, e como consequência no eixo HPA (O hipocampo tem um importante papel inibitório do eixo HPA como resposta a níveis plasmáticos elevados de corticosterona); ou 2) a uma acção directa nos GR. O

efeito de activar ou bloquear os receptores A_{2A} na actividade transcripcional e localização nuclear dos GR foram avaliados. O bloqueio dos receptores A_{2A} diminuiu a actividade transcripcional dos GR e impediu a sua translocação nuclear induzida por um agonista selectivo. As consequências desta interacção para fenómenos de plasticidade sináptica foram também avaliadas. Observou-se que os efeitos deletérios de activar os GR são prevenidos pelo bloqueio dos receptores A_{2A} e aumentados quando estes são sobreexpressos. Assim estes resultados revelam um papel crucial dos receptores A_{2A} nos efeitos dos GR sugerindo até que possa ser este o mecanismo pelo qual o seu bloqueio se tem revelado benéfico em patologias tão diversas.

Globalmente é agora possível concluir que alterações nos receptores A_{2A}, ao nível quer da densidade quer da sinalização, têm um papel instrumental nos efeitos do stresse e do envelhecimento na memória e na plasticidade sináptica. Estes dados apoiam a utilização terapêutica de antagonistas dos receptores A_{2A} em inúmeras patologias. Os resultados agora apresentados constituem também a primeira evidência de que os receptores A_{2A} tem um papel directo no controlo dos efeitos dos glucocorticoides e na função do eixo-HPA, sendo esta uma explicação totalmente nova do mecanismo subjacente aos efeitos benéficos dos antagonistas A_{2A}.

Publications related to this dissertation

Batalha VL, Pego JM, Fontinha BM, Costenla AR, Valadas JS, Baqi Y, Radjainia H, Müller CE, Sebastião AM, Lopes LV (2013) *Adenosine A(2A) receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation.* Mol Psychiatry.18(3):320-31.

Batalha VL, Ferreira DG, Coelho JE, Gomes RA, Valadas JS, Canas P, Cuvelier L, Shmidt T, Cunha RA, Schiffmann SN, Bader M, Blum D, Lopes LV**,** *Aging-like hippocampal deficits driven by overexpression of adenosine A2A receptors in forebrain neuron, s*ubmitted.

Batalha VL, Ferreira DG, Valadas JS, Coelho JE, Gomes RA, Shmidt T, Hamdane M, Buée L, Outeiro TF, Cunha RA, Bader M, Blum D, Lopes LV, *Adenosine A2A receptor regulates stress glucocorticoid receptor in the brain,* submitted

Other publications

Coelho JE, Alves P, Canas PM, Valadas JS, **Batalha VL**, Ferreira DG, Shmidt T, Ribeiro JA, Bader M, Cunha RA, Couto FS, Lopes LV (2014*) Overexpression of adenosine A2A receptors in rats: effects on depression, locomotion and anxiety* Frontiers in Psychiatry, *in press*

Jerónimo-Santos A, **Batalha VL,** Müller CE, Baqi Y, Sebastião AM, Lopes LV, Diógenes MJ (2014) *Impact of in vivo chronic blockade of adenosine A2A receptors on the BDNF-mediated facilitation of LTP.* Neuropharmacology 83C, 99-106.

Flaten V, Laurent C, Coelho JE, Sandau U, **Batalha VL**, Burnouf S, Hamdane M, Humez S, Boison D, Lopes LV, Buée L, Blum D. (2014) *From epidemiology to pathophysiology: what about caffeine in Alzheimer's disease?* Biochem Soc Trans. 42(2):587-92.

Sousa VC, Vital J, Costenla AR, **Batalha VL**, Sebastião AM, Ribeiro JA, Lopes LV. (2014) *Maternal separation impairs long term-potentiation in CA1-CA3 synapses and hippocampal-dependent memory in old rats.* Neurobiol Aging; 35(7):1680-5.

Valadas JS, **Batalha VL**, Ferreira DG, Gomes R, Coelho JE, Sebastião AM, Diógenes MJ, Lopes LV. (2012) *Neuroprotection afforded by adenosine A2A receptor blockade is modulated by corticotrophin-releasing factor (CRF) in glutamate injured cortical neurons.* J Neurochem; 123(6):1030-40.

Couto FS, **Batalha VL**, Valadas JS, Data-Franca J, Ribeiro JA, Lopes LV. (2012) *Escitalopram improves memory deficits induced by maternal separation in the rat.* Eur J Pharmacol.695(1-3):71-5.

Marques S, **Batalha VL**, Lopes LV, Outeiro TF (2011) *Modulating Alzheimer's disease through caffeine: a putative link to epigenetics.* J Alzheimers Dis.24 Suppl 2:161-71.

Table of Contents

Abbreviation list

A_1 - Adenosine A_1 Receptor

A_{2A} H – Human Adenosine A_{2A} Receptor

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

Act-B - Actin-β

ACTH - Adrenocorticotrophin

AD - Alzheimer Disease

ADA - Adenosine deaminase

AMPA - α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATP – Adenosine triphosphate

Aβ - amyloid-beta

BDNF- brain-derived neurotrophic factor

BSA -bovine serum albumin

CA1 - corpus ammonium 1

CA2 - corpus ammonium 2

CA3 - corpus ammonium 3

CaMKII - Calcium calmodulin dependent Protein Kinase II

cAMP – 3'-5'-cyclic adenosine monophosphate

CGS 21680 - 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride ($A_{2A}R$ selective agonist),

ChIP - chromatin immunoprecipitation

CPA - N6-cyclopentyladenosine (A1selective agonist),

CREB - cAMP response element-binding protein

CRH - corticotrophin-releasing-hormone,

CTR - Control

CTR KW - control treated with KW6002

CypA – cyclophilin A

[^{3}H]DPCPX - [propyl-^{3}H]8-cyclopentyl-1,3-dipropylxanthine (A_1R selective antagonist)

DG -Dentate Gyrus

DMEM - Dulbecco's modified Eagle's medium

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

DPCPX - 8-Cyclopentyl-1,3-dipropylxanthine (A_1R selective antagonist)

DTT - Ditiotreitol

EC - entorrhinal cortex

EDTA - Ethylenediaminetetraacetic acid

EPM - elevated plus maze

EPSP- excitatory postsynaptic potential

FBS - fetal bovine serum

fEPSP - field excitatory post-synaptic potentials

GABAAR - gamma-aminobutyric acid receptor A subunit

GF 109203X - 2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide (PKC inhibitor)

Gi - adenylate cyclase inhibitory G α protein

$GluR_1$ - AMPA receptors containing glutamate receptor one subunit

Go - adenylate cyclase other G α protein

GR - glucocorticoid receptor

GRE – Glucocorticoid response element

GRE_Luc - pGL3(GRE)3_TK_Luc plasmid

Gs - adenylate cyclase excitatory G α protein

H89 - N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (PKA inhibitor)

hACTB –human Actin-β

HBSS - Hanks' Balanced Salt Solution

HF- hippocampal formation

HPA-axis - hypothalamic pituitary adrenal axis

HPLC - High-performance liquid chromatography

HRP- horseradish peroxidsse

I/O - Input-output

KW6002- istradefylline, (*E*)-8-[2-(3,4-dimethoxyphenyl)vinyl]-1,3-diethyl-7-methyl-3,7-dihydropurine-2,6-dione) ($A_{2A}R$ selective antagonist)

LTD – long-term depression

LTP - long-term totentiation

MAPK - mitogen-activated protein kinase

MBD - methyl-CpG-binding domain

meDIP - Methyl DNA immunoprecipitation

MR - mineralocorticoid receptor

MS - Maternal separation

MS KW - maternal separated treated with KW6002

MWM- Morris water maze

NCAM - Neural cell adhesion molecule

NMDA - N-methyl-D-aspartate

NMDAR2B - - N-methyl-D-aspartate receptor 2B subunit

OF - Open Field test

PBS - phosphate buffered saline

PD - Parkinson's disease

Pgk1 - Phosphoglycerate kinase 1

PKA - protein kinase A

PKC- protein kinase C

PND - postnatal day

PPIA - peptidylprolyl isomerase A

RIPA - Immunoprecipitation-Assay

RNAse - Ribonuclease

Rpl13A - Ribosomal protein L13A

RT - room temperature

RT-qPCR - real-time quantitative PCR

SCH58261 - 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (A_{2A}R selective antagonist)

SDS - Sodium dodecyl sulfate

TBS - Tris-buffered saline

tg(CaMKII-hA2AR) - Transgenic rats with an overexpression of adenosine A_{2A} receptors under the control of the CaMKII promotor

WT - wild type

XAC - e 8-(4-[(2-minoethyl)amino]carbonylmethyloxyphenyl)xanthine (non-selective adenosine receptor antagonist)

[^{3}H]ZM 241385, - 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a] [1,3,5]triazin-5-ylamino]ethyl)phenol (A_{2A}R selective antagonist)

Preface

In the following pages you will find the description of the work leading to this PhD dissertation. First, a brief background that I consider essential to lay the foundations of this work is presented, which is Chapter 1. It is not exhaustive, since my aim is solely to transmit the scientific knowledge that gave rise to the work performed, as well as provide tools to understand the work itself. For this reason I divided the background in two sections, one being the state of the art and the second, the technical approaches. The first will hopefully help the reader to contextualize the work, understand its importance and the reasons why it was performed. The second aims at giving the reader the tools required to understand the methodological approach and the reasons of choice. Then, I present the results in the form of three scientific papers, which constitute Chapters 2, 3 and 4:

- **Chapter 2: Adenosine A_{2A} receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation**
  Pages 27 to 54 - published in Molecular Psychiatry (2013)
- **Chapter 3: Aging-like hippocampal deficits driven by overexpression of adenosine A_{2A} receptors in forebrain neurons**
  Pages 55 to 76 – Submitted for publication
- **Chapter 4: A novel role of adenosine A_{2A} receptor in the modulation of the stress glucocorticoid receptor in the brain**
  Pages 77 to 101 - Submitted for publication

In the end, I included a 'General Discussion' integrating all the data and finally, a more personal perspective of the impact of the research data obtained and future directions under 'Future Perspectives'.

Chapter 1: *Background*

State of the art

The brain, like all other organs from the simplest to the most complex organism, is composed of cells. These cells communicate between each other and are organized in circuits. Which are, in turn, organized in areas with specific functions. This is a simplified image of the brain. However, as science addresses each one of these "simple" dogmas, one understands that brain is all but simple. Whenever it was clear that only neurons communicated, data show that astrocytes also have an active role in neuronal communication. When a circuit was thought to be "isolated", evidence reveals that it is constantly receiving inputs from other circuits. And rarely one brain area is restricted to a function or a function requires solemnly one brain area. The most exciting thing about the brain is its complexity. And the more we know the more complex it becomes.

Memory and the hippocampus

Memory can be very complex, as in humans, that can even recall events that never happened and were only thought, or can be very simple, like conditioned behavior, that is present in the simplest brains as in those of flyes (Quinn et al., 1974). The concept that multiple types of memory could exist and be attributed to different brain areas was very controversial (Gabrieli, 1992; Squire, 1992; Warrington, 1979), however it is now known and accepted that many different types of memory exist and that they are associated in a large extent with the use of specific brain areas (Squire, 2004). While explicit memory, as spatial memory, largely relies in the hippocampus, implicit memory, as working memory, relies mostly on dorsal striatum (Milner et al., 1998). One of the most widely studied types of memory is the hippocampal-dependent spatial memory.

The **hippocampus**, or *cornu ammonis* as was previously named, is located on the medial temporal lobe of the cerebral cortex in close contact with the lateral ventricles. With its unique and organized structure this brain area closely interacts with many

different and distinct areas of the brain, receiving inputs from all the sensory areas of the cortex and also from limbic structures (Lopes da Silva et al., 1990). The first studies regarding the organization of the hippocampal formation are from Cammilo Golgi 1886 (Golgi, 1989). However it was Santiago Ramon Y Cajal that in 1893 described the stratification of the different afferent pathways as well as the different types of neurons in each region of the hippocampus. Later the hippocampus was divided in different sub areas: the DG (Dentate Gyrus) the CA3 (*corpus ammonium* 3), CA2 (*corpus ammonium* 2) and CA1 (*corpus ammonium* 1). These areas form a mainly unidirectional circuit, in which glutamate is the main neurotransmitter and that integrates the inputs from the different areas of the brain (Lopes da Silva *et al.*, 1990; Teyler and DiScenna, 1985). Most of this inputs come from the entorinal cortex that channels the information from the cingulate cortex, temporal lobe cortex, amygdala, orbital cortex, and olfactory bulb (Sewards and Sewards, 2003). This structure has a pivotal role in different types of memory has olfactory memory or fear memory, however its main function is the spatial location and thus the spatial memory. There is now scientific evidence for the existence of what are called place cells, hippocampal neurons that encode for a given spatial location and that are activated when the animals are in that specific location (O'Keefe and Conway, 1978; O'Keefe and Dostrovsky, 1971) .

Interestingly, the hippocampus is one of the brain areas most susceptible to external insults, being one of the most and primarily affected brain areas upon normal ageing. Hippocampal dependent tasks, as spatial memory, are one of the first to be compromised by ageing, and changes in hippocampal excitability are also widely described to occur (Barnes, 1988; Rosenzweig *et al.*, 2003).. Finally, multiple pathologies characteristically affect this brain area, such as epilepsy or Alzheimer Disease (AD). Despite having a totally distinct etiology and outcome, both have a profound impact in the brain by drastically modifying hippocampal function (impairing hippocampal dependent memory tasks), excitability (impairing both neuronal properties and circuit excitability) and neuronal survival and morphology (Lado *et al.*, 2002; Marchetti and Marie, 2011).

There are many common features between normal ageing and pathologies, such as AD, concerning their impact in the brain and particularly on hippocampal formation. Both are, for example, associated with memory and synaptic plasticity impairments, dendritic retraction or even cell death (Keller, 2006). Moreover both present elevated plasmatic corticosterone levels (Lupien *et al.*, 1998; Rothman and Mattson, 2010) and increased brain adenosine levels (Mackiewicz *et al.*, 2006). Knowing that stress and stress hormones, as corticosterone, also have a profound impact in hippocampal formation (Reviewed by Miller and O'Callaghan, 2005) and that adenosine is crucial to regulate brain homeostasis in the next pages I would like to focus on stress and the main mediators of stress response, the glucocorticoids, and also on adenosine.

Stress and the HPA-axis system

Stress has been studied due to its importance in the adaptative response of the organism to the environment. Any type of threat that compromises homeostasis, requiring a compensatory response of the organism 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 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.

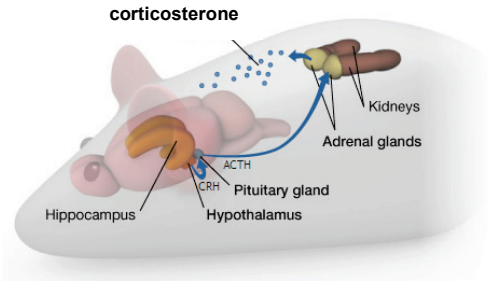

Figure 1.1: Schematic representation of the HPA-axis. The hypothalamus releases CRH that will activate pituitary neurons to release ACTH. This hormone will enter the bloodstream and induce the release of corticosterone by the adrenal glands.
(Adapted from learn.genetics.utah.edu /content/epigenetics/rats)

Stressful events are present throughout life, triggering peripheral and central physiological responses coordinated by the central nervous system, mostly through the activation of the **Hypothalamic-Pituitary-Adrenal axis system (HPA-axis,** Figure 1.1), (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 induce Adrenocorticotrophin (ACTH) secretion by pituitary neurons into the bloodstream. Circulating ACTH will stimulate the secretion of glucocorticoids from the adrenal cortex (cortisol in humans and corticosterone in rodents) being the latter the key mediators of the stress response (Tsigos and Chrousos, 2002). **Glucocorticoids** are 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 itself 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; McEwen, 2012).

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 classically cytoplasmatic receptors that upon binding to its ligand translocate to the nucleus and act as transcription factors (Zalachoras et al., 2013). Interestingly corticosteroid receptors present a distinctive distribution pattern among brain areas. Whereas GR are ubiquitously distributed in neurons and glial cells, being present in higher levels in the hippocampus, MR are mostly expressed in hippocampal and septal neurons and have 10 times more affinity for corticosterone than GR (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 (Joels, 2006; Sandi, 1998; Tsigos and Chrousos, 2002). This differential activation of corticosteroid receptors will lead to biphasic effects of stress hormones (Figure 1.2). For low or short term increases in corticosteroids there is an increase in general performance, memory, cognition and

attention; whereas for higher or prolonged exposure to glucocorticoids the effects are deleterious (Joels, 2006, Figure 1.2). 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 cell populations, depending on the activation status of the HPA-axis (Reviewed by Joels, 2006; Sousa *et al.*, 2008).

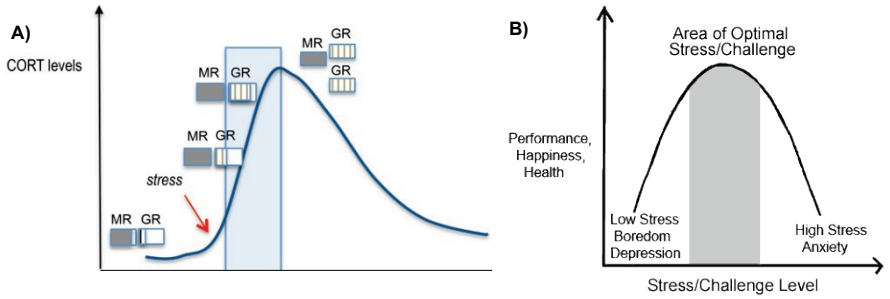

Figure 1.2: Schematic representation of the stress effects. A) presents the relationship between the levels of corticosterone and the differential occupation and activation of MR and GR. B) represents the outcome of this, the first beneficial effects of stress and the latter, deleterious effects, if corticosterone levels remain high for prolonged periods of time. Adapted from http://www.mindtools.com/stress/UnderstandStress/StressPerformance.htm and (Yau and Seckl, 2012)

The control of the HPA-axis activity is essential to maintain a functional and adaptative response to stress and multiple inhibitory pathways were developed 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. Lesions of the hippocampus lead to higher circulating levels of corticosteroids whereas it's stimulation decreases HPA-axis activity probably due to GR activation (Jacobson and Sapolsky, 1991). Therefore the increased plasma glucocorticoid levels, that result from stress, can impair the function of this brain area and consequently the stress response system can also be affected. Given the susceptibility of the hippocampal formation to stress, the control of the HPA-axis is particularly susceptible to situations that are characterized by persistently high 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) and later on impact in the stress response system.

Stress and the hippocampus

The hippocampus was the first higher brain center to be recognized as a target for stress hormones (McEwen et al., 1968). Latter, the effects of stress on hippocampal plasticity were explored by Foy and co-workers when studying stress–induced impairments in Long-Term Potentiation (LTP) (Foy et al., 1987). Nowadays multiple observations sustain that 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 biphasic effects of stress and stress hormones in the hippocampus were already described. Stress and corticosterone have been shown to impact upon neuroexcitability, regulation of glutamate release and response, synaptic plasticity, neuronal morphology and learning and memory. Stress has been shown to increase basal glutamate levels (Lowy et al., 1993) to induce neuronal death (Sapolsky, 1985) as well as neuronal atrophy of apical dendrites (Magarinos et al., 1996) and also to impair adult neurogenesis (Dagyte et al., 2009). Moreover stress was also shown to impair LTP while increasing long term depression (LTD) (Kim *et al.*, 1996; Xu *et al.*, 1997), the most characterized molecular models of memory. Changes in the levels of neurotransmitters receptors were also reported. The levels of $GABA_A$ and NMDA receptors can be decreased (Caldji *et al.*, 2000b; Roceri *et al.*, 2002) and the subunits content of AMPA receptor are also changed (Pickering et al., 2006). Changes in neurotrophins were observed in stressed animals, such as lower levels of brain-derived neurotrophic factor (BDNF) (Smith et al., 1995). All these profound changes, together with the acute effects mediated by GR activation, have implications for learning and memory. This is confirmed by the observed learning deficits (Diamond *et al.*, 1999; Okuda *et al.*, 2004; Sousa *et al.*, 2000) and anxious behavior (Caldji et al., 2000a)

characteristic of stressed animals. Figure 1.3 presents a summarized representation of the stress effects in the hippocampus (Sousa et al., 2008). Besides glucocorticoids, other molecules have an important role in the modulation of brain function, being one of them adenosine.

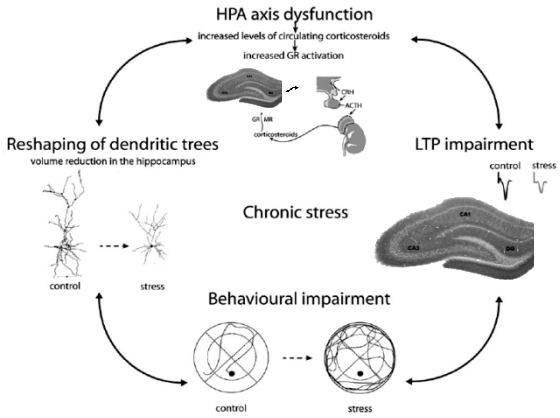

Figure 1.3: Schematic representation of the stress effects in the hippocampus. Changes in the balance between MR and GR activation will trigger cellular and molecular changes in the hippocampus, ultimately leading to behavioral impairment, adapted from Sousa (2008).

Adenosine and Adenosine A_{2A} receptors

Adenosine is a purine nucleotide that is mainly produced upon the degradation of ATP and therefore is present in all cells as a metabolite (Stone et al., 1985). In situations of compromised energy status or when cells require an augmented consume of ATP, the production of adenosine increases and this metabolite acts with an homeostatic role in the control of cellular metabolism (Arch and Newsholme, 1978). Adenosine has many important functions at cellular level in all tissues, but has broader modulatory roles in particular systems, such as the immune or nervous systems.

In the brain, adenosine has important neuromodulatory 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 Sebastiao and Ribeiro, 2009). Adenosine exerts its action through the activation of

different membrane receptors with opposite actions. Therefore the net effect of adenosine depends on the expression pattern of these receptors and differs among brain areas, cell type and even with ageing or pathology. The neuromodulatory role of adenosine in the brain is mediated by a balance between the inhibitory and excitatory actions of adenosine, via A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) respectively. Adenosine can also activate adenosine A_{2B} and A_3 receptors; however those receptors are mostly involved in the peripheral effects of adenosine (for a review see Cunha, 2001; Sebastiao and Ribeiro, 2009).

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). However some protein kinase C (PKC) dependent effects were already described (Lopes *et al.*, 1999a), thus pointing also to a Gq coupling of $A_{2A}R$. The distribution of adenosine receptors in the brain is crucial to understand adenosine differential modulation (Figure 1.4). While A_1 receptors are widely distributed, being more abundant in the cortex, cerebellum and hippocampus (Reppert et al., 1991), $A_{2A}R$ display a more restricted expression pattern. High expression levels of $A_{2A}R$ 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).

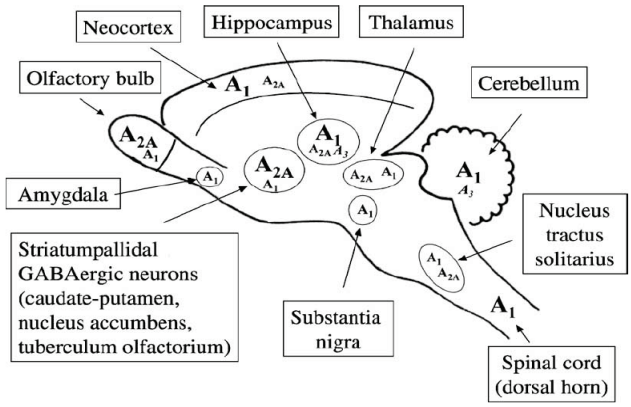

Figure 1.4: Schematic representation of the distribution of adenosine receptors in the brain. The hippocampus presents high levels of adenosine A_1 receptors and low levels of adenosine A_{2A} receptors. (Adapted from Ribeiro *et al.*, 2003)

In the hippocampus, the effects of adenosine under physiological conditions are mostly mediated by A_1R activation and consequent inhibition of glutamate release. Adenosine has therefore a tonic inhibitory effect on hippocampal synaptic transmission (Sebastiao et al., 1990). Interestingly, in spite of the low expression and density of $A_{2A}R$ in the hippocampus, these receptors also play an important role in the modulation of synaptic transmission. Different effects resulting from $A_{2A}R$ activation were observed, not only in neurons but also in astrocytes (reviewed by Sebastiao and Ribeiro, 2009). The first evidences of $A_{2A}R$ mediated effects on the hippocampus revealed a presynaptic modulatory effect of $A_{2A}R$ upon A_1R 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 later in the modulation of release or uptake of different neurotransmitters, such as glutamate (Lopes et al., 2002), GABA (Cristóvão-Ferreira *et al.*, 2009; Cunha and Ribeiro, 2000) or acetylcholine (Cunha et al., 1994b). Post-sinaptically, A_{2A} receptors are implicated in the modulation of AMPA mediated currents (Dias et al., 2012). Evidences also expand $A_{2A}R$ modulatory actions to neurotrophins. A crosstalk between $A_{2A}R$ and Trk_B receptor (neurotrophin receptor) was described; with important implications for brain derived neurotrophic factor (BDNF) effects (Assaife-Lopes *et al.*, 2013; Diogenes *et al.*, 2007; Diogenes *et al.*, 2004; Tebano *et al.*, 2008). In astrocytes, $A_{2A}R$ are involved in the modulation of glutamate release (Nishizaki et al., 2002) and GABA uptake (Cristovao-Ferreira et al., 2013).

Adenosine A_{2A} receptors pathology

The extracellular levels of adenosine are crucial for adenosine signaling. Under physiological conditions adenosine concentrations range from 140 to 200nM (Dunwiddie and Diao, 1994). However different noxious brain conditions, as hypoxia, ischemia, epilepsy, or other brain insults, raise these values from 3 to 10 fold (Reviewed by Dale and Frenguelli, 2009). In such situations $A_{2A}R$ are activated (Fredholm, 1997) and A_1R desensitized (Fernandez et al., 1996). If this is prolonged,

the levels of adenosine receptors may change. In different chronic noxious brain conditions the levels of $A_{2A}R$ in the hippocampus are usually increased, whereas those of A_1R are decreased (reviewed by Cunha, 2005). These changes in adenosine receptor levels may not only impact on the overall effect of adenosine, but also have consequences for the signaling pathway operated by these receptors. Aged animals, which also present high basal levels of adenosine and an imbalance in the levels of its 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. In aged rats, $A_{2A}R$ dependent activation of glutamate release becomes more pronounced and shifts from a PKC mediated signaling to protein kinase A (PKA), c-AMP dependent effects (Lopes *et al.*, 1999a). Moreover this effect, that in young animals is dependent on A_1R activation, is observed even when these receptors are blocked revealing an effect that is independent of A_1R (Lopes *et al.*, 1999a). In summary, $A_{2A}R$ are over activated in deleterious brain conditions and being so, the blockade of these receptors has been proposed to be therapeutically useful in such situations (see Figure 1.5). $A_{2A}R$ blockade has been shown to prevent amyloid-beta (Aβ) toxicity in the hippocamapus (Dall'Igna et al., 2007), as well as the memory impairments induced by infusion of this peptide in the brain (Canas *et al.*, 2009; Cunha *et al.*, 2008a). It was also shown to improve memory performance and decrease Aβ levels in different animal models of Alzheimer disease (Arendash *et al.*, 2006; Espinosa *et al.*, 2013). Finally, it has also been proven beneficial in Parkinson's disease (PD) models, and $A_{2A}R$ antagonists were already in clinical trials for the treatment of this pathology (Lopes *et al.*, 2011; Muller, 2013). In other models of pathology, as in convulsive episodes (Cognato et al., 2010), LPS administration (Rebola et al., 2011b) or hypoxia (Pugliese *et al.*, 2009; Von Lubitz *et al.*, 1995), $A_{2A}R$ blockade can prevent or even revert the deficits observed.

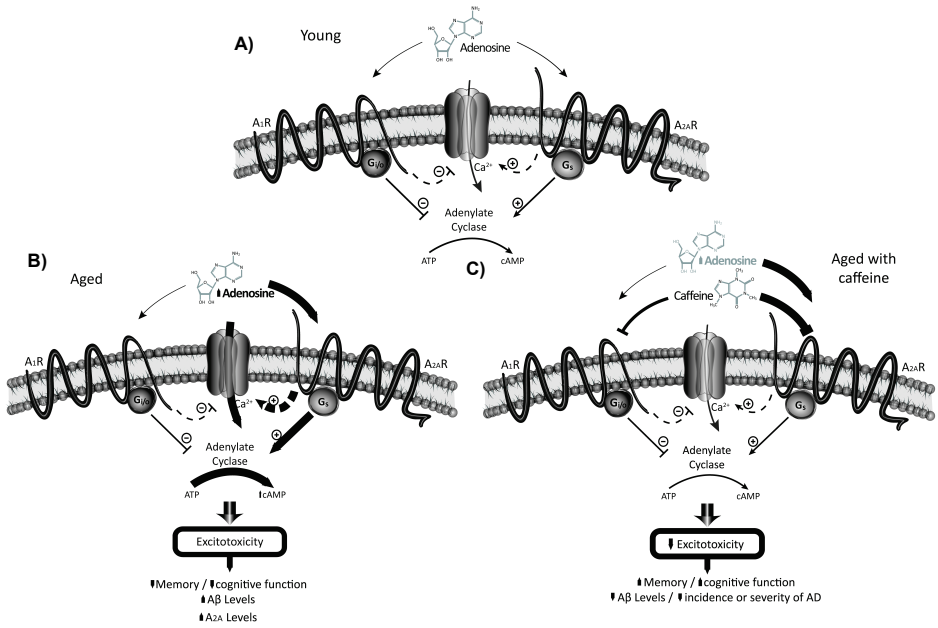

Figure 1.5: Effects of aging in adenosine neuromodulation and the effects of caffeine, an adenosine $A_{2A}R$ antagonist. A) In Young animals adenosine neuromodulation is based mainly in A_1R mediated inhibition and $A_{2A}R$ mediated excitation. B) Along the aging process, the increase in the levels of both Adenosine and $A_{2A}R$, shifts this balance into a stage in which more excitation occurs and thus excitotoxicity is observed. C) Whenever caffeine is present, and by blocking $A_{2A}R$, it will decrease excitation and thus protect from brain damage.(Marques *et al.*, 2011)

Adenosine A_{2A} receptors and stress

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 in the levels of adenosine receptors in a similar pattern to what happens in noxious brain conditions (Cunha et al., 2006). In this model the blockade of $A_{2A}R$ has been shown to prevent the synaptic loss presented by stressed animals (Cunha et al., 2006). Interestingly, both stress and adenosine are being implicated in psychiatric disorders as depression, anxiety, post-traumatic stress dirsorder, among others. Adenosine receptors are being studied as possible therapeutic

targets for its treatment (Cunha et al., 2008b); and several stress related 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). These models mimic, among others, one of the main features of psychopathologies, a dysfunctional stress response system that leads to a tonic activation of hippocampal GR due to permanent high circulating levels of corticosteroids.

As mention above many evidences are accumulating for a role of hippocampal $A_{2A}R$ in stress induced deficits however it is not yet clear how this is taking place.

Technical Approaches

Maternal separation stress model

Maternal separation (MS) protocol is a neonatal chronic stress model that has been proposed for the study of mood disorders. There are multiple models of maternal separation that vary according to multiple factors: the duration of each separation from (15 min to 24 hours); the number of separations (only once or repeatedly); the duration of the protocol (2 weeks or 3 weeks for instance) or the starting date (post natal day 2 or 1 week old for instance). Depending on the model used the effects are different (Reviewed by Marco *et al.*, 2011; Nishi *et al.*, 2013). For example, 15 min to 180 min separations have been described to be beneficial (Banihashemi et al., 2011), while separations longer than 180min are deleterious (Marco et al., 2011). These models are based on the modification of the natural behavior of the mother, the liking/grooming behavior, induced by the separation (Newport et al., 2002). When the separation is short (15 min), maternal care increases, if it is prolonged (180 min), the periods of linking/grooming are decreased. Natural differences in the periods of licking/grooming have also been described to change stress susceptibility, with increased anxiety associated with lower licking/grooming periods (Champagne et al., 2003). One of the most robust models is the daily separation of the litter from their mother for 180 minutes each day during postnatal days 2–14 (Ladd et al., 2000). In this model the parent offspring interaction is altered, not only the pups are deprived from maternal care reducing the linking/grooming period, maternal behavior also remains aberrant after the reunion.

Among different stress models, MS is the one thought to be more physiological, since stress is induced by modifying maternal care which 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 occurrence of an early trauma as parental loss, sexual abuse or physical assault in childhood, and the chance of developing affective

disorders later in life (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. Increased corticosterone levels in this period of brain development lead to permanent changes at the levels of gene expression, neurochemistry, electrophysiology, and morphology that are particularly profound in hippocampal formation (Bakshi and Kalin, 2000; Kaufman *et al.*, 2000).

Learning and memory behavioral assessment

The **Morris water maze (MWM)** test is used to investigate hippocampal-dependent spatial memory in rodents and relies on the use of distal cues to navigate in an open swimming arena with the aim of locating a submerged platform (Sweatt, 2003). It was first described by Richard Morris (Morris, 1981), and has been applied with different modifications to multiple means throughout the years (D'Hooge and De Deyn, 2001; Terry, 2009). In the classical paradigm the device consists of a large circular pool filled with water, made opaque to hide the escape platform. The pool is positioned in a room with external distal cues, visible to the swimming animal, to allow his spatial location. The animals are trained to find the hidden platform with 4 trials/day (60 seconds maximal duration/ trail) for several days, the latency to find the platform evaluates learning performance. Latter a probe test is performed to evaluate memory retrieval, in this test the platform is removed and the time spent searching for the platform in its location (in a 60 second trial) is indicative of memory retention and requires information retrieval. In Figure 1.6 is a schematic representation of the MWM apparatus. Several characteristics of this test contributed for its widespread use, it presents several advantages not only in terms of experimental constrains but also of applicability. This test is highly reliable across procedures or

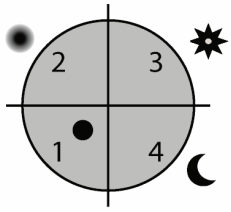

Figure 1.6: Graphic representation of the MWM swimming arena with the 4 quadrants signed, the plataform and the visual ques used for spatial orientation.

species, even humans (Kallai et al., 2005), and is relatively immune to motivational or locomotor differences. MWM learning impairments are independent on locomotor effects since land-based locomotor changes do not affect the swimming speed. Moreover, even if the learning curve is affected by the swimming speed, the probe test is insensitive to this parameter (Fitzgerald and Dokla, 1989). This task has been extensively validated as a measure of hippocampal dependent spatial navigation and reference memory and has been closely linked with hippocampal long-term-potentiation (LTP) and NMDA receptor function (Morris et al., 1986), therefore this is the test of excellence to study hippocampal function.

The **Y maze** test is a memory test that evaluates Spatial Recognition Memory and is based on the innate tendency of rats to explore novelty. It was first developed in 1992 by Dellu (Dellu et al., 1992) mainly because of its simplicity. It's a two trial test, performed in a Y shaped maze (see figure 1.7 for a schematic representation). In the first trial the animal is allowed to explore two of the Y-maze arms, the 3rd arm is blocked. After a delay period (from 15 min to 6h) the animal performs the second trial with all the three arms available. It is then possible to evaluate the discrimination between the novel and the familiar environment, revealing or not the recognition of the spatial locations already visited. Since the three arms of the maze are identical the discrimination between novel and familiar relies on the different aspects of the environment that the animal can perceive. Therefore, this task tests both working and spatial memory (Dellu *et al.*, 2000; Dellu *et al.*, 1997; Dellu *et al.*, 1992). There are many advantages of this test, the fact that it does not require the learning of a rule enables the specific testing of working memory. Moreover, because it is based in innate behavior, unlike most other memory tests, it is less affected by motivational or emotional states like food deprivation, water avoidance or foot shock (Dellu *et al.*, 2000; Dellu *et al.*, 1997; Dellu *et al.*, 1992). Again, because it evaluates working

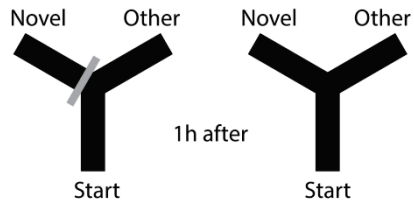

Figure 1.7: Graphic representation of the Y maze apparatus.

memory and its retention does not last longer than some hours, the same animal can be tested multiple times if the delay is sufficient to extinguish the memory. Finally, the influence of locomotor activity is minimized since the main parameter is the choice between the novel and the familiar arms; still locomotor activity is evaluated by measuring the total number of transitions (Dellu *et al.*, 2000; Dellu *et al.*, 1997; Dellu *et al.*, 1992).

Exploratory behaviour and anxiety

The **Open Field test (OF)** was first described for the study of emotional behavior in rats, by evaluating the amount of time that animals spent in the periphery of the apparatus (Hall, 1934). The test is based on the evaluation of the spontaneous activity of the animal, in an open arena that is a novel environment to the animal (Prut and Belzung, 2003). The normal behavior, average speed and time spent in the periphery (preferable environment) versus the center (avoidable environment) are evaluated for a given period of time (usually 5 minutes) and evaluated for gross changes in animal locomotion or anxiety state (Crawley, 1985; Prut and Belzung, 2003; Stone, 1932). Figure 1.8 presents a schematic representation of the arena and the different areas that are used to evaluate thigmotactic behavior (time spent In the periphery of the maze). Nowadays, this test is mostly used to evaluate locomotor and exploratory behavior, since emotional behaviors, as anxiety or depression like behavior, can be more precisely evaluated by other tests.

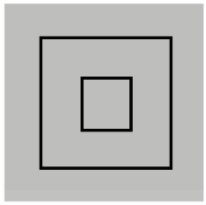

Figure 1.8: Graphic representation of the Open Field arena with the different zones delimitated.

The **Elevated Plus Maze (EPM)** is probably the most popular of all currently available tests to evaluate anxiety in animal models. One of its great advantages is the fact that it is based on an unconditioned or spontaneous behaviour, namely the natural tendency that rodents display of avoiding bright open spaces and preferring dark closed spaces. This type of behavior was first described by (Montgomery, 1958) but the actual plus maze was only designed later (Handley, 1984)

and has now been validated by different pharmacological tests. Namely, the administration of anxiolytic drugs produces a reduction of anxious behavior in the EPM (Pellow et al., 1985). It was also shown that once exploring the open arms, the corticosterone levels in the plasma rise, an indication that this is indeed a stressful environment (File *et al.*, 1994; Rodgers *et al.*, 1999).

The maze consists of two open arms and two closed arms disposed in a plus sign shape (Figure 1.9). The animals naturally prefer the closed arms, however due to their tendency to explore the environment, they also display an exploratory behavior towards the open arms. If the animals are more anxious then they will spent less time in the open, avoidable, bright arms. The percentage of time spent in the open arms is therefore used as a measure of the anxiety state. To evaluate effects in locomotor performance the total number of entries in the open and closed arms is evaluated (Reviewed in Walf and Frye, 2007). Evidences that subsequent testing in the same environment lead to an abolishment of anxious behavior in a second trial (File *et al.*, 1990; Lister, 1987; Pellow *et al.*, 1985) raised the hypothesis that EPM behavior is not restricted to anxiety and can require other memory strategies. To overcome this, the validity of the test is only guaranteed in a single trial testing of 5 minutes (see Rodgers and Dalvi, 1997; Walf and Frye, 2007). Since the test is based on variation of the baseline anxious behavior of the animals many factors can influence the testing, namely those that affect directly the stress response, as animal housing or handling prior to testing (Reviewed in Hogg, 1996).

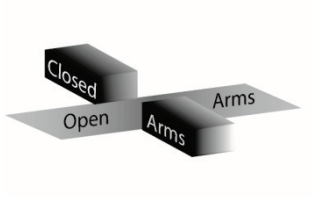

Figure 1.9: Graphic representation of the Elevated Plus maze.

Electrophysiological recordings

The discovery that the function of the nervous system was tightly linked with electrical activity was made back in the 1700's by Galvani (Piccolino, 1998). Since then, the goal of many scientists was to be able to measure that electrical activity. Electrophysiology is what made it possible, the ability to study the electrical properties of cells and tissues

by measuring voltage changes or electrical current. Nowadays electrophysiological techniques allow scientists to measure from single channel conductivities to whole cells and cell population responses. It was the development of electrophysiology that allowed many of the now basic concepts of neuroscience to be discovered and its trough electrophysiological techniques that many are still revealed. To be able to develop these techniques it was necessary to have cells and circuits available, and for this the discovery of the brain slice preparation was crucial. Back in the 1950's McIlwain was able to show, for the first time, that under controlled conditions it was possible to keep brain slices alive, metabolic active and with healthy neurons (Li and Mc, 1957). From that time onwards scientists used brain slices as a tool to study neuronal properties and responses in an accessible and controlled environment. However, the understanding of synaptic connectivity and neuronal communication was only possible after the introduction and development of the hippocampal slice preparation by Skrede and Westgaard in 1971 (Skrede and Westgaard, 1971). They showed that 400-500 μM hippocampal slices present a preserved circuitry and remain alive for several hours if kept in oxygenated artificial cerebrospinal fluid. As previously mentioned (page 9) the hippocampus has a very particular structure that is composed by a major unidirectional circuit (Lopes da Silva et al., 1990 - Figure 1.10). The information input arises from the entorrhinal cortex (EC), which neurons project into the hippocampal dentate gyrus (DG) granule cells. These, in turn, send afferent fibers (mossy fibers) into the CA3 pyramidal cells which through the Schaffer collaterals innervate CA1 neurons. These cells project back to the enthorrinal cortex, closing the hippocampal circuitry. There are other intermediate pathways; however is through this mainly unidirectional circuitry that it is proposed that information is integrated and stored into the nervous system, namely spatial information. With this preparation, and due to its organized structure, it is possible to stimulate the different afferent fibers, as the Schaffer collaterals, and record in the innervated cells, in this case CA1 cells, either single cell recording, by patch clamp techniques, or extracellularly, thus performing extracellular recordings. In figure 1.10 is a schematic representation of the hippocampus and how the electrodes are

positioned when performing extracellular recording of field excitatory post-synaptic potentials (fEPSP) in the CA3-CA1 synapse.

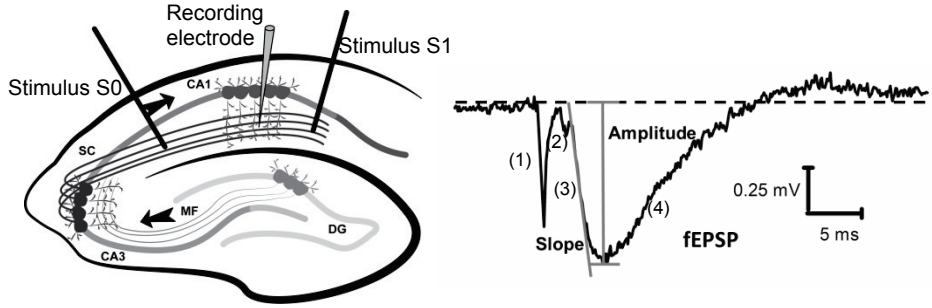

Figure 1.10: Schematic representation of the simplified circuitry of the hippocampus. Left: DG: Dentate Gyrus; MF: mossy fibers; SC: Schaffer Collaterals; CA3: *cornu ammonis* 3; CA1: *cornu ammonis 1,* Adapted from (Kesner, 2013). Two independent stimulation pathways are signed as S0 and S1 as well as the recording electrode in CA1 dendritic area. Right: a representative field Excitatory Post Synaptic Potential (fEPSP). The Slope and the Amplitude are the parameters normally used to evaluate synaptic transmission. (1) Stimulus artifact; (2) Fiber volley; (3) early EPSP; (4) late EPSP.

To perform **extracellular recordings** the recording electrode is placed in the dendritic area of the innervated cells and the response of the population of cells that is being stimulated is measured. The result is a fEPSP (Figure 1.10). The main neurotransmitter released by hippocampal neurons is glutamate. Therefore, the fEPSP obtained when performing extracellular recordings in the hippocampus, results from the depolarization of the post-synaptic population as a result of ion influx caused by the glutamate release. In figure 1.10 it is possible to observe a typical waveform of the fEPSP. It has mainly three components, the stimulus artifact (1), followed by the "fiber volley" (2) and finally the (EPSP) itself (3) and (4). This is composed by two phases, the first (3), a result of the post-synaptic response to glutamate and the second (4) the repolarization part of the EPSP in which the main neurotransmitter involved is GABA. The "fiber volley" (2) results from the presynaptic action potential arriving at the recording site, and is therefore the first to be recorded. This can give an indication of how healthy are the slices, since a small fiver valley amplitude means that less afferent fibers are being recruited to obtain a given fEPSP. The EPSP itself is the manifestation of the post-

synaptic depolarization induced by the glutamate released from the stimulated fibers and its slope and amplitude can be used to evaluate glutamate release. Usually the parameter evaluated is the slope since the peak amplitude of the fEPSP is more prone to contamination and more affected by the GABAergic contribution. (Sweatt, 2003). This technique is widely used to evaluate the efficiency of synaptic transmission, and the way it is modified in different situations. The evaluation of basal synaptic transmission is mainly achieved by performing **input-output curves.** In this protocol the response of the circuitry to step by step increments in intensity of stimulation is evaluated: thus, for higher stimulations a bigger fEPSP will be obtained, until a plateau is reached. It is then possible to infer how efficiently the information is being transmitted.

Another property of the nervous system that was crucial to the development of neurosciences was the concept of synaptic plasticity, that is, the idea that a synapse is not static, that it is modified by different stimuli. This was first proposed by Konorski (1948) however only in 1973 Tim Bliss and Terje Lomo reported long lasting changes in synaptic efficiency (Bliss and Lomo, 1973). In the following years different electrophysiological paradigms for synaptic plasticity arise, namely **long-term potentiation** (LTP) and long-term depression (LTD). These are thought to be the molecular basis for learning and memory and evidence shows that learning and memory require processes as LTP or LTD (Cantarero *et al.*, 2013; Fedulov *et al.*, 2007) and impairments in this paradigms are usually associated with poorer memory performances (For ex. Kinney et al., 2009). The first protocol described for LTP induction was using high frequency stimulation, 100 pulses at a 100Hz during 1s frequency. However, nowadays, multiple paradigms for LTP induction arose, with different types of stimulation, namely the Theta-burst stimulation that is thought to be more similar to the physiological process of learning and memory. It has also been debated that depending on the age of the animals, or the type of stimulation, the mechanisms underlying this permanent potentiation of synaptic transmission are different. Some are protein synthesis dependent, and in theory more stable and prolonged in time, others are not, and usually the potentiation originated is smaller and lasts for a shorter period of time

(Park et al., 2014). The great advantage of these molecular models for memory is that they allow the *in vitro* assessment of how plastic the brain is and this can in turn be correlated with learning and memory performance.

1.2) Aim

Chronic stress is known to induce a dysfunction in the HPA-axis leading to higher circulating levels of stress hormones, which has deleterious effects on brain function. The hippocampus is one of the primary brain areas affected by stress hormones, leading to memory and synaptic plasticity deficits. Since the blockade of A_{2A} receptors was proven beneficial against synaptic loss associated to acute stress in the hippocampus, one can imagine a deleterious contribution of A_{2A} receptors to stress-related impairments. However, this has never been tested directly.

I now tested the hypothesis that A_{2A} receptors are directly controlling stress-mediated effects, possibly by interacting with glucocorticoid receptor (GR). To clarify this, three specific tasks were designed:

- Evaluate if A_{2A} receptors are involved in the effects of chronic stress, using an early-life stress model in rodent, and evaluating the outcome in hippocampal-dependent function.

- Test whether A_{2A} receptors overexpression drives stress/ageing like modifications in memory performance and synaptic plasticity, by using a transgenic approach.

- Assess whether A_{2A} receptors can directly control GR-mediated effects.

Chapter 2:Adenosine A_{2A} receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation.

Adenosine A_{2A} receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation.

Vânia L. Batalha[1], José M. Pego[2,3] Bruno Fontinha[1], Ana Rita Costenla[1], Jorge Valadas[1], Younis Baqi[4], Hamid Radjainia[4], Christa E. Müller[4], Ana M. Sebastião[1] and Luísa V. Lopes[1]

[1]Institute of Pharmacology and Neurosciences, Faculty of Medicine and Neurosciences Unit, Instituto de Medicina Molecular, University of Lisbon, Portugal.

[2]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal

[3]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

[4]PharmaCenter Bonn, Pharmazeutische Chemie I, Pharmazeutisches Institut, University of Bonn, Germany.

VLB (Vânia L. Batalha) has written the draft, designed and performed all the experimental work, except the synthesis of KW 6002 done by CEM, YB and HR, the hippocampal volume analysis done by JMP and input/output curves plus SCH effects in MS animals done by BF, ARC and JV. The morphology experiments were made in ICVS, Braga by VLB under JMP supervision.

Abstract

Maternal separation (MS) is an early-life stress model that induces permanent changes in the central nervous system, impairing hippocampal long-term potentiation (LTP) and spatial working memory. There are compelling evidences for a role of hippocampal adenosine A_{2A} receptors in stress-induced modifications related to cognition, thus opening a potential window for therapeutic intervention. Here we submitted rats to maternal separation and evaluated the long lasting molecular, electrophysiological and behavioural impairments at adult age. We then assessed the therapeutic potential of blocking endogenous activation of A_{2A} receptors, by administering a selective antagonist, KW6002, orally for one month to stress-impaired animals. We report that the blockade of A_{2A} receptors was efficient in reverting the behaviour, electrophysiological and morphological impairments induced by MS. In addition, this effect is associated with the reestablishment of the Hypothalamic-Pituitary-Adrenal axis function, since both the plasma corticosterone levels and hippocampal glucocorticoid receptor expression pattern returned to physiological-like status after the treatment.

These results reveal the involvement of A_{2A} receptors in the stress-associated impairments and directly in the stress response system. Moreover, provide the first evidence that the dysfunction of the HPA-axis as well as the long-lasting synaptic and behavioural effects of MS can be reverted by targeting adenosine A_{2A} receptors. These findings provide a novel evidence for the use of adenosine A_{2A} receptor antagonists as potential therapy against psychopathologies.

Introduction

Exposure to stress has deleterious effects on brain structure and function, which could be manifested either immediately after stress(McEwen, 1999a), as a long term vulnerability to cognitive deficits (McEwen, 1999b) or even as an increased susceptibility to neuropsychiatric disorders, where stress plays a major role (McKinney, 1984; Willner, 1997).

Mother-infant interaction is a key factor for brain maturation and disease susceptibility which in humans can manifest in cognitive and behavioural disorders later in life (Heim and Nemeroff, 2001; Sullivan *et al.*, 2006). In rats, the daily separation of the litter from their mothers for 180 minutes each day during postnatal days 2-14 will result in an alteration of maternal behaviour, namely with a significant reduction licking/grooming duration (Ladd et al., 2000). During this period the hippocampus, which is critically involved in long-term memory formation (Morris, 2003) and is also a primary target for stress hormones in the central nervous system (de Kloet *et al.*, 1999; McEwen, 1999a) goes through great development. The majority of hippocampal granule neurons develop and extend their axons between postnatal day (PND) 1 and 21 (Amaral and Dent, 1981) and the peak period of neurogenesis and mossy fiber outgrowth overlaps with the stress hyporesponsive period (PND 4-14) in neonatal rats (Sapolsky and Meaney, 1986). This will induce changes that persist throughout adult life at the level of gene expression, neurochemistry, electrophysiology proprieties, and morphology (Bakshi and Kalin, 2000; Kaufman *et al.*, 2000) with behavioural and neuroendocrine signs of cognitive deficits and over-activation of the Hypothalamic-Pituitary-Adrenal axis (HPA-axis) as adults (Aisa *et al.*, 2007; Anisman *et al.*, 1998; Ladd *et al.*, 2000; Lehmann *et al.*, 2000).

Adenosine receptors in the hippocampus are important modulators of synaptic transmission and neuronal excitability. Glutamatergic synaptic transmission in physiological conditions is controlled negatively by the dominant adenosine A_1 receptors, and positively to a lesser extent by A_{2A} receptors (Cunha et al., 1994a). Interestingly, this pattern appears to be modified in the aged hippocampus, with a

marked increase in the expression of A_{2A} receptors and a decrease in the expression of A_1 receptors (Cunha *et al.*, 1995; Lopes *et al.*, 1999b). These changes are accompanied by a strong direct facilitatory effect of A_{2A} receptors on the release of glutamate (Rebola et al., 2003). This is also observed in other situations associated to neuronal dysfunction, such as epilepsy, acute stress or animal models of Alzheimer's disease (Cunha, 2005), which suggests a deleterious contribution of A_{2A} receptors to these conditions. The blockade of A_{2A} receptors was proven beneficial against synaptic loss associated to acute stress in the hippocampus (Cunha et al., 2006). Interestingly cognitive impairments also occur when excessive levels of corticosteroids are attained due to disease, or due to hypersecretion in response to a stressor (Belanoff *et al.*, 2001a; Belanoff *et al.*, 2001b).

However, it is still unknown the extent to which A_{2A} receptors are involved in the long-term effects of early life stress. Here we submitted rats to maternal separation and evaluated the long lasting molecular, electrophysiological and behavioural impairments at adult age. We then assessed the therapeutic potential of blocking endogenous activation of A_{2A} receptors, by administering a selective antagonist, KW6002 (istradefylline), orally for one month to stress-impaired animals. We report that the blockade of A_{2A} receptors was efficient in reverting the long-lasting behaviour, morphological and electrophysiological impairments induced by MS. We also show that this effect is associated with the reestablishment of the HPA-axis activity, since both the plasma corticosterone levels and hippocampal glucocorticoid receptor expression pattern returned to physiological-like status after the treatment.

Material and methods

Animals: Pregnant Wistar rats were purchased (Harlan, Barcelona) in mid-gestation and were due in our animal facility. All animals were handled according to European Community guidelines and Portuguese law on animal care (1005/92). The animals that were sacrificed by decapitation were anesthetized under halothane atmosphere.

Maternal Separation Protocol: The protocol used has been previously validated and described (Daniels et al., 2004). Wistar dams and their litters were assigned either to the control (CTR – non-separated) or to the maternal separated (MS) groups as described before (Lopes et al., 2008). To exclude artifacts from genetic background, at post natal day (PND) 2 all the litters were collected together, gender assessed and the pups were randomly distributed to foster dams (gender proportion maintained). MS pups were removed from their cages as a group from PND 2 to 14, for 180 minutes, daily, at 9 am, 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 with the mother. CTR pups were not handled and were maintained in their home-cages until weaning. At day 21 the pup's gender was confirmed, they were weaned and housed in groups of 5-8 animals per cage until use at adult age (8-14 weeks; according to diagram in Figure S2.1).

Oral administration of the drug: KW6002 (istradefylline), a selective adenosine A_{2A} receptor antagonist (Yang et al., 2007) was orally administered diluted in the drinking water, being continuously available. The weight of the animals and the volume intake were assessed twice a week and the concentration of the solution adjusted so that the drug intake was maintained at 3mg/Kg/day. Animals were divided in 4 groups: CTR or MS, drinking vehicle (0.025% methylcellulose) and CTR KW or MS KW drinking KW6002 (3mg/kg/day, 0.025% methylcellulose). The treatment started at 4 – 6 weeks old, and was prolonged for one month until sacrifice. The KW6002 administration was kept throughout the behavioural assessments.

Corticosterone quantification: Blood was extracted from the tail, in animals previously handled to minimise stress and without anaesthesia, at two different time points, 8 am (nadir) and 8 pm (zenith). The plasma was isolated by centrifugation at 2000g, 4ºC for 15 minutes and corticosterone quantified by radioimmunoassay using the rat corticosterone ^{3}H kit from MP Biomedicals, UK according to the manufacturer's protocol.

Behavioural assessments: CTR, MS, CTR KW, MS KW were first handled for five days before testing in the behaviour assays, that were performed in the following sequence: open-field (OF), Elevated plus maze (EPM) and Morris water maze (MWM) (Morris et al., 1982): Rats were given spatial acquisition training consisting of four trials/day for four consecutive days, as performed before (Diogenes et al., 2011). On the fifth day a probe test was given in which the platform was removed and animals were allowed to swim freely for 60s while recording the percentage of time spent on each quadrant. The latency to found the plataform during acquisition and the percentage of time in the platform quadrant in probe test were used to evaluate hipppocampal dependent memory. Elevated plus maze: The maze is shaped like a plus sign and consists of two "open" and two "closed" arms, arranged perpendicularly, and elevated 50 cm above the floor. Each animal was placed on the center of the equipment, facing an open arm. Each test lasted 5 minutes and all testing sessions were performed between 10:00 am and 17:00 pm in a sound attenuated room. The maze was cleaned with a 70% ethanol solution between each animal. The total time spent in the open arms and the total arms entries (number of entries in open + closed arms) were used as anxiety and locomotor measures (Pellow et al., 1985). Open field: The animals were placed in the center of the arena (66x66 cm) and allowed to explore for 5 minutes. Changes in mean speed and path length of the subjects were continuously monitored by an automated tracking system (Smart 2.5, PanLab, Barcelona). The maze was cleaned with a 70% ethanol solution between each animal. *Histological procedures*: The day after the last testing session, five rats from each experimental group were perfused transcardially with PBS, under deep pentobarbital anesthesia. Brains were removed and

split into two hemispheres, and processed either for stereology, or for Golgi-Cox staining according to the procedures previously described (Gibb and Kolb, 1998; Keuker *et al.*, 2001). Briefly, for stereology the left hemispheres were included in glycolmethacrylate (Tecnovit 7100; Heraeus Kulzer, Werheim, Germany) and every other microtome-cut section (30 μm) was then collected on a gelatinized slide, stained with Giemsa, and mounted with Entellan New (Merck, Darmstadt, Germany). The shrinkage factor was calculated according to Madeira et al (1990) (Madeira et al., 1990). For 3D neuronal reconstructions, hemispheres were removed and immersed in Golgi-Cox solution (a 1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate (Glaser and Van der Loos, 1981)) for 14 days; hemispheres were then transferred to a 30% sucrose solution (3 days), before being cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed in Kodak Rapid Fix (prepared as manufacturer instructions), dehydrated through a graded series of ethanols, and cleared in xylene before being mounted and coverslipped. Slides were coded before morphometric analysis in both sets.

Region and layer boundaries: We analyzed the following regions of the hipocampal formation (HF): the dentate gyrus (including polymorphic, granule cell layer, and molecular layer), CA1 (strata oriens, pyramidale, radiatum and lacunosum-moleculare) and CA3 (strata oriens, pyramidale, lucidum and radiatum). The above mentioned regions were outlined according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998), based on noticeable cytoarchitectural differences (Palomero-Gallagher and Zilles, 2004).

Stereological procedures: Volume estimations were performed using StereoInvestigator software (MicroBrightField, Williston, VT) and a camera (DXC390; Sony, Tokyo, Japan) attached to a motorized microscope (Axioplan 2; Zeiss, Oberkochen, Germany). Cavalieri's principle (Gundersen et al., 1988) was used to assess the volume of each region. Briefly, every 10th section was used and its cross-sectional area was estimated

by point counting at a final magnification of 112x. For this, we randomly superimposed onto each area a test point grid in which the interpoint distance, at tissue level, was as follows: (1) 150μm for the three layers of the dentate gyrus, (2) 250 μm for the three layers of CA1 and CA3. The volume of the region of interest was calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

Dendritic tree analysis: Three-D reconstructions of representative Golgi-impregnated neurons from CA1 were made. The criteria used to select neurons for reconstruction were as follows: (i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) dendrites without significant truncation of branches; (iii) relative isolation from neighboring impregnated neurons to avoid interference with the analysis; (iv) no morphological changes attributable to incomplete dendritic impregnation of Golgi–Cox stain. Golgi-impregnated pyramidal-like neurons of the CA1 region were readily identified by their characteristic pyramidal or piriform soma, spine-sparse primary dendrites and spine-dense secondary dendrites (Fig. 2e for representative reconstructions). For each selected neuron, all branches of the dendritic tree and the location of all dendritic spines were reconstructed at 600X magnification, using a motorized microscope (Carl Zeiss Axioplan 2, Hamburg, Germany, with oil-objectives), attached to a camera (DXC-390, Sony Co., Japan) and Neurolucida software (Microbrightfield, VT, USA). Three-D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). In each hemisphere, 10 CA1 pyramidal neurons were reconstructed; as a result in this study we have analyzed 200 neurons. Several aspects of dendritic morphology were examined. To assess overall changes, total dendritic length, number of ramifications and number of dendrites were compared between groups. Sholl analysis was performed to assess changes in the ramification pattern.

Electrophysiological recordings: After decapitation the brain was rapidly removed and the hippocampi were dissected free in ice-cold Krebs solution composed of (mM): NaCl 124; KCl 3; NaH2PO4 1.25; NaHCO3 26; MgSO4 1; CaCl2 2; and glucose 10,

previously gassed with 95% O2 and 5% CO2, pH 7.4. 400 µM slices were obtained with a McEwen tissue shopper and Field excitatory postsynaptic potentials (fEPSPs) were recorded as previously (Diogenes et al., 2011) in *stratum radiatum* of the CA1 area. Input output curves and long term potentiation (LTP, 100 Hz, 1s, 100 pulses induced at 0.5mV/ms; <50% max) were recorded as previously. The second hippocampus was rapidly frozen in liquid nitrogen for further analysis.

Tissue processing: Samples were homogenized either in Immunoprecipitation-Assay (RIPA) buffer (50 mM Tris, 1 mM EDTA,150 mM NaCl 0,1% SDS, 1% NP 40, pH 8; (Palacios et al., 2004) or in 0.32 M sucrose solution with 50 mM Tris at pH 7.6 (Lopes *et al.*, 1999b) supplemented with protease inhibitors (ROCHE). The first were centrifuged at 14000g during 15 minutes, and the second at 1000g during 10 minutes, at 4ºC. The supernatant was collected, corresponding to whole tissue lysate and whole tissue homogenate respectively. For membrane isolation the whole tissue homogenate was centrifuged at 14 000g during 12 minutes, at 4ºC, the pellet is the membrane fraction. Protein was quantified using the BioRad Protein or DC Protein based on procedures previously described (Bradford, 1976; Lowry *et al.*, 1951).

Saturation binding assays: The radioligand binding experiments were performed as described (Lopes *et al.*, 1999a) with membrane fractions. Briefly, [^{3}H]ZM 241385 binding (0-10 nM) was for 1 hour with 20-35 µg of protein/well for striatum membranes and [^{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. Specific binding was determined subtracting the non-specific binding, measured in the presence of 2 µM of XAC and normalized for protein concentration. Radioactivity was determined after 12 hours with an efficiency of 55-60% for 2 minutes. All binding assays were performed in triplicate.

Immunoblotting: Lysates or homogenates were denatured with 5x sample buffer (350 mM Tris pH 6.8, 30% glycerol, 10% SDS, 600 mM DTT and 0,012% Bromophenol blue, pH6.8) and heated either at 95º for 5 minutes or at 60-70ºC for 30 minutes, respectively, and further processed as before (Diogenes et al., 2011). $A_{2A}R$ and

$GABA_AR$ antibodies (Upstate/Millipore, 05-717 and 05-474) were at 1:2000, GR, MR (Sta. Cruz Biotechnology, sc-1004and sc-11412) at 1:1000 and 1:200, NMDAR2B (Cell Signaling, D15B3) at 1:1000 and GluR1 (Millipore, 05-855) at 1:6000. Optical density was determined with Image-J software and normalized to the respective β-actin or α-tubulin band density.

Drugs: $A_{2A}R$ selective antagonist, 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH58261) and the non-selective adenosine receptor antagonist 8-(4-[(2-minoethyl)amino]carbonylmethyloxyphenyl)xanthine (XAC) were purchased from Tocris Cookson, UK. These solutions were diluted in the assay solution from 5 mM stock aliquots made in DMSO stored at -20ºC. $A_{2A}R$ selective antagonist, (*E*)-8-[2-(3,4-dimethoxyphenyl)vinyl]-1,3-diethyl-7-methyl-3,7-dihydropurine-2,6-dione (KW6002, istradefylline) was synthesized according to a published procedure (Hockemeyer et al., 2004). The purity of the product was determined by HPLC analysis coupled to electrospray ionization mass spectrometry and was greater than 98%. Adenosine deaminase (ADA, from calf intestine 10 mg/2 mL, EC 3.5.4.4) was from ROCHE; A_1R selective antagonist, [propyl-^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX, specific activity 100 Ci/mmol) was from Amersham, Buckinghamshire UK, and $A_{2A}R$ selective antagonist, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a] [1,3,5]triazin-5-ylamino]ethyl)phenol, ([^{3}H]ZM 241385, specific activity 27.4 Ci/mmol) was from ARC Inc, St. Louis, USA. All these drugs were diluted directly in the incubation solution each day. HRP-coupled secondary antibodies were from Santa Cruz Biotechnology. All other reagents used were of the highest purity available either from Merck, Germany or Sigma Aldrich, Spain.

Statistics: Values presented are mean ±SEM of *n* experiments. To test the significance of the differences between CTR and MS groups, an unpaired Student's *t* test was used. When comparing CTR, MS, CTR KW and MS KW groups a one-way ANOVA was used, followed by a Bonferroni's Multiple Comparison post hoc test. For the Sholl analysis of reconstructed neurons a repeated measures analysis was used. For the saturation binding curves an F-test 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. For the analysis of the Morris water maze acquisition curve and corticosterone circadian oscilation the statistical differences were evaluated using two-way ANOVA repeated measures test. Values of $P<0.05$ were considered to be statistically significant.

Results

Maternal separation induces long-lasting regional effects in the brain.

Maternal separation induces numerous changes in the brain particularly in the hippocampus (Aisa *et al.*, 2009; Pickering *et al.*, 2006). The balance between mineralocorticoid and glucocorticoid receptors (MR and GR respectively) can determine the impact of stress in different brain areas (Joels, 2006). We quantified the levels of GR and MR in the hippocampus, cortex and striatum of adult animals previously subjected to maternal separation (Figure 2.1 a). Maternal separation has lead to a long lasting decrease of the GR levels that was more evident in the hippocampus (0.74 ± 0.04 of CTR, n=8, $P<0.05$) than in cortex (0.86 ± 0.03 of CTR, n=4, $P<0.05$) or striatum (0.90 ± 0.03 of CTR, n=4; $P<0.05$). MR levels were not modified in any of the brain areas analyzed (Figure 2.1 b). Concomitant changes on the levels of adenosine receptors were observed. Comparing to CTR, MS animals presented a 1.49 ± 0.04 fold increase (n=9; $p<0.05$) in the levels of $A_{2A}R$ that was restricted to the hippocampus (Figure 2.1 c). A hippocampal specific decrease in the A_1R levels was also observed; B_{max} values were of 1202 ± 33 fmol/mg protein (n=4) for CTR animals and 1073 ± 23 fmol/mg protein for MS animals, (n=4; $P<0.05$; Figure 2.1 d). MS animals also presented a sustained increase in plasmatic corticosterone levels (Figure 2.1 e).

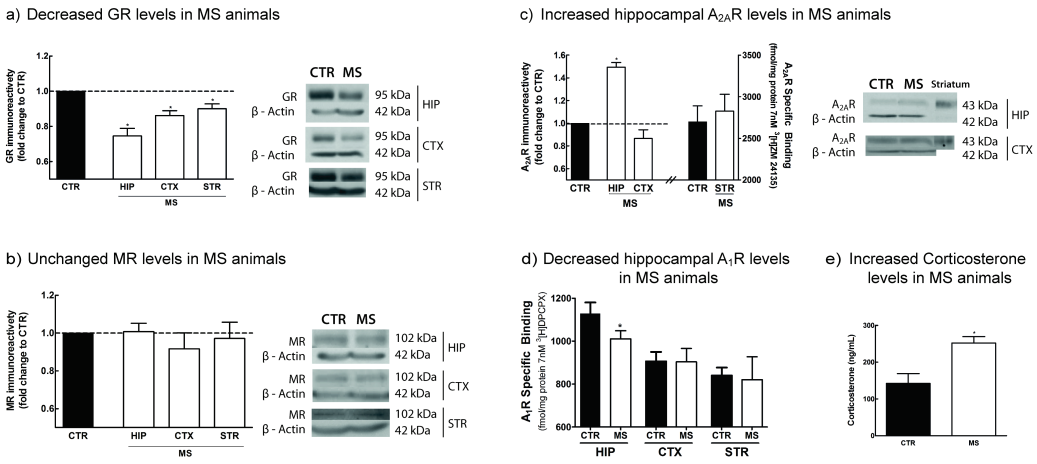

Figure 2.1 - Region specific effects of Maternal Separation.
MS induced region specific changes in, GR **(a)** MR **(b)** $A_{2A}R$ **(c)** and A_1R **(d)** and an increase in plasmatic corticosterone levels **(e)**. Protein levels of GR, MR (in all brain areas) and $A_{2A}R$ (in hippocampus and cortex) were evaluated by western blotting. Specific immunoreactivity was normalized to that of β-Actin or α-tubulin. For $A_{2A}R$ immunoreactivity 5μg of striatum were used as positive control. Results are the mean ±SEM of 3 to 9 experiments; (*):P<0.05, comparing to CTR, calculated using an unpaired Student t-test. A_1R levels in all areas and $A_{2A}R$ levels in striatum were measured by saturation binding curves with the A_1 or A_{2A} receptor selective antagonist [^{3}H]DPCPX or [^{3}H]ZM 24135 respectively. [^{3}H]DPCPX or [^{3}H]ZM 24135 (7 nM) were incubated with 20-100 μg of membranes in a final volume of 300 μL for 2h/1h at room temperature. The ordinates represent the specific binding obtained upon subtraction of the non-specific binding, determined in the presence of 2 μM of XAC, from total binding. Values are the mean ± SEM of 4-5 experiments performed in triplicate. (*): P<0.05 calculated using an F-test compared to control. Corticosterone levels in the morning period (8 am) were measured by radioimmunoassay using the rat corticosterone [^{3}H] kit. Results are mean ±SEM of 9 experiments; (*):P<0.05 obtained using a unpaired Student t-test.

Adenosine A_{2A} receptors are involved in synaptic changes induced by MS.

To evaluate the impact of stress in synaptic transmission and plasticity, field excitatory postsynaptic potentials were measured in the CA1 area of the dorsal hippocampus. Basal synaptic transmission was accessed by performing Input-output (I/O) curves, whereas synaptic plasticity was evaluated by LTP induced by High Frequency Stimulation (HFS, 100Hz,1s).

The I/O curve was not modified by maternal separation (Figure 2.2 a, n=3). However, LTP magnitude (Figure 2.2 b) was reduced in MS animals to 34.4 ± 2.7 % from 50.7 ± 3.4 % of potentiation obtained in CTR (n=7, $P<0.05$).

To evaluate if the increase in A_{2A} adenosine receptors was involved in the impairments observed in synaptic plasticity, LTP was induced in the presence of SCH58261 (50 nM), a selective $A_{2A}R$ antagonist. The *ex vivo* blockade of $A_{2A}R$ reverted the LTP deficits induced by maternal separation without affecting LTP magnitude in control animals (53.1±3.7% and 57.3 ±1.7% of potentiation respectively, n=4-7, $P>0.05$; Figure 2.2 c).

Moreover, the *in vivo* administration of the A_{2A} selective antagonist KW6002 for one month to adult MS animals was also able to revert the LTP deficits observed (47.6±3,9% of potentiation in MS KW animals, n=4, $P>0,05$ *versus* CTR; Figure 2.2 d).

In order to better characterize the plastic changes observed in the electrophysiological studies, a 3D morphological analysis of dendritic arborizations of CA1 pyramidal neurons was performed. Data revealed a significant treatment effect in the total length of apical dendrites of pyramidal neurons ($F = 7.371$, $P < 0.001$), and in the total number of apical dendrite ramifications ($F = 9.272$, $P < 0.001$); post-hoc analysis showed that MS induced a significant decrease in the total length of apical dendrites when compared to CTR ($P<0.05$) (Figure 2.2 e). Similarly, MS pyramidal neurons had significantly less ramifications in apical dendrites when compared to CTR ($P <0.05$). Both parameters were restored by KW6002 treatment ($P>0.05$ *vs* CTR). There were no significant differences in the structure or number of basal dendrites. Sholl analysis showed coherent changes in the pattern of ramification ($F = 5.691$, $P<0.001$). The changes in apical dendrite arborizations observed in MS animals when compared to CTR ($P<0.05$) were reverted by KW6002 (Figure S2.2).

To further evaluate the consequences to the overall morphology we undertook an estimation of hippocampal formation volumes. Our data revealed that neither MS nor KW6002 treatment significantly affected volumetric estimates (Table S2.1).

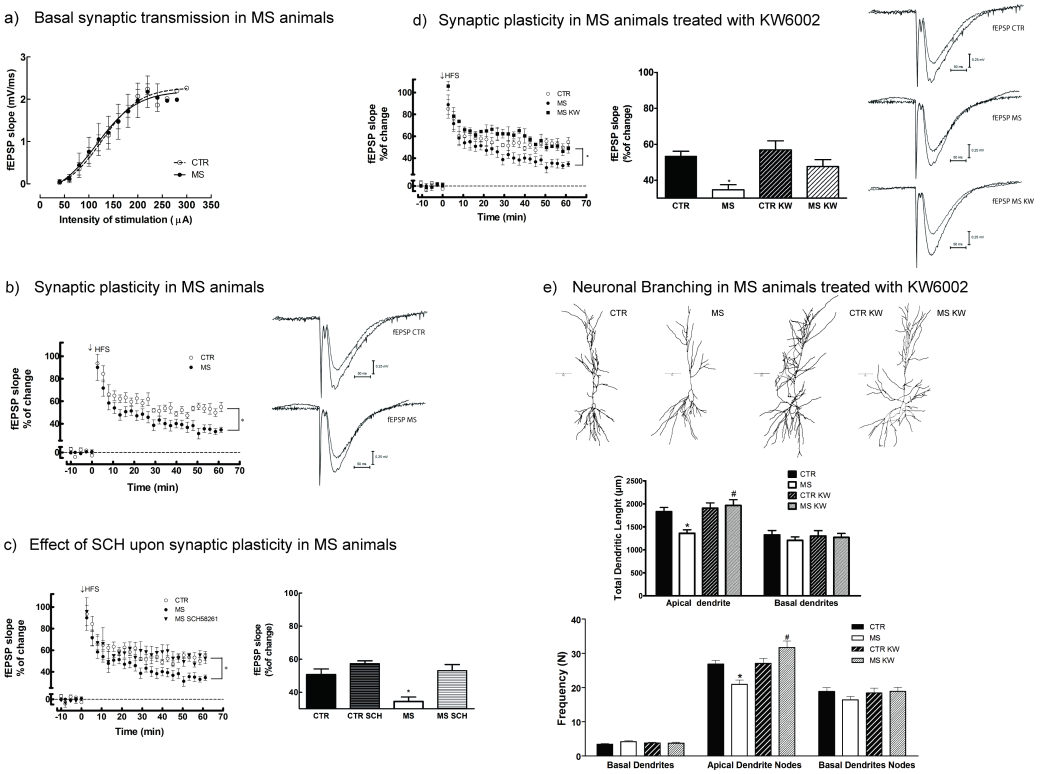

Figure 2.2. Involvement of adenosine A_{2A} receptors in the synaptic changes induced by maternal separation.

(a) Input-output curves performed to evaluate synaptic transmission in CTR and MS animals and (b) LTP (HFS,100 Hz,1 s), used to evaluate synaptic plasticity. Representative recordings of the fEPSPs obtained both for CTR and MS animals prior LTP induction and in the last 10 minutes are presented. The effect of SCH58261 (50 nM) application for 30 minutes prior to LTP induction and throughout the protocol in shown in (c). The outcome of KW6002 treatment upon LTP is in (d) with representative recordings of the fEPSPs obtained for CTR, MS and MS+KW6002 animals, prior to LTP induction and one hour after LTP. Bar graphs are obtained by making the average of the last 5 timepoints of each experiment. Results are the mean ±SEM of 3 (a) or 4-7 experiments (*):P<0.05, comparing to CTR. (e) Administration of KW6002 reverses dendritic atrophy induced by MS in CA1 pyramidal neurons. Upper panel depicts representative schematics of 3D reconstructions of CTR, MS, CTR KW and MS KW CA1 neurons. (*):P<0.05, comparing to CTR, (#):P < 0.05, comparing to MS, calculated using a 1way ANOVA followed by a Bonferroni's Multiple Comparison Test.

Oral administration of a selective A_{2A} receptor antagonist reverts the stress induced anxious behaviour and learning-deficits.

We then evaluate the extent to which $A_{2A}R$ are involved in the stress induced behaviour alterations, by the administration of the $A_{2A}R$ selective antagonist, KW6002, to adult MS animals.

Anxious behaviour and hippocampal dependent memory were evaluated by the elevated plus maze (EPM) and the Morris Water Maze (MWM) paradigms, respectively. In the EPM, MS animals presented a higher anxious related behaviour (spent less time in the open arms, 11.4 ±2.0% *versus* 28.9± 5.3% in CTR, n=8-11, $P<0.05$), validating the MS stress induction. The hyperanxious behaviour in MS animals, was reverted upon treatment with KW6002 (time in open arms: 27.3±4.4%, n=8, $P>0.05$ *versus* CTR; Figure 2.3 a). KW6002 treatment by itself had no effect in the anxious behaviour of CTR animals (26.5±3%, n=7, $P>0.05$), neither had an impact in locomotor performance in EPM (Figure 2.3 b). On the MWM, the learning ability (Figure 2.3 c) of MS animals was impaired, so that at day 3 MS performed worse than CTR animals ($F(3,132)=8.56$, n=6, $P<0.0001$). These deficits were reverted by blocking $A_{2A}R$ *in vivo* (n=10, $P<0.05$). The retention ability of MS animals was also compromised, since in the probe test MS animals spent less time in the platform quadrant than CTR (36.8±3.9%, n=8; $P<0.05$ *versus* 51.3±5.0%, n=7; Figure 2.3 d). When treated with the $A_{2A}R$ antagonist the retention ability of MS animals was restored (52.8±4.2% of the time in the platform quadrant; n=8, $P>0.05$; Figure 2.3 d). KW6002 by itself had no effect in the performance of CTR animals.

$A_{2A}R$ are highly abundant in striatum exerting important effects in motor control (Janusz and Berman, 1992). To evaluate directly locomotor activity, the open field arena test was used. In accordance with data obtained in the EPM, neither MS nor the KW6002 treatment induced changes in the locomotor performance of the animals, since

no alterations were observed in the mean speed on the open field arena (Figure 2.3e).

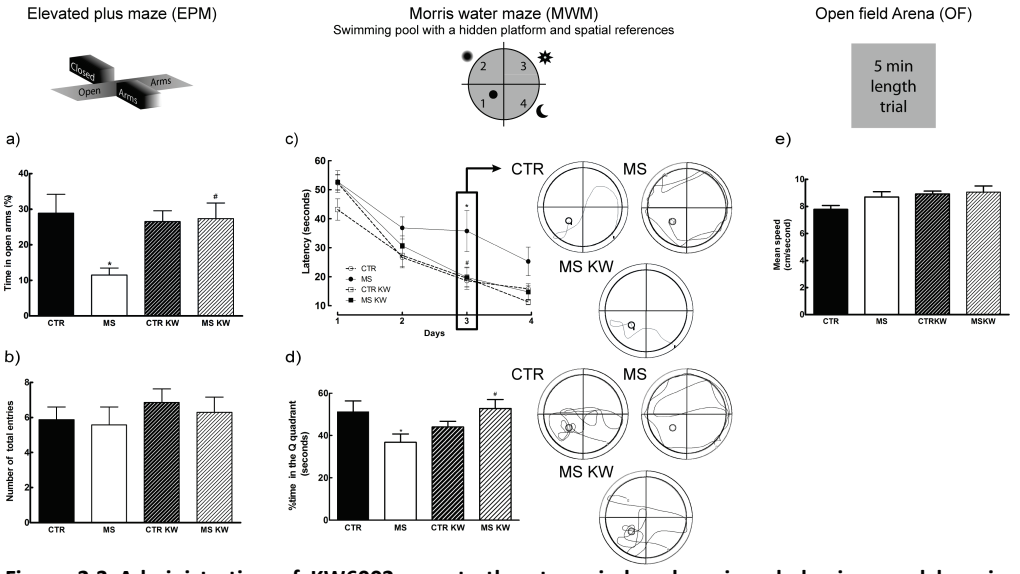

Figure 2.3 Administration of KW6002 reverts the stress induced anxious behaviour and learning deficits.

Anxious behaviour (a,b) and locomotor activity (e) were evaluated by the elevated-plus-maze-test and open-field, respectively. Hippocampal dependent memory performance was assessed by the Morris water maze test, in which acquisition (c) and retention (d) were evaluated. Results are the mean ±SEM of 6-9 animals; (*):P<0.05, comparing to CTR, (#):P<0.05 comparing to MS, calculated using 2way ANOVA repeated measures (a) or 1way ANOVA followed by a Bonferroni's Multiple Comparison Test.

Oral administration of a selective A_{2A} receptor antagonist reestablishes stress-induced modifications on synaptic markers.

Given the positive effects of the *in vivo* KW6002 treatment in behaviour, in *ex vivo* synaptic plasticity and in neuronal morphology, we next explored the molecular changes that could underlie the observed therapeutic effects. The levels of $A_{2A}R$ and GR were measured in the hippocampus of CTR and MS animals treated with KW6002. Given the role of AMPA, $GABA_A$ and NMDA receptors in synaptic transmission and plasticity, AMPA-GluR1, $GABA_A$-$\beta_{2/3}$ and NMDAR2B subunits levels were also evaluated. AMPA-GluR1 levels in MS animals were significantly decreased comparing to CTR (0.81± 0.02, n=9, P<0.05; Figure 2.4 a). These values were reestablished by

KW6002 (0.98 ± 0.03, n=4,P>0.05 *versus* CTR). $GABA_A$-$\beta_{2/3}$ levels decreased in MS animals (0.78± 0.02, n=8; P<0.05; Figure 2.4 b) and increased to 1.15 ± 0.06 of CTR (n=6, p<0.05) upon KW6002 treatment. The levels of the NMDAR2B subunit were not altered by MS nor by KW6002 treatment (n=5; Figure 2.4 c). Furthermore, the increased levels of $A_{2A}R$ observed in MS animals (1.49 ± 0.04 fold to CTR, n=9; p<0.05) were maintained in MS KW animals (1.56± 0.05 fold to CTR, n=5, P>0.05; Figure 2.4d). The KW6002 administration alone increased the levels of $A_{2A}R$ to 1.25± 0.09 of CTR (n=5, P<0.01; Figure 2.4 d), as could be expected from a chronic administration of receptor antagonist. The GR levels (Figure 2.4 e) were not changed by KW6002 in CTR animals (n=7, P>0.05); however when KW6002 was administered to MS animals the levels of GR increased to values similar to CTR (1.033 ± 0.04, n=6, P>0.05)

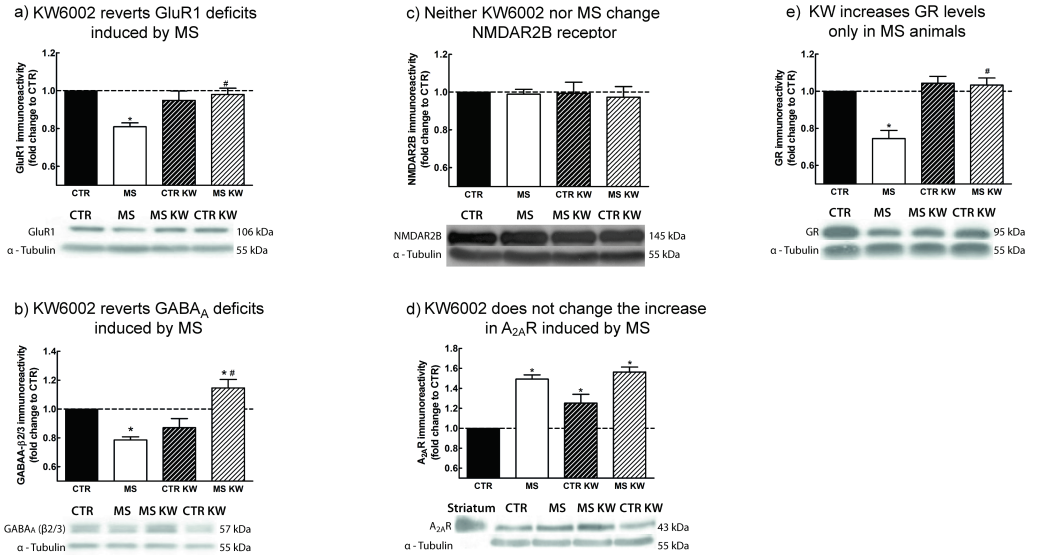

Figure 2.4. Administration of KW6002 reverts the synaptic changes induced by MS.
The effects of the treatment with KW6002 in the levels of GluR1 subunit of AMPA receptors (a), β2/3 subunit of $GABA_A$ receptors (b), NMDAR2B (c), $A_{2A}R$ (d), and GR (e) were evaluated by Western blotting. Specific immunoreactivity normalized to that of α-tubulin. Results are the mean ± SEM of 4 to 9 experiments; (*):P<0.05, comparing to CTR and (#):P<0.05, comparing to MS, calculated using a 1way ANOVA followed by a Bonferroni's Multiple Comparison Test.

The $A_{2A}R$ antagonist reestablishes the corticosterone circadian oscillation.

Since the blockade of $A_{2A}R$ reestablishes the GR levels in the hippocampus, we hypothesized that this could involve a regulation of the HPA-axis function, which is compromised due to the early life stress (Aisa et al., 2007). HPA-axis activity was evaluated by measuring circadian changes in plasmatic corticosterone levels (Figure 2.5).

CTR animals had the expected circadian oscillation, with corticosterone levels significantly elevated at 8 p.m comparing to those measured at 8 a.m. MS animals present significantly higher corticosterone levels already at 8 am (234 ± 13 ng/mL *versus* 142±27 ng/mL; P<0.05, n=9) comparing to CTR at the same time of the day, and the absence of a circadian oscillation. Animals treated with KW6002 had a restored circadian variation, with plasmatic corticosterone levels at 8 am similar to CTR (63±5 ng/mL), (F(3;24)=9.04, P=0.0003). KW6002 alone did not affect corticosterone levels, neither at zenith nor at nadir.

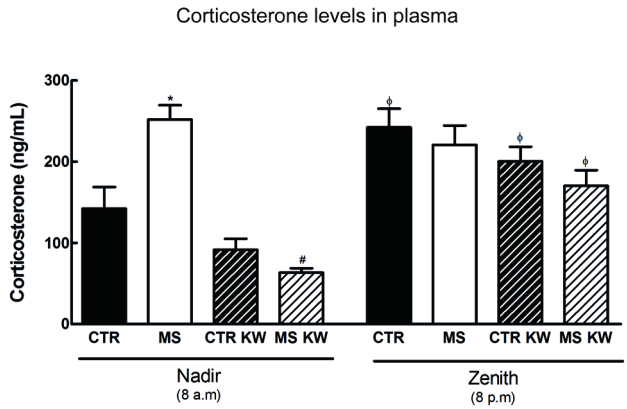

Figure 2.5. The KW6002 administration reestablishes the corticosterone circadian oscillation. Corticosterone levels in the plasma measured at nadir and zenith. Results are mean of 6-9 animals.(*):P<0.05, comparing to CTR, (#):P<0.05 comparing to MS, (ϕ):P<0.05, comparing to a.m values, calculated using a 1way ANOVA followed by a Bonferroni's Multiple Comparison Test.

Discussion

The data now reported reveal that adenosine A_{2A} receptor activation is directly involved in the stress deleterious effects in the brain. We show, for the first time, that the administration of a selective adenosine A_{2A} receptor antagonist reverts the long-lasting consequences of stress on spatial memory, synaptic plasticity and neuronal morphology in the hippocampus. Moreover our data indicate that these effects are associated with the reestablishment of the HPA-axis activity.

An imbalance in adenosine receptors has been observed in multiple conditions (Cunha, 2005), particularly with progressive aging (Cunha *et al.*, 1995; Lopes *et al.*, 1999b), which has consequences to their modulatory effects (Lopes *et al.*, 1999a, b). In the aged rat brain, adenosine A_1 receptor density is decreased (Meerlo et al., 2004), particularly in hippocampus and cortex (Cunha et al., 1995). However, A_{2A} receptor levels are differently affected: they decrease in striatum, but in contrast there is an increase in their expression in cortical and hippocampal areas (Cunha et al., 1995). As we now show, the changes in adenosine receptor levels induced by MS, follow a close pattern to the one occurring in the aged brain, i.e., an increase in A_{2A} and a decrease in A_1 receptor levels. The modifications observed are however restricted to the hippocampus, probably due to the changes in GR levels that are more profound in this brain area. Thus, as observed in aging(Lopes *et al.*, 1999b; Pardon and Rattray, 2008), MS induces a decrease in GR levels, increasing the MR/GR ratio, an increase in plasma corticosterone levels and changes in adenosine receptor levels. Thereupon our data reinforce the hypothesis that stress is associated with an early aging in the hippocampal area (McEwen, 1999b; Miller and O'Callaghan, 2005). Different brain regions have a distinct vulnerability to stress due to the differential expression of GR and MR in the brain (Reul and de Kloet, 1985). In the hippocampus, corticosterone is able to trigger signaling pathways activated by both GR and MR due to their particular high affinity ratio for GR, which does not occur in other brain areas (Reul and de Kloet, 1985). This confers to the hippocampus a particular susceptibility to stress effects and consequent deficits. Additional region-specific effects were reported previously, such as stress-

induced alterations in $GABA_A$ receptor levels and MAP kinase activity (Gruen *et al.*, 1995; Meller *et al.*, 2003).

We have observed a sustained increase in the plasmatic levels of corticosterone, a feature that is also shared with ageing (Mizoguchi et al., 2009). Such an increase is usually associated to a down-regulation in GR (Sapolsky *et al.*, 1984; Sapolsky and Meaney, 1986; Sapolsky *et al.*, 1985), as a way to limit their action. This is generally an isolated and reversible effect, reverted whenever the plasma levels of corticosterone return to baseline (Sapolsky et al., 1984). However, as we show, MS animals exhibit elevated plasma corticosterone levels throughout life and an associated sustained downregulation of GR in the hippocampus. These receptors regulate memory and synaptic plasticity (Joels, 2006; Sousa *et al.*, 2008). Accordingly, we found that long-term potentiation (LTP) is impaired in MS animals and this is accompanied by a poorer performance in a spatial memory task, the Morris water maze. The observed changes in synaptic plasticity can be related to the altered levels in $GABA_A$ and AMPA receptors, reported here. Others have described that MS induces a decrease in markers of synaptic plasticity, such as NCAM or synaptophysin (Aisa et al., 2009), as well as in the levels of NMDAR2B, AMPAGluR1 and GluR2 (Pickering et al., 2006) in the hippocampus. Changes now observed in glutamate receptor levels had, however, no impact upon basal synaptic transmission, possibly because they are accompanied by a decrease in $GABA_A$ receptors, which will result in a final compensatory balance in order to maintain homeostasis.

The observed impairments in LTP were overcome by blocking adenosine A_{2A} receptors. These receptors are known to have stimulatory effects on basal synaptic transmission in the hippocampus (Cunha *et al.*, 1994a; Lopes *et al.*, 1999b) by promoting glutamate release (Lopes et al., 2002), and were recently shown to potentiate LTP when exogenously activated (Dias *et al.*, 2012). Accordingly, the acute treatment of slices with the A_{2A} receptor antagonist, SCH58261, may instead cause a LTP drop, in particular under overexcitability conditions, such as ageing, in which LTP is enhanced (Costenla *et al.*, 2011; Diogenes *et al.*, 2011); due to an age-induced shift in A_{2A}

receptor signaling (Lopes *et al.*, 1999a, b). However, in chronic patho-physiological situations, in which LTP is decreased, SCH58261 is able to promote its restoration (Rebola et al., 2011a), in accordance with what we now report for stress-induced deficits. More importantly, the chronic administration of a selective A_{2A} receptor antagonist, KW6002, for one month, clearly reestablished the MS-driven impairment in LTP, while not affecting LTP in CTR animals. This suggests that, rather than having a direct effect on glutamatergic transmission, A_{2A} receptors may be instead modulating the GR-mediated effects. Indeed we have recent data showing the ability of A_{2A} receptors to influence GR transcriptional activity and nuclear translocation (Batalha et al., 2011), suggesting that the chronic blockade of A_{2A} receptor may decrease GR transcriptional activity and thereby the overall GR driven effects.

Genetic deletion of A_{2A} receptors affects anxiety and aggressive behaviour (Ledent et al., 1997), and this constituted the first evidence that A_{2A} receptors could be implicated in stress. The subsequent report that A_{2A} receptor antagonists applied prior and during a single episode of acute stress prevented synaptic loss (Cunha et al., 2006), suggested that A_{2A} receptors overactivation could underlie the genesis of stress-induced changes. Nonetheless, the question whether this overactivation is a consequence of the stress paradigm or a triggering factor to the observed deficits has never been addressed before.

We now explored the possibility that blocking the action of A_{2A} receptors would restore pre-existing stress-associated impairments. The advantage of using KW6002 over other antagonists for A_{2A} receptors is its enhanced bioavailability, permeability to the brain blood barrier, having a longer half-life and high affinity and selectivity towards A_{2A} receptors (Yang et al., 2007). Additionally, KW6002 has undergone clinical trials for Parkinson's and therefore its safety has been established (Hauser et al., 2003). We now report that oral administration of KW6002, for one month, to adult animals previously subjected to MS, reestablishes impaired hippocampal dependent memory, synaptic plasticity and morphology, and reverts the anxious behaviour. The learning ability of MS animals was restored by the treatment, as well as the retrieval, evaluated by the

time spent in the previous retained platform quadrant. This is associated with a reestablishment of the hippocampal CA1 induced-LTP. 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). We found that GluR1-subunit is decreased by maternal separation, which may explain the decreased LTP. Moreover, the LTP reestablishment is accompanied by a concomitant restoration of GluR1 levels upon KW6002 treatment. Accordingly, MS leads to a decrease in apical dendritic length, as described using other stress models (Bessa et al., 2009), but this structural effect is reverted by the blockade of A_{2A} receptors.

The HPA-axis maintains the physiological circadian oscillation of corticosterone levels, which reach their maximum at zenith (8 pm) and the minimum at nadir (8 am), for rodents (Allen and Kendall, 1967). The hippocampus is crucial in the negative feedback required to limit HPA-axis activation, particularly in stressful situations (McEwen, 1999a, b). However this function can be compromised when glucocorticoid levels are persistently high as in chronic stress, aging or in psychopathologies (Pardon and Rattray, 2008). The observation that the A_{2A} receptor antagonist was able to reestablish the decreased GR levels in the hippocampus lead us to test whether the observed effects were related to a modification of the HPA axis, by measuring the circadian levels of corticosterone in plasma. MS animals present not only higher plasmatic levels of corticosterone, but also an impaired circadian fluctuation. Corticosterone levels were chronically higher in MS animals and did not decrease along the night. This is probably associated to an impaired inhibition of the HPA-axis, which is consequence of a decrease in hippocampal GR levels. Interestingly, by blocking A_{2A} receptors, the basal levels of corticosterone were reestablished, so as the circadian rhythm and the GR levels in the hippocampus. Altogether these data suggest that A_{2A} receptors have a role in the regulation of the HPA-axis, either directly or by regulating hippocampal function. This effect may be due to interference with the release of corticotrophin releasing hormone (CRH), adrenocorticotrophin (ACTH), which is known to be affected by

adenosine (Anand-Srivastava et al., 1989), or by modulating glucocorticoids. The beneficial effect resulting from A_{2A} receptor antagonism may derive instead from a reestablishment of hippocampal excitability, which in turn would restore the inhibitory tonus onto the HPA-axis. Overall the blockade of adenosine A_{2A} receptors by KW6002 has a beneficial effect in overcoming the hippocampal related deficits induced by MS. Interestingly, this effect of KW6002 *in vivo* does not result from a decrease in $A_{2A}R$ levels which remain high in MS animals under KW6002. Indeed it would be unlikely that KW6002 would cause a decrease in $A_{2A}R$ levels since prolonged blockade of receptors usually leads to either no change or upregulation of receptor levels due to compensatory mechanisms following restraining from receptor activation by the endogenous ligand. Remarkably, the findings that blockade of $A_{2A}R$ overcomes the synaptic and memory deficits associated to MS, strongly suggests that A_{2A} receptors overactivation is the cause rather than the consequence of the herein reported changes associated with chronic stress.

In conclusion, our results show, for the first time, that the changes induced by stress are reverted by the *in vivo* blockade of A_{2A} receptors. Moreover they imply a role of $A_{2A}R$ in the HPA-axis regulation revealing that its blockade is efficient in reestablishing the compromised HPA-axis, which has clinical implications for the treatment of psychopathologies. This provides a potential alternative to the established therapies against stress related pathologies, by targeting a modulatory system rather than interfering directly with neurotransmitters, and thereby limiting the associated side effects.

Supplementary information

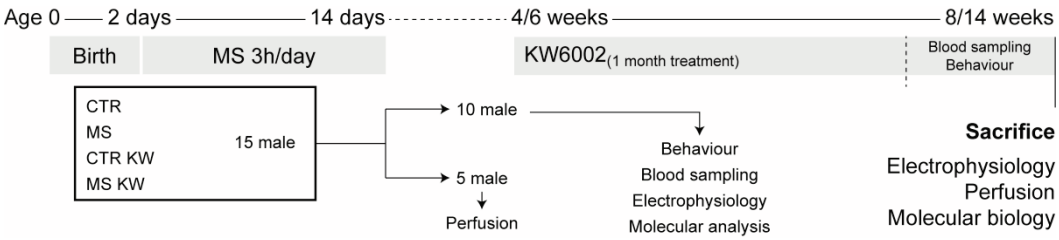

Figure S2.1: Schematic timeline of the MS protocol and KW6002 treatment.

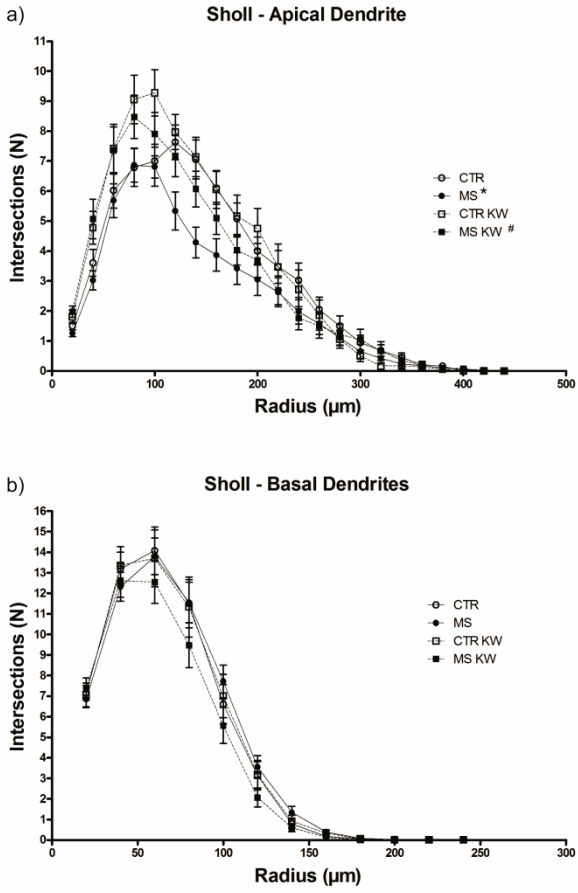

Figure S2.2: Sholl analysis data of apical (a) and basal (b) dendrites of CA1 neurons. MS induces a significant change ($P < 0.001$) in the structure of apical dendrites which is reversed by KW6002. (*):$P < 0.05$, comparing to CTR, (#):$P < 0.05$, comparing to MS, calculated using a 1way ANOVA followed by a Bonferroni's Multiple Comparison Test.

Table S2.1. Estimates of volumes of hippocampal formation subregions (in mm3). No significant differences were found in any of the assessed subregions. All data presented as Mean ± SEM. CA1 LMol – CA1 molecular layer; CA1 Or – CA1 stratum oriens; CA1 Pyr – CA1 stratum pyramidalis; CA1 Rad – CA1 stratum radialis; CA3 Or – CA3 stratum oriens; CA3 Pyr – CA3 stratum pyramidalis; CA3 Rad – CA3 stratum radialis; CA3 – stratum lucidum; DG Gr – dentate gyrus granular cell layer; DG Mol – dentate gyrus molecular layer; DG Pol – dentate gyrus polymorphic layer

	CTR			**MS**			**CTR KW**			**MS KW**			**F**	**Sig.**
CA1 LMol	4,691	±	0,70	4,619	±	0,54	4,180	±	0,06	3,888	±	0,15	1,378	0,297
CA1 Or	3,761	±	0,55	2,945	±	0,19	2,955	±	0,30	3,008	±	0,12	1,403	0,29
CA1 Pyr	1,945	±	0,21	2,089	±	0,26	2,147	±	0,24	2,213	±	0,09	0,291	0,831
CA1 Rad	6,662	±	0,61	6,556	±	0,61	6,413	±	0,45	6,431	±	0,22	0,055	0,982
CA3 Or	4,185	±	0,07	3,494	±	0,10	3,366	±	0,03	3,484	±	0,02	2,881	0,08
CA 3 Pyr	2,243	±	0,02	2,247	±	0,07	2,159	±	0,04	2,322	±	0,03	0,22	0,881
CA3 Rad	2,668	±	0,15	2,605	±	0,34	2,103	±	0,23	2,675	±	0,05	0,886	0,476
CA3 SLu	1,074	±	0,20	0,954	±	0,31	0,918	±	0,24	0,838	±	0,03	1,119	0,38
DG Gr	1,637	±	0,08	1,865	±	0,26	1,840	±	0,10	1,830	±	0,07	0,463	0,713
DG Mol	7,943	±	0,10	7,124	±	0,23	6,788	±	0,12	6,601	±	0,04	1,025	0,416
DG Pol	1,882	±	0,25	1,877	±	0,48	1,861	±	0,21	1,656	±	0,08	0,267	0,848

Chapter 3:Aging-like hippocampal deficits driven by overexpression of adenosine A_{2A} receptors in forebrain neurons.

Aging-like hippocampal deficits driven by overexpression of adenosine A_{2A} receptors in forebrain neurons.

Vânia L. Batalha[a], Diana G. Ferreira[a,c,d], Jorge S. Valadas[a] Joana E. Coelho[a], Rui Gomes[a,j], Paula M. Canas[g], Laetitia Cuvelier[f], Valerie Buée-Scherrer[e], Sandrine Humez[e], Tatiana Shmidt[b], Serge N. Schiffmann[f], Tiago F. Outeiro[c], Rodrigo A. Cunha[g,h], Michael Bader[b,i], David Blum[e#] and Luísa V. Lopes[a#]

[a]Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Portugal

[b]Max-Delbrück-Center for Molecular Medicine (MDC), Berlin, Germany

[c]Department of Neurodegeneration and Restorative Research, University Medizin Göttingen Germany

[d]Instituto de Farmacologia e Terapêutica, Faculdade de Medicina da Universidade do Porto, Portugal

[e]Inserm, U837, Lille, France

[f]Laboratory of Neurophysiology, ULB Neuroscience Institute, Université Libre de Bruxelles (ULB), Brussels, Belgium

[g]CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Portugal

[h]Faculty of Medicine, University of Coimbra, Portugal

[i]Charité-University Medicine Berlin, Germany[i]Institute of Biology, University of Lübeck, Germany

[j]Faculdade de Ciências da Universidade de Lisboa, Portugal

VLB (Vânia L. Batalha) has written the draft, designed and performed all the experimental work, except the generation and in situ hybridization of tg(CaMKII-$hA_{2A}R$) done by MB, TS, LC and SS; the qPCRs made by RG. DF, JV and JC helped in the behavior and electrophysiology experiments.

Abstract

Ageing is associated with cognitive decline both in humans and animals. Among brain structures, the hippocampus is particularly vulnerable to senescence and degeneration. Adenosine A_{2A} Receptors ($A_{2A}R$) are constitutively activated G-protein coupled-receptors, and the main brain targets of the homeostatic neuromodulator adenosine. Our team and others have found recurrent cortical and hippocampal upsurge of $A_{2A}R$ expression/function associated to cognitive deficits and alterations in their signaling properties. We recently reported in an early-aging model, with increased $A_{2A}R$ hippocampal expression, that $A_{2A}R$ blockade could restore synaptic and cognitive dysfunction. However the question whether $A_{2A}R$ overexpression is also present in the human aged brain and if it is the trigger rather than the consequence of cognitive dysfunction remains to be addressed. We have now generated transgenic rats overexpressing the human $A_{2A}R$ under the control of the calcium calmodulin dependent Protein Kinase II (CamKII) promoter. We describe for the first time that a forebrain neuronal selective increase in $A_{2A}R$ drives aging-like hippocampal deficits, such as impairments in memory tasks and synaptic plasticity, and impacts on adenosine receptor signaling. More importantly, human brain from aged and demented subjects presents a clear $A_{2A}R$ overexpression compared to young subjects. Finally we show that changes in $A_{2A}R$ signaling can be the trigger for the observed hippocampal deficits and that upon $A_{2A}R$ blockade normal $A_{2A}R$ signaling is reestablished.

Introduction

Ageing is associated with cognitive decline both in humans and animals. Among brain structures, the hippocampus appears to be particularly vulnerable to senescence and degeneration. Importantly, ageing is the main risk factor for Alzheimer's disease (AD) (Reitz et al., 2011), which targets primarily the temporal lobe and hippocampal formation. Age and AD-related cognitive impairments are accompanied by structural and functional alterations in the hippocampus, that directly affect neural plasticity (Burke and Barnes, 2006), leading to synaptic dysfunctions and, subsequently, memory deficits (Diogenes et al., 2011).

A_{2A} Receptors ($A_{2A}R$) are one of the main brain targets of the homeostatic neuromodulator adenosine (Fredholm et al., 2007). $A_{2A}R$ are constitutively activated G-protein coupled-receptors, preferentially expressed by the striatopallidal medium spiny striatal neurons (Blum *et al.*, 2003; Schiffmann and Vanderhaeghen, 1993b). They exhibit however a very distinct pattern of expression in the hippocampus and cortex where their expression is very low in physiological conditions (Cunha *et al.*, 1995). Our team and others have found compelling evidence of cortical and hippocampal upsurge of $A_{2A}R$ expression/function associated to cognitive deficits. Specifically, in the hippocampus of aged rats, $A_{2A}R$ expression is nearly two fold than of young ones (Cunha *et al.*, 1995; Lopes *et al.*, 1999b). More importantly, the $A_{2A}R$-dependent activation of glutamate release becomes more pronounced as ageing progresses and shifts from a protein kinase C-mediated signaling to a cAMP-dependent effect (Lopes *et al.*, 1999a; Rebola *et al.*, 2005). This is accompanied by clear behavioural deficits in hippocampal-dependent tasks, such as spatial memory in rats (Diogenes *et al.*, 2011; Sousa *et al.*, 2014). Interestingly, other detrimental conditions associated to cognitive impairments, such as hypoxia, diabetes, stress or epilepsy share similar $A_{2A}R$ overactivation (Batalha *et al.*, 2013; Lopes *et al.*, 1999a; Lopes *et al.*, 2011). Recently, we described impairments in long-term potentiation (LTP) and hippocampal dependent tasks in an early-aging model, in association with increased $A_{2A}R$ hippocampal expression (Batalha et al., 2013). Strikingly, in adults, we were able to restore synaptic

and cognitive dysfunction by blocking A_{2A}R with the selective antagonist KW6002 (Batalha et al., 2013) orally administered for one month. This supports an instrumental role of A_{2A}R dysregulation in the genesis of synaptic dysfunction underlying cognitive impairments. However, the mechanism involved or whether A_{2A}R upsurge is sufficient to accelerate hippocampal aging is yet unknown.

We have now generated transgenic rats expressing the human A_{2A}R driven by the CaMKII promoter. We describe for the first time that a forebrain neuronal selective increase in A_{2A}R impacts on receptor signaling and drives aging-like hippocampal deficits, such as impairments in memory task and synaptic plasticity deficits. More importantly, we probed human brain from aged and demented subjects and found a clear A_{2A}R overexpression compared to young subjects. Furthermore, we found that the rescue of synaptic and memory impairments achieved by blocking A_{2A}R activation is due to the reestablishment of adenosine neuromodulation and downstream A_{2A}R signaling in the hippocampus.

Material and methods

Human samples: Human AD samples were provided by Valerie Buée (INSERM U735 "Alzheimer & Tauopathies") Jean-Pierre Aubert (Research Centre Univ. Lille-Nord de France, France) or collected by Beatriz S. da Silva (National Institute of Legal Medicine and Forensic Sciences, Coimbra, Portugal) and prepared by Paula M. Canas (CNC-Center for Neurosciences and Cell Biology, Univ. Coimbra, Coimbra Portugal).

Animals: Animal procedures were performed in accordance with the European Community guidelines (Directive 2010/63/EU), Portuguese law on animal care (1005/92), and approved by the *Instituto de Medicina Molecular* Internal Committee and the Portuguese Animal Ethics Committee (*Direcção Geral de Veterinária*). Environmental conditions were kept constant: food and water ad lib, 21±0.5°C, 60±10% relative humidity, 12 h light/dark cycles. Male rats were killed by decapitation after anesthesia under halothane atmosphere.

Maternal separation and KW6002 treatment: Maternal separation protocol and treatment was performed as described before, (Batalha et al., 2013). Briefly Wistar dams and their litters were assigned either to control (CTR – non-separated) or to maternal separated (MS) groups at post natal day (PND) 2 all the litters were collected together, gender assessed and the pups were randomly distributed to foster dams (gender proportion maintained). MS pups were removed from their cages as a group from PND 2 to 14, for 180 minutes, daily, at 9 am, 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 with the mother. CTR pups were not handled and were maintained in their home-cages until weaning. At day 21 the pup's gender was confirmed, they were weaned and housed in groups of 5-8 animals per cage. At 4-6 weeks one started the oral administration of KW6002 (istradefylline), a selective adenosine A_{2A} receptor antagonist (Yang et al., 2007) at 3mg/Kg/day. Animals were divided in 4 groups: CTR or MS, drinking vehicle (0.025% methylcellulose) and CTR KW or MS KW drinking KW6002 (3mg/kg/day, 0.025% methylcellulose).

Generation and maintenance of transgenic animals: Transgenic rats with an overexpression of adenosine A_{2A} receptors (A_{2A}R) under the control of the CaMKII promotor, tg(CaMKII-hA2AR), were generated by microinjection of a linearized DNA construct into the male pronucleus of Sprague–Dawley rat zygotes with established methods (Popova *et al.*, 2002). The construct contained a full-length human A_{2A} cDNA cloned into an expression vector with the 8.5 kb mouse CaMKIIα promoter (Mayford *et al.*, 1996) and a polyadenylation cassette of bovine growth hormone (see Fig. 3.1A). Sprague Dawley wild type (WT) rats were used as controls. Genotyping: Transgenic rats were identified by PCR (30 cycles, 58 °C annealing temperature) of their genomic DNA isolated from ear biopsies by the use of the following transgene-specific primers: CaMKII-hA2A and rat Act-B primers as an internal control (Invitrogen, UK, see Table 3.1 page 70).

In situ hybridization: The *in situ* hybridization technique was adapted from previously described methods (Schiffmann and Vanderhaeghen, 1993a). The sections mounted on RNAse free poly-L-lysine-coated slides were fixed in freshly prepared 4% paraformaldehyde solution for 30 min and rinsed in 0.1 M phosphate buffered saline (PBS:130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4). All sections were dehydrated and dipped for 3 min in chloroform. After air drying, the sections were incubated overnight at 42°C with $0.35x10^6$ cpm per section of ^{35}S-labelled probes diluted in hybridization buffer, which consisted of 50% formamide, 4xSSC (1xSSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), 1 x Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin-BSA, 0.02% Ficoll, 1% sarcosyl, 0.02 M sodium phosphate at pH 7.4, 10% dextran sulfate, 500 µg/mL yeast tRNA, 100 µg /mL salmon sperm DNA, and 60 mM dithiothreitol). After hybridization, the sections were rinsed for 4x15 min in 1xSSC at 55°C, dehydrated and covered with Hyperfilm-βmax film (Amersham, Belgium) for 2 or 3 weeks. The oligonucleotide probes were synthesized on an Applied Biosystems 381A DNA synthesizer or Eurogentec (Belgium) with a GC to AT ratio between 45 and 65%. The human A_{2A}R oligonucleotide probe (CAGCCCTGGGAGTGGTTCTTGCCCTCCTTTGGCTGACC-

GCA) is complementary to nucleotides 123-166 in a partial human cDNA sequence (Libert *et al.*, 1989) and has been previously used on human brain sections (Schiffmann *et al.*, 1991). The rat $A_{2A}R$ probe (CCGCTCCCCTGGCAGGGGCTGGCTCTCCATC-TGCTTCAGCTG) is complementary to nucleotides 604–645 of the rat cDNA sequence (Fink et al., 1992). Oligonucleotides were labelled with α-^{35}S dATP (DuPont-NEN, Belgium) at their 3' end by terminal DNA deoxynucleotidylexotransferase (Gibco, Belgium) and purified with a G50 column (Pharmacia, Belgium) according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR analysis (RT-qPCR): Total RNA was extracted and purified using the RNeasy Lipid Tissue Mini Kit (Qiagen, Maryland, USA). RNA quality was assessed by NanoDrop 2000 (Thermo Scientific) analysis (A260/A280 $\approx$ 2; 260/235 >1.8). Total RNA (2 μg) was reverse-transcribed using random primers and SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, USA). RT-qPCR analysis was performed on a Corbett Rotor-gene 6000 apparatus (Qiagen, Germany) using Power SYBR Green PCR Master Mix (Applied Biosystems, UK), 0.2 μM of each primer and 1/20 dilutions of total cDNA (final concentration 0.4 ng/μl). The thermal cycler conditions were 10 min at 95°C, 40 cycles of a two-step PCR, 95°C for 15 s followed by 60°C for 25 s with a final thermal ramp from 72 to 95ºC. Primer efficiencies (E=1±0.02) were obtained from standard curves of serial dilutions (slope and R^2 respectively around -3.3 and 0.99). Sequences of primers used (all from Invitrogen, UK, HPLC purified) are listed in the Table 3.1 below. Reference genes were PPIA (cyclophilin A) and β-actin for human tissues and PPIA, Rpl13A (ribosomal protein L13A) and Pgk1 (phosphoglycerate kinase 1) for rat tissues. Amplifications were carried out in triplicate in two independent runs, and according to the MIQE guidelines (Bustin et al., 2009). The relative expression of target genes was determined by the comparative CT method (Schmittgen and Livak, 2008).

Table 3.1: Primers used for genotyping and qPCR.

Primer	Target Gene	Organism	Forward Primer	Reverse Primer	Amplicon Size
CypA	PPIA peptidylprolyl isomerase A (cyclophilin A)	rat, human	TATCTGCACTGCCAAGACTGAGTG	CTTCTTGCTGGTCTTGCCATTCC	126bp
Rpl13A	Ribosomal protein L13A	rat	GGATCCCTCCACCCTATGACA	CTGGTACTTCCACCCGACCTC	130bp
Pgk1	Phosphoglycerate kinase 1	rat	ATGCAAAGACTGGCCAAGCTAC	AGCCACAGCCTCAGCATATTTC	103bp
hACTB	Human Actin-β	human	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	233bp
A2AH	Human Adenosine A2A Receptor	human	AACCTGCAGAACGTCAC	GTCACCAAGCCATTGTACCG	245bp
A1	Adenosine A1 Receptor	rat	ACCTCCGAGTCAAGATCCCT	TTGGCTCTCCAGTCTTGCTC	160bp
Act-B	Actin-β	rat	AGCCATGTACGTAGCCAT	CTCTCAGCTGTGGTGGTGAA	228bp
CaMKII -hA2A	calmodulin-dependent protein kinase II promoter and human Adenosine Receptor A2A	transgene	GACTAAGTTTGTTCGCATCCC	GTGACACCACAAAGTAGTTGG	450bp

Behavioural assessments: WT and transgenic animals with 10-14 weeks were first handled for 5 days before testing in the behavior assays. For the Morris water maze (MWM) (Morris *et al.*, 1982), rats were given spatial acquisition training consisting of four trials/day for four consecutive days, as performed before (Batalha *et al.*, 2013); on the fifth day a probe test was given in which the platform was removed and animals were allowed to swim freely for 60 s while recording the percentage of time spent on each quadrant; the latency to find the platform during acquisition and the percentage of time in the platform quadrant during the probe test were used to evaluate hippocampal-dependent memory. The Y-maze was performed in a two-trial recognition test in a Y-shaped maze with 3 arms (each with 35 cm length x 10 cm width x 20 cm height), angled at 120º; on the first trial (learning trial), the animal explored the maze for 10 min with only two arms opened (start and other arm); after 1 h, the animal is exposed to the maze for 5 min (test trial) with the novel arm available, the preference for the novel arm is considered a measure of short-term reference memory. The number of transitions was used to evaluate motor performance. The maze was cleaned with a 70% ethanol solution between each animal. Rat tracings were continuously monitored by an automated tracking system (Smart 2.5, PanLab, Barcelona) in both learning tasks.

Electrophysiological recordings: After decapitation the brain was rapidly removed and the hippocampi were dissected free in ice-cold Krebs solution. 400 μM slices were obtained with a McIIwen tissue shopper and field excitatory postsynaptic potentials (fEPSPs) were recorded as previously in *stratum radiatum* of the CA1 area. For drug effects, CGS58261 (30nM), DPCPX (100nM), CPA (30nM), H89 (1μM) or GF109203X (1μM) were added to de perfusion after obtaining a stable 10 minutes

baseline. Long term potentiation (LTP, 100 Hz, 1s, 100 pulses induced at 0.5mV/ms) was recorded as previously (Batalha et al., 2013) . Recordings were performed at 30.5ºC, 3mL/min.

Samples preparation for Western blotting: Tissue was homogenized by sonication using RIPA buffer (50mM Tris, 1mM EDTA, 150mM NaCl, 0.1% SDS, 1% NP40, pH 8). The protein concentration was determined using a BioRad DC Protein assay Kit (based on Lowry, (1951) due to the high levels of detergents present in the sample).

Western blotting: The appropriate volume of each sample was diluted in water and sample buffer (350 mM Tris pH 6.8, 30% glycerol, 10% SDS, 600 mM DTT and 0.012% Bromophenol blue). The samples were denatured at 70°C for 20 minutes. Based on the protocol of Towbin et al. (Towbin *et al.*, 1979). Samples and molecular weight markers were separated by SDS-PAGE (10% for resolving and a 5% for stacking gels) in denaturing conditions and electro-transferred to PVDF membranes (Millipore). Membranes were blocked with 5% non-fat dry milk for 1 hour, washed with TBS-T 0.1% (Tris Buffer Saline with 0.1% Tween-20 solution, 200 nM Tris, 1.5 M NaCl) and incubated with primary antibody (diluted in TBS-T, 3% Bovine Serum Albumin and 0.1% NaN3) overnight at 4°C. After washing with TBS-T for 30 minutes, the membranes were incubated with horseradish peroxidise (HRP, EC 1.11.1.7) conjugated secondary antibody (in 5% non-fat dry milk) for 1 hour at room temperature. Primary antibodies were mouse anti-$A_{2A}R$ (1:2000, Upstate/Millipore - 05-717, Darmstadt, Germany), rabbit anti α-Tubulin specific (1:2000, abcam, ab4074) and rabbit anti-pan-cadherin (1:20000, abcam ab6529, UK). Secondary antibodies were goat anti-rabbit and anti-mouse (Santa Cruz Biotechnology, Heidelberg, Germany). After 40 minutes of washing with TBS-T, chemoluminescent detection was performed with ECL western blotting detection reagent (GE Healthcare) using X-Ray films (Fujifilm). Optical density was determined with Image-J software and normalized to the respective pan-chaderin band density.

Drugs: A_{2A}R selective antagonist, 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]¬pyrimi¬din-5-amine (SCH58261), A_{2A}R selective agonist 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS 21680), A_1 selective agonist N6-cyclopentyladenosine (CPA), A_1R selective antagonist 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and PKC inhibitor 2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide (GF 109203X) were purchased from Tocris, Bristol, UK. PKA inhibitor N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89) was purchased from Sigma Aldrich, Spain. These drugs were diluted in the assay solution from 5 mM or 1mM stock aliquots made in DMSO stored at -20ºC. A_{2A}R selective antagonist, (E)-8-[2-(3,4-dimethoxyphenyl)vinyl]-1,3-diethyl-7-methyl-3,7-dihydropurine-2,6-dione (KW6002, istradefylline) was synthesized according to a published procedure (Hockemeyer et al., 2004). The purity of the product was determined by HPLC analysis coupled to electrospray ionisation mass spectrometry and was greater than 98%. HRP-coupled secondary antibodies were from Santa Cruz Biotechnology. All other reagents used were of the highest purity available either from Merck, Germany or Sigma Aldrich, Spain.

Statistics: Values presented are mean ± SEM of *n* independent experiments. In statistical analysis for every two comparisons a Student T-test was used while for three or more conditions, a one way ANOVA test followed by a Bonferroni's Multiple Comparison post hoc test was used. For the analysis of the Morris water maze acquisition statistical differences were evaluated by using two-way ANOVA test while for the probe test and for the Y maze a one way ANOVA test followed by a Bonferroni's Multiple Comparison *post hoc* test was used within groups. Values of $p<0.05$ were considered as statistically significant.

Results

Hippocampal spatial memory deficits induced by neuronal increase in $A_{2A}R$

To test whether $A_{2A}R$ upsurge by itself is able to induce hippocampal deficits we generated animals that present a neuronal selective overexpression of human $A_{2A}R$ under the control of the CaMKII promoter (Figure 3.1 A - tg(CaMKII-hA2AR). Overexpression is more evident in the forebrain areas (Figure 3.1 B and C), mainly in the hippocampus and cortex, though we also detected increased $A_{2A}R$ mRNA levels in other areas (Figure 3.1 C). These animals have the great advantage of overexpressing $A_{2A}R$ from 2 weeks-old onwards. This is visible by the increased $A_{2A}R$ immunoreactivity along age in the hippocampal area (Figure 3.1 D). No changes in hippocampal adenosine A_1 receptor (A_1R) levels were observed upon overexpression of $A_{2A}R$ (Figure 3.1 E).

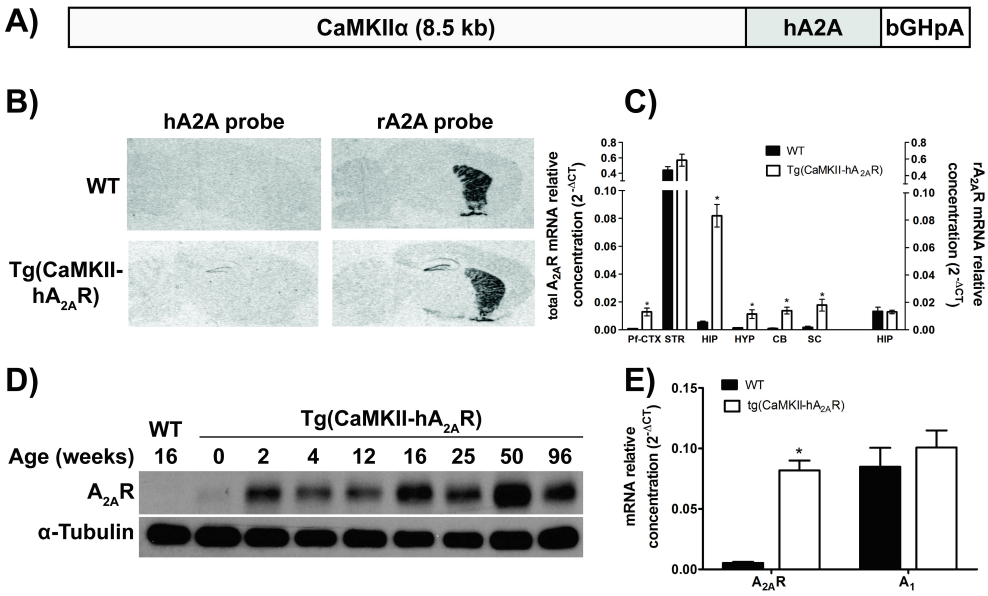

Figure 3.1: Tg(CAMKII-hA2AR) rats overexpress $hA_{2A}R$ mainly in the forebrain.
A) Construct used to generate tg(CaMKII-$hA_{2A}R$) rats. B) These animals present an overexpression of $A_{2A}R$ in the forebrain visible by *in situ* hybridization C) confirmed by qPCR. The endogenous (right axis; $rA_{2A}R$ mRNA) levels were not modified in the hippocampus. D) $A_{2A}R$ protein levels increase from 2 weeks old onwards in the hippocampus, but E) no changes were detected in Adenosine A_1R mRNA levels at 12-16 weeks old. Results were analyzed using an unpaired Student t-test for each brain area or gene, *P<0.05 compared to CTR.

We then evaluated hippocampal dependent spatial memory using the Morris water maze (MWM) test. Transgenic animals presented a slower learning during acquisition (Figure 3.2 A) and lack of preference by the target quadrant during probe test (Figure 3.2 B). Accordingly, when tested for short term working memory, using the Y-maze test, tg(CaMKII-hA2AR) animals performed worse than WT, revealing no preference for the novel arm (Figure 3.2 D). No changes were observed at a locomotor level that could compromise the tests either in the swimming speed (Figure 3.2 C) or in the total number of transitions (Figure and 3.2 E).

Changes in synaptic plasticity induced by neuronal increase in A_{2A}R

To further explore the consequences of A_{2A}R overexpression for hippocampal function we then evaluated long-term potentiation (LTP), a form of synaptic plasticity that has been associated to different forms of learning (Lynch, 2004). Strikingly, A_{2A}R overexpressing animals displayed enhanced LTP when compared with WT animals (Figures 3.2 F-H). To further confirm that this was dependent on A_{2A}R overactivation LTP was induced in the presence of a selective antagonist for A_{2A}R, SCH 58261 (50 nM). This blockade of A_{2A}R reverted the LTP back to WT-like levels (Figures 3.2 F-H).

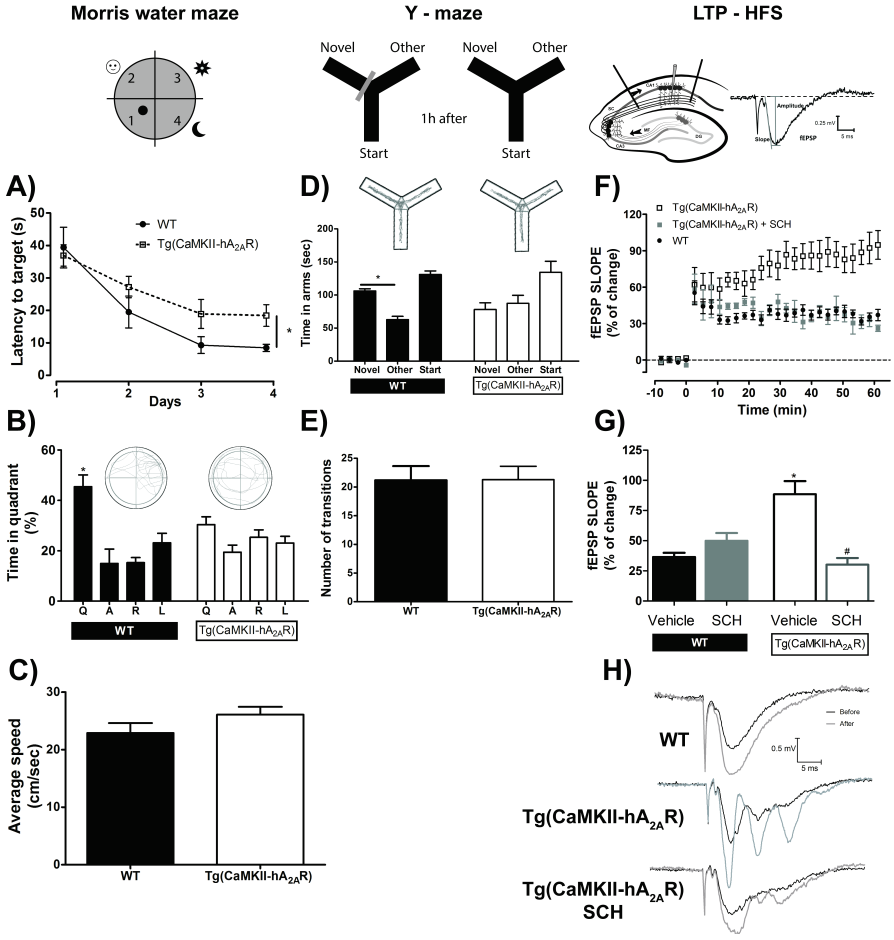

Figure 3.2: Overexpression of $A_{2A}R$ induces hippocampal deficits.
In the morris water maze Tg(CaMKII-hA_{2A}R) animals present: A) a slower learning performance to find the hidden platform (open squares) than WT (closed circles), results analyzed using a TWO-way anova (n=6/8, F(1, 44)=4.66,* P=0.036); B) no preference for the plataform quadrant during probe test (n=7, P>0.05) while WT clearly prefer the Q quadrant (n=5, P<0.05 comparing all quadrants) results analyzed using a One-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test within groups; and C) no changes in swimming speed during probe test, results analyzed using a unpaired Student t-test (n=5/7 P>0.05). In the Y maze test D) Tg(CaMKII-hA_{2A}R) animals present no preference by the novel arm (n=7, P>0.05 comparing to the other arm) while WT animals prefer the novel arm (n=6, P<0.05 comparing to the other arm). Results were analyzed using a One-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test within groups and E) no changes in the total number of entries were observed, results analyzed using unpaired Student t-test (n=6/7, P<0.05). Long term potentiation (LTP) was induced by High frequency stimulation (HFS: 100Hz, 1s) and used to evaluate synaptic plasticity in hippocampal rat slices. Tg(CaMKII-hA_{2A}R) animals (n=4) present a higher increase in fEPSP after HFS than WT (n=7) an effect that was prevented by superfusion of the A_{2A}R antagonist SCH 58261 (n=4). F) Raw data of HFS, G) quantification of the magnitude of changes of the fEPSP in the last 10 minutes of LTP and H) representative tracings prior and in the last 10 minutes of LTP, results were analyzed using a one-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test. *P<0.05 comparing to WT and $^{\#}$P<0.05 comparing to Tg(CaMKII-hA_{2A}R) .

Increased levels of A_{2A}R in human aged and Alzheimer's disease brain

Our findings suggest that upregulation of A_{2A}R triggers neuronal dysfunction. We tested whether this upsurge is detectable in human brain. A_{2A}R immunoreactivity was measured in young (20-40 years old), aged (50-60 years old) and AD (50-60 years old, Braak stages 5-6) human cortex. Aged brains have a clear upregulation of A_{2A}R levels, that was further enhanced in AD brains (Figure 3.3 A and B). We also confirmed this increase by comparing A_{2A}R mRNA levels from AD to aged-matched healthy brains. (Figure 3.3C)

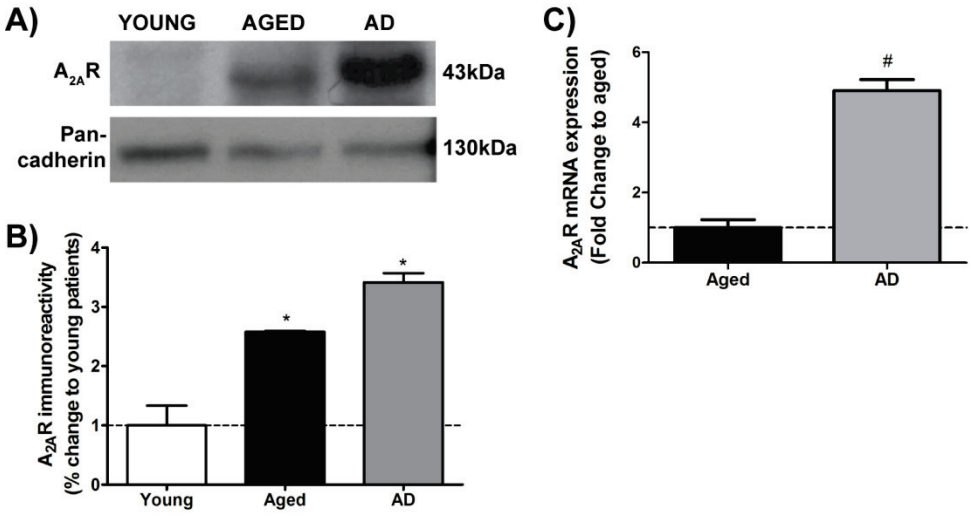

Figure 3.3: Increased levels of A_{2A} receptors in aged and AD human brain.
A) Representative image of the western blot for A_{2A}R in human prefrontal cortex and the internal control Pan-cadherin. B) A_{2A}R immunoreactivity in young, aged and AD human cortex (n=2/3), results were analyzed using a one-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test, *P<0.05 compared to young subjects. C) Increase in A_{2A}R mRNA in AD human brain when compared with age-matched control subjects (n=3), results were analyzed using an unpaired Student t-test #P<0.05 compared to age-matched subjects.

Neuronal increase in $A_{2A}R$ induces a PKC-PKA shift in signaling

Associated with ageing and with an upregulation of $A_{2A}R$ it was observed a shift in $A_{2A}R$ signaling pathways from PKC to PKA activation (Lopes *et al.*, 1999a). We further explored the $A_{2A}R$ signaling and efficiency associated to the observed hippocampal dysfunction to evaluate if it followed an age like pattern. The effect of CGS 21680, a selective $A_{2A}R$ agonist on basal synaptic transmission was much higher in transgenic than in WT animals (Figure 3.4 A and B). This effect was abolished in the presence of H89, a PKA blocker, but not by GF 109203x, a PKC blocker (Figure 3.4 C). To evaluate if $A_{2A}R$ effect is mediated by $A_{2A}R$ alone or requires A_1R activation, we activated $A_{2A}R$ while blocking A_1R with a selective antagonist, DPCPX. A_1R blockade did not prevent $A_{2A}R$ effect on basal synaptic transmission (Figure 3.4 D). Finally we explored if the $A_{2A}R$ crosstalk with A_1R, shown to disappear in normal ageing (Lopes *et al.*, 1999a; Rebola *et al.*, 2005), is lost in the rats that overexpress $A_{2A}R$. While in WT animals A_1R activation by CPA causes a strong inhibition of synaptic transmission that is attenuated when $A_{2A}R$ are simultaneously activated with CGS 21680; in transgenic animals, the $A_{2A}R$ activation did not modify A_1R mediated effects (Figure 3.4 E and F).

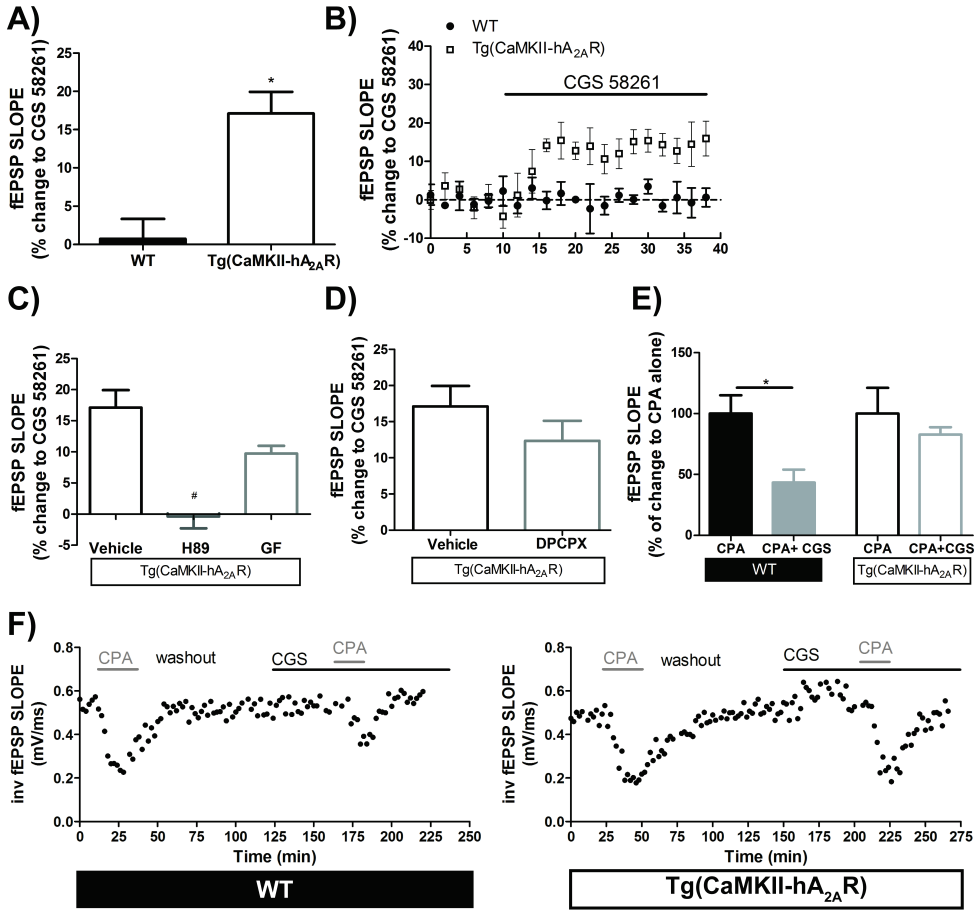

Figure 3.4: Overexpression of A_{2A}R induces age like modification in adenosine neuromodulation.
A) The A_{2A}R selective agonist CGS58261, 30nM, has an effect on basal fEPSP slope, B) for *row data*, results were analyzed using a unpaired Student t-test (n=7/4,*P<0.05 comparing to CTR). C)The effect of CGS58261, 30nM is blocked by H89 (1µM), a PKA antagonist, but not GF (1µM) a PKC antagonist, results were analyzed using One-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test (n=3/6, #P<0.05 comparing with Tg(CaMKII-hA$_{2A}$R). D)The effect of A_{2A}R activation is the same in the presence or absence of the A_1 selective antagonist DPCPX (100nM) an A_1R selective antagonist (n=3/4, P>0.05 using a unpaired Student t-test analysis). E) The effect of CGS58261 upon CPA, 30nM, on fEPSP is lost in Tg(CaMKII-hA$_{2A}$R) animals, results were analyzed using a paired Student t-test comparing to CPA alone (n=4/7, *P<0.01 in WT animals comparing to CPA alone). F) Representative *row data* of one experiment for WT (left panel) and Tg(CaMKII-hA$_{2A}$R) (right panel).

$A_{2A}R$ blockade reestablishes $A_{2A}R/A_1R$ crosstalk

Previously, we were able to restore synaptic and cognitive dysfunction induced by chronic stress, which leads to $A_{2A}R$ overexpression, by blocking $A_{2A}R$ with the selective antagonist KW6002 (Batalha et al., 2013) orally administered for one month. We now evaluated whether these benefits would be associated with a signaling shift and $A_{2A}R$ underactivation. So, we tested $A_{2A}R$ function in control (CTR) and chronically stressed animals, using the same maternal separation stress paradigm as before (Batalha et al., 2013). Interestingly, in maternally separated (MS) animals, $A_{2A}R$ activation by CGS 21680 has a higher effect on basal synaptic transmission compared to CTR (Figure 3.5 A). Moreover, the characteristic $A_{2A}R$-induced attenuation of A_1R function is lost (Figure 3.5 B). To evaluate if this was due to the $A_{2A}R$ overactivation, MS animals were treated for 1 month with an $A_{2A}R$ antagonist (KW 6002; 3 mg/Kg/day) and the same parameters evaluated. MS treated animals (MS KW) recovered from the $A_{2A}R$ overactivation (see Figure 5A), whereas treatment in CTR animals did not affect function (CTR KW). More interestingly, the $A_1R/A_{2A}R$ crosstalk was reestablished in treated animals (Figure 5B, MS KW), since CPA effect was again attenuated in the presence of CGS 58261, similar to the CTR situation, which was absent in MS animals.

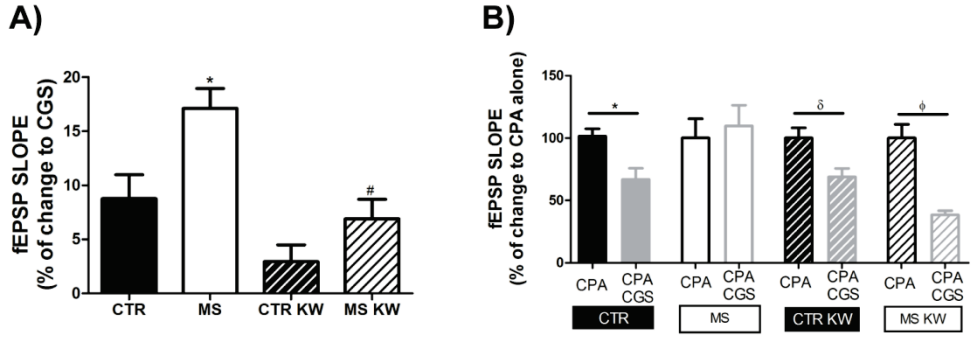

Figure 3.5: Stress induces age like modifications in adenosine neuromodulation that are reverted by chronic in vivo treatment with KW6002 a selective $A_{2A}R$ antagonist.
A) The increased effect of CGS58261 30nM, a selective $A_{2A}R$ agonist, on fEPSP observed in MS is reverted in MS KW animals, results were analyzed using a one-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test (n=6/8,*P<0.05 comparing to CTR; #P<0.01 comparing to MS). B)The effect of CGS58261 upon CPA on fEPSP is lost in MS animals and recovered in MS KW animals, results were analyzed using a paired Student t-test comparing to CPA alone (n=5/6, *P=0.008 in CTR animals; δP=0.01 in CTR KW animals; ϕP=0.0003 in MS KW animals).

Discussion

Here we show for the first time that the $A_{2A}R$ upsurge described in different pathological situations in rodent models, such as hypoxia, ischemia, stress, diabetes and even upon ageing, is also characteristic of the human aged brain and is aggravated in AD. Moreover, we give the first evidence that an increase in neuronal $A_{2A}R$ is sufficient to drive deficits in synaptic plasticity and impairments of hippocampal-dependent learning and memory. The present results also indicate that a shift in $A_{2A}R$ signaling is associated to pathological situations.

Multiple pathologies displaying cognitive deficits are associated with increased levels of $A_{2A}R$. Namely, in acute or chronic stress (Batalha *et al.*, 2013; Cunha *et al.*, 2006), in rodent models of Parkinson's Disease (PD) (Varani et al., 2010) or even in normal ageing (Cunha et al., 1995). However, there was no evidence that this upsurge of $A_{2A}R$ was instrumental in driving pathology. We now show, not only that a forebrain selective neuronal increase in $A_{2A}R$ is sufficient to drive memory impairments, but also that $A_{2A}R$ are increased also in human aging and pathology. Previously, it has been shown that increasing $A_{2A}R$ under the control of the neuron-specific enolase promoter could lead to working memory deficits (Gimenez-Llort et al., 2007). However, in that report the promoter drives $A_{2A}R$ overexpression since embryonic stage and throughout the whole brain. So a definite conclusion that the $A_{2A}R$ overexpression is triggering memory deficits was not possible. Now, we generated $A_{2A}R$ overexpressing animals under the control of a CaMKII promoter. This renders $A_{2A}R$ overexpression selectively in forebrain neurons and only postnatally. Importantly, the fact that the observed alterations in synaptic plasticity are reverted when $A_{2A}R$ are blocked, reinforces $A_{2A}R$ involvement in the observed deficits.

The magnitude of long-term potentiation (LTP) in CA1/CA3 hippocampal synapses has been correlated with memory performance for a long time (Lynch, 2004). However, and being learning a highly complex process, sometimes data contradict this paradigm. Indeed, it has already been described an increase in LTP magnitude associated with age-related memory deficits (Diogenes et al., 2011). Interestingly, tg(CaMKII-h$A_{2A}R$)

animals present a very similar profile, that is memory impairments in behavioral tasks together with an increase in LTP magnitude. Furthermore, the features of adenosine neuromodulation evaluated in tg(CaMKII-hA_{2A}R) also follow a similar pattern to that observed upon aging suggesting that A_{2A}R upregulation mimics hippocampal aging. Indeed this hypothesis is supported by the observation that A_{2A}R activation in tg(CaMKII-hA_{2A}R) animals has a direct effect in basal synaptic transmission, which is no longer PKC mediated as in WT, and is independent on A_1R inhibition, which is only observed upon aging (Lopes *et al.*, 1999a). Additionally we've shown that in a chronic stress model of maternal separation, that present increased levels of A_{2A}R in the hippocampus plus memory deficits, A_{2A}R blockade restoration of memory deficits (Batalha et al., 2013) was accompanied by a reestablishment of adenosine neuromodulation. Indeed, MS animals treated with the A_{2A}R selective antagonists present a reestablishment of a normal A_{2A}R signaling via A_1R inhibition, and loss of A_{2A}R direct effect on basal synaptic transmission. Taken together, the results now presented reveal that an increase in neuronal A_{2A}R is sufficient to drive changes in adenosine neuromodulation. This imbalance is associated with impaired synaptic plasticity, sustaining an instrumental role of adenosine neuromodulation in learning and memory processes, particularly those related to ageing.

The therapeutic interest of using selective A_{2A}R antagonists against multiple pathologies is increasing (Lopes *et al.*, 2011; Muller, 2013). Some adenosine-based drugs were already in clinical trials for PD (Lopes *et al.*, 2011; Muller, 2013), however the lack of knowledge on their mechanism of action compromised their acceptance for clinical use. In AD, multiple groups have shown protective effects of A_{2A}R blockade (Rivera-Oliver and Diaz-Rios, 2014). Caffeine consumption, a non-selective A_{2A}R antagonist, has been reported to decrease the risk of developing AD (Arendash and Cao, 2010; Maia and de Mendonca, 2002). Recent work also revealed that in humans, A_{2A}R antagonists can even have pro-cognitive effects (Borota et al., 2014). Finally, A_{2A}R antagonism was also proposed for the treatment of depression and anxiety-like disorders (Batalha *et al.*, 2011; Cunha *et al.*, 2008b). However, there was so far no

direct evidence that A_{2A}R overactivation could trigger or increase the susceptibility for this multiple pathologies. Our results show that A_{2A}R overexpression and consequent overactivation shifts adenosine neuromodulation towards a pathology-related status, leading to impairments in synaptic plasticity and consequent deficits in memory outcome.

The targeting of A_{2A}R always generated some controversy since A_{2A}R activation promotes Brain derived neurotropic factor (BDNF) actions (Diogenes *et al.*, 2007; Diogenes *et al.*, 2004) which could be beneficial. Therefore some observations point A_{2A}R activation rather than its blockade as the solution. By now showing that an alteration in adenosine signaling is associated with dysfunction, and that upon blockade of A_{2A}R with KW6002, the physiological actions of A_{2A}R are reestablished without any side effects in control animals, we offer a new possibility for research. The challenge is now in developing new antagonists or to evaluate the already available regarding their selectivity for A_{2A}R according to their different signaling and effects.

In summary, we report that a post-natal, neuronal selective, overexpression of A_{2A}R drives age-like alterations in hippocampal function. This is associated with an altered adenosine neuromodulation, LTP dysfunction and memory deficits. These observations are crucial at clarifying the instrumental role A_{2A}R in pathology and strongly support the development of new A_{2A}R antagonists in therapeutics.

Chapter 4: Adenosine A_{2A} receptor regulates stress glucocorticoid receptor in the brain

Adenosine A_{2A} receptor regulates stress glucocorticoid receptor in the brain

Vânia L. Batalha[a], Diana G. Ferreira[a,c], Joana E. Coelho[a], Rui Gomes[a,g], Tatiana Shmidt[b], Malika Hamdane[e], Ghazaleh Sadri-Vakili[h], Luc Buée[e], Michael Bader[b,f], David Blum[e#] and Luísa V. Lopes[a#]

[a]Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Portugal

[b]Max-Delbrück-Center for Molecular Medicine (MDC), Berlin, Germany

[c]Department of Neurodegeneration and Restorative Research, University Medizin Göttingen Germany

[e]Inserm, U837, Lille, France

[f]Charité-University Medicine Berlin, Germany[i]Institute of Biology, University of Lübeck, Germany

[g]Faculdade de Ciências da Universidade de Lisboa, Portugal

[h]MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital, Boston, MA, United States of America.

VLB (Vânia L. Batalha) has written the draft, designed and performed all the experimental work, except the qPCRs made by RG. The epigenetic experiments were done by VLB at Harvard Medical School, Boston under GSV supervision. The N1E115 experiments were performed by VLB in INSERM, Lille under DB and MH supervision. DF and JC helped in the electrophysiology experiments.

Abstract

The hippocampus was the first higher brain area to be recognized as a target for stress hormones. It is known that corticosteroids by activating glucocorticoid receptor (GR) receptors have a great impact on synaptic plasticity, learning and memory in this brain area.

We have recently revealed that oral administration of an adenosine A_{2A} receptor ($A_{2A}R$) blocker restores hippocampal morphological, behavioral and synaptic deficits induced by HPA-axis dysfunction in rodents. However, there is no evidence for a direct role of $A_{2A}R$ in GR effects, or whether the benefits of blocking $A_{2A}R$ are mediated by a GR-dependent effect.

We generated transgenic rats that overexpress $A_{2A}R$ under the control of the CaMKII promoter, tg(CaMKII-h$A_{2A}R$), in order to evaluate its impact on HPA-axis and GR-dependent effects. $A_{2A}R$ overexpression in forebrain neurons was sufficient to induce HPA-axis dysfunction, namely loss of plasmatic corticosterone circadian oscillation. Moreover, $A_{2A}R$ overactivation modulated GR-induced deficits in hippocampal synaptic plasticity, increasing susceptibility to activation by the GR agonist, dexamethasone. Conversely, blockade of $A_{2A}R$ prevented dexamethasone-induced GR transcriptional activity and nuclear translocation. Finally we show that $A_{2A}R$ blockade therapy *in vivo* increased histone H3 acetylation of the *Nr3c1* gene encoding GR therefore impacting in GR mRNA levels.

Together, our results suggest that $A_{2A}R$ directly modulate GR, unveiling an important therapeutic alternative to GR antagonists for clinical applications. These findings are significant for the treatment not only of psychopathologies but can also be extended to the multiple age-related conditions where glucocorticoid response is impaired.

Introduction

Stressful events have a role in the development and/or susceptibility for psychiatric disorders (McKinney, 1984; Willner, 1997; Willner *et al.*, 1997) such as anxiety, depression or posttraumatic stress disorders. These events are present throughout life, triggering peripheral and central physiological responses coordinated by the central nervous system, mostly through the activation of the Hypothalamic-Pituitary-Adrenal axis system (HPA-axis) (Herman and Cullinan, 1997) and consequent production of glucocorticoids, which are stress hormones and play a vital role in stress response.

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 classically cytoplasmic receptors that upon ligand binding, translocate to the nucleus and act as transcription factors (Zalachoras et al., 2013).

The hippocampus was the first higher brain area to be recognized as a target for stress hormones (McEwen et al., 1968). It is known that corticosteroids by activating GR receptors have a great impact on synaptic plasticity, learning and memory in this brain area (reviewed by Kim et al., 2006).

Adenosine is an important neuromodulator that acts through the activation of the G coupled receptors A_1 and A_{2A} (A_1R and $A_{2A}R$). In the recent years multiple evidences suggest an association between adenosine modulation and stress response, mainly by $A_{2A}R$ mediated effects. $A_{2A}R$ activation seems to contribute to stress response, by inducing corticosterone secretion (Chen et al., 2008) and mimicking GR effects (Okonkwo et al., 2006). Moreover we have recently revealed that oral administration of an antagonist for the receptor, which blocks $A_{2A}R$ activation, is able to restore morphological, behavioral and synaptic deficits induced by HPA-axis dysfunction in rodents (Batalha et al., 2013). Multiple situations where $A_{2A}R$ are overactivated or overexpressed are associated with impaired GR function in the brain. In Alzheimer's disease (AD) both $A_{2A}R$ and GR antagonists ameliorate cognitive deficits and decrease

amyloid-β levels (Arendash *et al.*, 2006; Baglietto-Vargas *et al.*, 2013). This supports the instrumental role of A_{2A}R in GR response and suggests that A_{2A}R effects may be mediated by reestablishing GR dysfunction.

However, there is no evidence so far for a direct role of A_{2A}R in GR effects, or that the beneficial effects achieved by blocking A_{2A}R in multiple pathologies are mediated by a GR-dependent effect. Since A_{2A}R-GR interaction is not exclusive of the nervous system, such an interaction could have far reaching implications in many clinical situations where corticosteroids play a pivotal role.

We therefore evaluated whether A_{2A}R overactivation impacts on HPA-axis function and GR effects upon transcriptional activity and synaptic plasticity. We report, for the first time, that A_{2A}R overexpression in forebrain neurons is sufficient to induce HPA-axis dysfunction, namely loss of plasmatic corticosterone circadian oscillation. We show that A_{2A}R blockade prevents GR transcriptional activity and nuclear translocation. Moreover, A_{2A}R activation modulates GR-induced deficits in hippocampal synaptic plasticity, increasing susceptibility to GR activation.

Material and methods

Animals: Animal procedures were performed in accordance with the European Community guidelines (Directive 2010/63/EU), Portuguese law on animal care (1005/92), and approved by the *Instituto de Medicina Molecular* Internal Committee and the Portuguese Animal Ethics Committee (*Direcção Geral de Veterinária*). Environmental conditions were kept constant: food and water ad lib, 21±0.5°C, 60±10% relative humidity, 12h light/dark cycles. The animals were killed by decapitation after anesthesia under halothane atmosphere. Transgenic rats with an overexpression of adenosine A_{2A} receptors ($A_{2A}R$) under the control of the Calcium calmodulin dependent Protein Kinase II (CaMKII) promoter, tg(CaMKII-h$A_{2A}R$), were generated by microinjection of a linearized DNA construct into the male pronucleus of Sprague–Dawley rat zygotes with established methods (Popova *et al.*, 2002). The construct contained a full-length human A_{2A} cDNA cloned into an expression vector with the 8.5 kb mouse CaMKIIα promoter (Mayford *et al.*, 1996) and a polyadenylation cassette of bovine growth hormone (see Fig. S4.1). Sprague Dawley wild type (WT) rats were used as controls.

Genotyping of rats: Transgenic rats were identified by PCR (30 cycles, 58 °C annealing temperature) of their genomic DNA isolated from ear biopsies by the use of the following transgene-specific primers: CaMKII-hA2A and rat Act-B as an internal control (Invitrogen, UK, see Table below).

Primer	Target Gene	Organism	Forward Primer	Reverse Primer	Amplicon Size
Act-B	Actin-β	rat	AGCCATGTACGTAGCCAT	CTCTCAGCTGTGGTGGTGAA	228bp
CaMKII -hA2A	Calcium calmodulin dependent Protein Kinase II promoter and human Adenosine Receptor A2A	transgene	GACTAAGTTTGTTCGCATCCC	GTGACACCACAAAGTAGTTGG	450bp

Cell culture: N1E-115 mouse neuroblastoma cells (CRL-2263) were cultured in Dulbecco's modified Eagle's medium (DMEM) without Pyruvate supplemented with 10%(v/v) fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin, and 2 mM L-glutamine (Gibco). For transfections, cells were plated into 6 well plates for 24h to reach 60% confluency before transfection with Exgene 500 (Euromedex, France). Briefly 4ug of pGL3(GRE)3_TK_Luc (GRE_Luc) plasmid were mixed in 400µL of

non suplemented DMEM with 20µL of Exgene 500 (the mix volume/well) and incubated for 15 minutes at room temperature. Cells were incubated for 3h with the transfection mix before completing the volume to 3mL. Drug treatments were performed 24h after transfection. Primary neuronal cultures. Cortical neurons from 18 days Sprague Dawley rat embryos (Harlan, Barcelona) were cultured according to Valadas (2012). Briefly the embryos were collected in Hanks' Balanced Salt Solution (HBSS) and rapidly decapitated. Meninges and white mater were removed and whole cortices were fragmented and cells were isolated by trypsinization HBSS Ca^{2+}/Mg^{2+} (1 mM/ 1 mM, 0.025% trypsine) and centrifugation at 200 rpm. Cells were washed with HBSS Ca^{2+}/Mg^{2+} supplemented with 10% FBS and resuspended in Neurobasal Medium. Cells were plated on poly-L-lysine-coated coverslips in 6-well plates at density of $1x10^6$ cells/well. Neurons were grown in Neurobasal medium with 2% B-27 supplement, glutamate 25 µM, glutamine 0.5 mM and 2U/mL Penicillin/Streptomycin, in the absence of any positive selection for neurons. Medium was totally replaced at day 4 (without glutamate). Drug treatments were performed at day 8, 1 hour after replacing the medium by neurobasal without B27. All cells were kept in a 5% CO_2 humidified incubator at 37 °C.

Dissection and tissue collection: After decapitation the brain was rapidly removed and the hippocampi were dissected free in ice-cold Krebs solution composed of (mM): NaCl 124; KCl 3; NaH2PO4 1.25; NaHCO3 26; MgSO4 1; CaCl2 2; and glucose 10, previously gassed with 95% O2 and 5% CO2, pH 7.4). One hippocampi was used for electrophysiological recordings, the remaining tissue was separated by areas and rapidly frozen in liquid nitrogen for further analysis.

Sample preparation: Tissue homogenates of WT and tg(CaMKII-hA_{2A}R) were prepared from frozen samples. Briefly samples were homogenized by sonication in immunoprecipitation-assay (RIPA) buffer (50 mM Tris, 1 mM EDTA, 150 mM NaCl 0.1% SDS, 1% NP 40, pH 8.0) (Palacios et al., 2004). Protein was quantified using the BioRad Protein DC assay based on (Lowry et al., 1951). The appropriate volume of sample was completed with water and 5x sample buffer. Nuclear/cytoplasmic fraction

enrichment was performed by differential centrifugation. Samples were homogenized with a 28G syringe and centrifuged at 1000 *g* for 10 min. The supernatant is the cytoplasmic fraction; the pellet was resuspended in 100 µL of sucrose buffer (0.32 M sucrose, 50 mM Tris, pH 7.6), homogenized and centrifuged again to ensure a minimum contamination with cytoplasm. 150 µL of 1.5x sample buffer (350 mM Tris, 30% glycerol, 10% SDS, 600 mM dithiothreitol and 0,012% bromophenol blue, pH 6.8) were added to the nuclear fraction and 15 µL were used for immunoblot detection. The cytoplasmic fraction was prepared with 20 µL of sample and 5 µL of 5x sample buffer.

Western Blotting: Samples were denatured by heating at 95ºC for 5 minutes or at 70º for 30 minutes for A_{2A}R. Samples and molecular weight markers were resolved by SDS-PAGE (8% or 10% for resolving and a 5% for stacking gels) in denaturing conditions and electro-transferred to PVDF membranes (Millipore). Membranes were blocked with 5% non-fat dry milk in TBS-T 0.1% (Tris Buffer Saline with 0.1% Tween-20 solution, 200 nM Tris, 1.5 M NaCl). After washing with TBS-T 0.1% membranes were incubated with primary antibody in 3% Bovine Serum Albumin (BSA). Secondary antibody incubation was in 5% non-fat dry milk in TBS-T 0.1%. Primary antibodies were rabbit anti-GR M20 (1:750/1:1000 sc-1004, Santa Cruz Biotechnology, Heidelberg, Germany), Rabbit anti-Lamin A/C (1:2000, cell signaling – 2032, Danvers, MA, USA) mouse anti-A_{2A}R (1:2000, Upstate/Millipore - 05-717, Darmstadt, Germany), mouse anti-GAPDH (1:1000, ambion AM4300) and rabbit anti-αTubulin (1:2000, abcam, ab4074, UK), secondary antibodies conjugated with horseradish peroxidase were goat anti-rabbit and anti-mouse (Santa Cruz Biotechnology, Heidelberg, Germany). Chemoluminescent detection was performed with ECL-PLUS western blotting detection reagent (GE Healthcare) using X-Ray films (Fujifilm). Optical density was determined with Image-J software.

Corticosterone quantification: Blood was collected from the tail in animals previously handled to minimize stress and without anesthesia at two different time points, 8 AM, and 8 PM (as in Batalha *et al.*, 2013). The plasma was isolated by centrifugation at 2000 *g*, 4ºC for 15 min and corticosterone quantified by radioimmunoassay using the

rat corticosterone ^{3}H kit (MP Biomedicals, UK), according to the manufacturer's protocol.

Drug treatments: Cell treatments were performed as in Valadas et al. (2012). Briefly, N1E115 cells were treated with dexamethasone 100 nM for 24 h; antagonists (SCH58261 10-100 nM, KW6002 30 nM and RU486 50 mM) were applied 15-20 min before treatment and agonists (CGS21680, 10-50 nM) were co-applied with dexamethasone. After treatment cells were washed in ice-cold PBS and processed for luciferase assay. Primary neuronal cultures were treated with dexamethasone 100 nM for different periods of time 0, 5, 10, 15, 30, 60, 90 min; the $A_{2A}R$ antagonist SCH58261 (50 nM) was applied 15-20 min before dexamethasone. After treatment, cells were washed with ice-cold PBS and resuspended in 200 µL of sucrose solution (0.32 M sucrose, 50 mM Tris, pH 7.6) supplemented with protease inhibitors (ROCHE). Hippocampal slices were incubated with dexamethasone 100 nM for 20 min or 1 h at 32ºC. Antagonists (50 nM SCH58261; 50 mM RU486) were applied 15-20 min before treatment and agonists (CGS21680, 30 nM) at the same time. *In vivo* therapy: KW6002 (istradefylline, a selective $A_{2A}R$ antagonist) or vehicle were orally administered in the drinking water (3 mg/kg) to WT male rats as before (Batalha et al., 2013).

Luciferase assay: Luciferase activity was evaluated with the luciferase assay system (Promega, USA) according to the manufacture procedure. Briefly, N1E115 cells were lysed in 150 µL of luciferase cell culture lyses reagent for 15 min at 4ºC. The supernatant was collected after 2 min, centrifuged at 12,000 *g* at 4ºC and 5µL were used for the assay. Luciferase activity was measured on a Mithras Microplate Reader LB 940 (Berthold Technologies).

Electrophysiological recordings: Slices (400 µm thick) were obtained with a McIlwain tissue chopper, left to recover for at least 1 h at 32ºC in Krebs solution and field excitatory postsynaptic potentials (fEPSPs) were recorded as previously described (Diogenes et al., 2011) in the CA1 *stratum radiatum*. Long term potentiation (LTP, 100 Hz, 1s) was recorded as previously described (Batalha et al., 2013). Slices were

incubated with different drugs or as a control at 32ºC and kept at 32ºC until recording. All recordings were performed at 32ºC with a constant flux of 3mL/minute.

Chromatin and methyl DNA immunoprecipitation: Samples (1 hippocampus) were cut into small pieces (1x1 mm) and incubated for 30 min with 1% formaldehyde. After washing with PBS, the sample was lysed in SDS lysis buffer (0.05 M Tris, 1% SDS 1%, 0.01 M EDTA), 200 µL of the lysate were used for phenol/clorophorm DNA extraction and 400 µL aliquots were used for chromatin immunoprecipitalion (ChIP). Purified DNA and ChIP samples were fragmented by sonication until DNA size was 200-500 kb. Chromatin IP (ChIP) was performed as previously described (McFarland *et al.*, 2012). Briefly, fragmented DNA/protein complexes were incubated with 5 µg of AcH3K9K14 (Millipore 06599) and magnetic beads overnight at 4ºC. After washing, the immunoprecipitated DNA/protein complexes were digested with proteinase K and the DNA purified for downstream analysis. Methyl DNA immunoprecipitation (meDIP) was performed using MethylMiner™ Methylated DNA Enrichment Kit (Invitrogen) according to manufacturer's instructions. Briefly, beads were incubated with MBD (methyl-CpG-binding domain) protein for 1 h at room temperature (RT); after washing, the beads-MBD protein complexes were incubated with 1 µg of DNA for 1 h at RT, the non-captured DNA was recovered and the methylated DNA was eluted with high salt solution (2M NaCl) and purified for downstream analysis (UltraClean PCr Clean-Up Kit, Mo Bio laboratories, inc). Following ChIP or meDIP, gene specific changes were evaluated by qPCR targeting two specific CpG islands of the *Nr3c1* gene (CpG147 and CpG11, found using the UCSC genome browser) using the following primer sequences: CpG147 forward: CGGAGAAGGAAGTCAACAGT; reverse: GGTGACTTTCAGCG-CTAGG, and CpG11 forward: CGGTCTGGCTTTTTCGATTT; reverse: CAGAG-AACCCCAAGAGTTCA. Threshold amplification cycle numbers (T_c) using iCycler software were used to calculate IP DNA quantities as percentage of corresponding inputs.

Drugs: $A_{2A}R$ selective antagonist, 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]-pyrimidin-5-amine (SCH58261), $A_{2A}R$ selective agonist 4-[2-

[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzene propanoic acid (CGS21680) and the GR antagonist (11β,17β)-11-[4-(Dimethylamino)phenyl]-17-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one (RU486) were purchased from Tocris Cookson, UK. These drugs were diluted in the assay solution from 5 mM or 100mM (for RU486) stock aliquots made in DMSO stored at -20ºC. GR agonist (1β,16α) -9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione, 9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione, 9α-Fluoro-16α-methylprednisolone (Dexamethasone) was purchased from Sigma (Spain), diluted to 10mM stock in DMSO and stored at -20ºC. A_{2A}R selective antagonist, (E)-8-[2-(3,4-dimethoxyphenyl)vinyl]-1,3-diethyl-7-methyl-3,7-dihydropurine-2,6-dione (KW6002, istradefylline) was synthesized according to a published procedure (Hockemeyer et al., 2004). The purity of the product was determined by HPLC analysis coupled to electrospray ionization mass spectrometry and was greater than 98%. For in vitro assays a stock solution at 10mM in DMSO was prepared and used only for 1 week and stored at -20ºC. All other reagents used were of the highest purity available either from Merck, Germany or Sigma Aldrich, Spain.

Statistics: Values presented are mean ±SEM of n experiments. To test the significance of the differences between groups in Western Blotting experiments, a paired Student's T test was used. In all other experiments, when comparing 3 or more groups a one-way ANOVA was used, followed by a Bonferroni's Multiple Comparison *post hoc* test. For the analysis of the primary neuronal cultures results and corticosterone levels, a two-way ANOVA followed by a Bonferroni's Multiple Comparison *post hoc* test was used. Values of $P<0.05$ were considered to be statistically significant.

Results

Overexpression of $A_{2A}R$ in forebrain neurons impairs HPA-axis

A possible explanation for the beneficial effects achieved by blocking $A_{2A}R$ is the reestablishment of the HPA-axis function and the circulating levels of corticosteroids (Baglietto-Vargas *et al.*, 2013; Coogan *et al.*, 2013), as observed upon the treatment of chronic stressed animals with the $A_{2A}R$ antagonist KW6002 (Batalha et al., 2013). We therefore evaluated if the overexpression of $A_{2A}R$ alone was sufficient to drive a dysfunction of the HPA-axis.

We generatered transgenic rats that overexpress $A_{2A}R$ under the control of the CaMKII promoter, tg(CaMKII-h$A_{2A}R$). tg(CaMKII-h$A_{2A}R$) animals present an overexpression of $A_{2A}R$ in the hippocampus, hypothalamus and pituitary (Figure 4.1 A - C) compared to WT. $A_{2A}R$ overexpression drives a decrease in hippocampal GR levels in the hippocampus and an increase in hypothalamus and pituitary gland (Figure 4.1 D - F). To assess if the stress response was compromised, blood was collected from tg(CaMKII-h$A_{2A}R$) and wild-type animals at two time points to evaluate plasmatic corticosterone levels (Figure 4.1 G). Control animals present a normal variation of corticosterone levels throughout the day, whereas tg(CaMKII-h$A_{2A}R$) present elevated corticosterone levels in the morning and loss of normal circadian oscillation (58±8 ng/mL in WT at a.m comparing with 92±12 in tg(CaMKII-h$A_{2A}R$)).

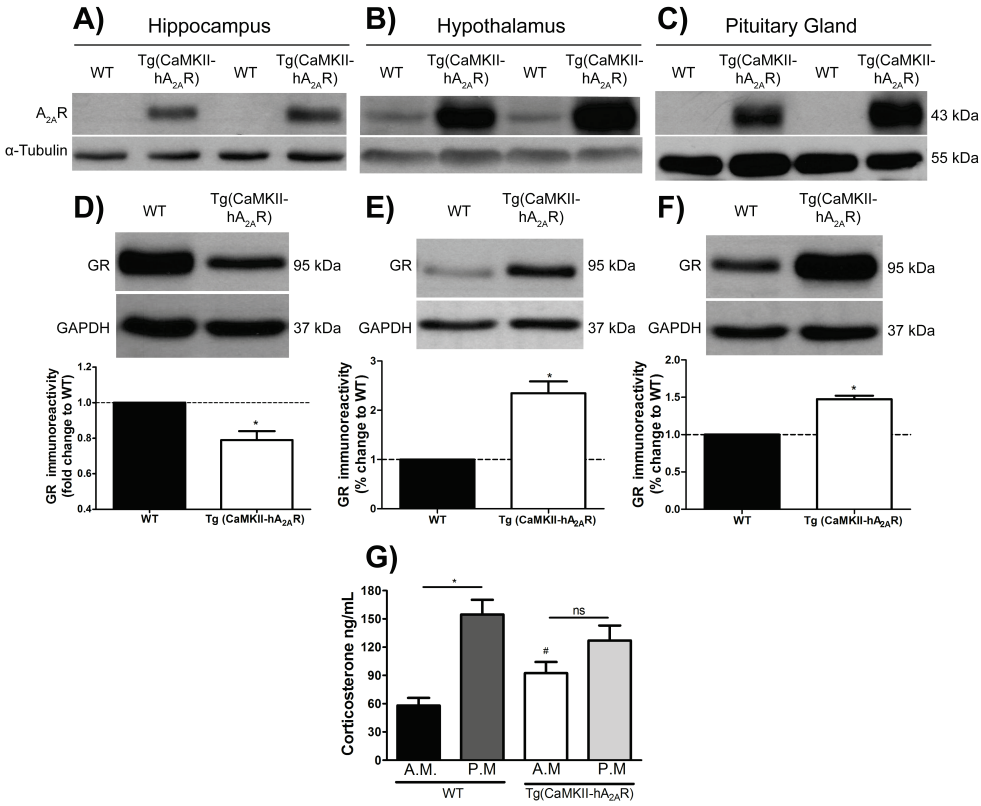

Figure 4.1. Neuronal overexpression of adenosine A_{2A} receptor (A_{2A}R) disrupts HPA-axis function. A_{2A}R overexpression in tg(CaMKII-hA_{2A}R) was confirmed in the A) hippocampus, B) hypothalamus and C) pituitary gland by Western Blotting. D) GR protein levels are decreased in the hippocampus (n=5) and increased in the E) Hypothalamus (n=5) and F) pituitary gland (n=4) of Tg(CaMKII-hA_{2A}R) compared to WT animals. G) Corticosterone levels evaluated at 8 a.m and 8 p.m are elevated in Tg(CaMKII-hA2AR) and do not oscillate in a circadian manner (n=6-9). Results are presented as mean ± SEM of n experiments.. *P<0.05 compared to WT, $^{\#}$P<0.05 compared with WT at a.m, calculated using a paired Student or a Two-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test.

Adenosine $A_{2A}R$ activation induces GR transcriptional activity

We then tested whether $A_{2A}R$ could directly modulate GR transcriptional regulation. So, N1E115 cells were transiently transfected with the plasmid pGL3(GRE)3_TK_Luc (GRE_Luc) that contains the glucocorticoid response element (GRE:promoter region at which GR bound to regulate gene transcription) coupled to the luciferase gene. To test the luciferase response to GR activation, cells were incubated for 24 hour with dexamethasone (100 nM). Upon exposure to dexamethasone, luciferase expression increased (212.5±20,2%, n=17, P<0.0001), an effect that was blocked by the GR antagonist RU486 (100 nM) (n=8, P<0.0001, Figure 4.2 A). To assess whether $A_{2A}R$ activation could modulate GR, $A_{2A}R$ agonist or antagonist were applied together with dexamethasone. $A_{2A}R$ blockade with SCH58261 (10 – 100 nM) reduced dexamethasone induced increase in luciferase expression, an effect also observed when a different antagonist, KW6002, was used (Figure 4.2 B). On the other hand, activation of $A_{2A}R$ with the agonist CGS21680 (10-50 nM), increased dexamethasone induced luciferase expression in a concentration-dependent manner (Figure 4.2 C).

To evaluate the $A_{2A}R$ effect on endogenous GR activity, $A_{2A}R$ agonist and antagonist were applied in the absence of GR activation by dexamethasone. Even in the absence of exogenous GR activation, $A_{2A}R$ increased luciferase expression (Figure 4.2 D) whereas $A_{2A}R$ blockade decreased it (Figure 4.2 E). This effect of $A_{2A}R$ was prevented in the presence of RU486 (Figure 4.2 F).

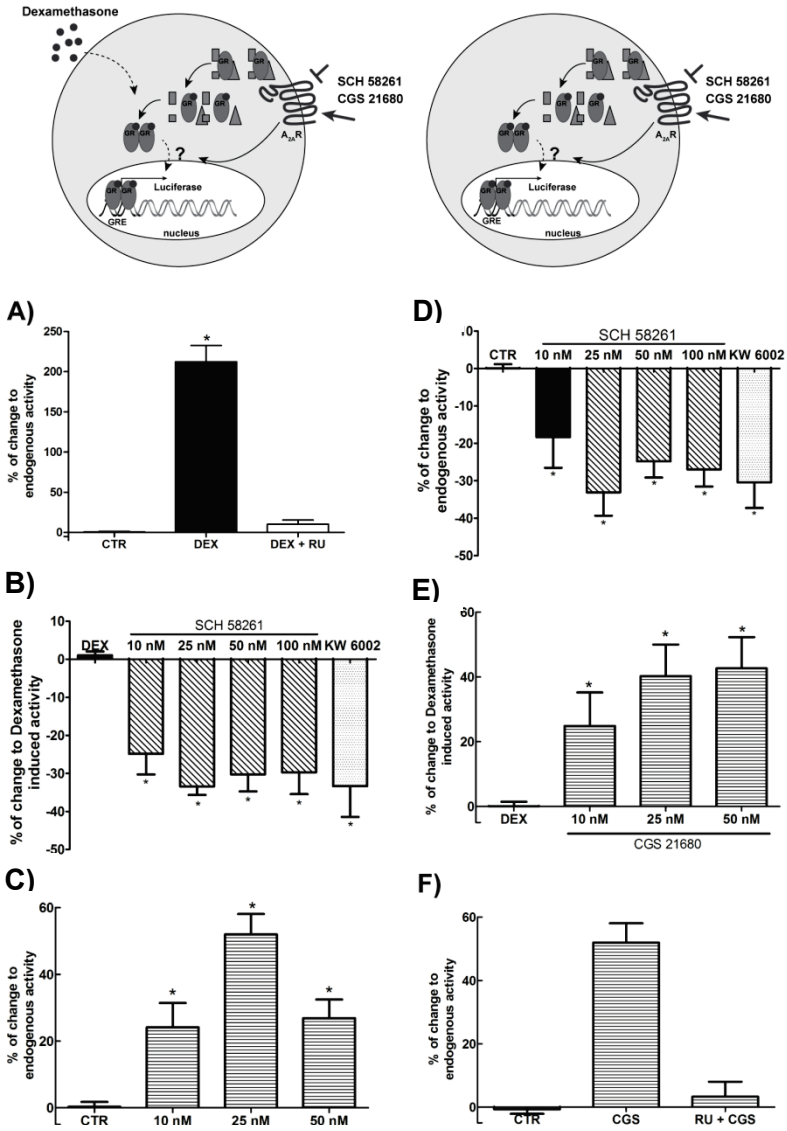

Figure 4.2: Adenosine A_{2A} receptors (A_{2A}R) modulate glucocorticoid response element (GRE) regulated luciferase expression in N1E115 cells. A) Dexamethasone induced increase in luciferase activity (n= 8-17) B) is decreased upon A_{2A}R blockade by two antagonists, SCH 58261 (10-100 nM) and KW 6002 (50 nM) (n=5-11) C) and increased upon direct A_{2A}R activation with CGS 21680 (10-50 nM) (n=3-9). Activation of A_{2A}R alone is sufficient to modulate endogenous GR transcriptional activity, D) A_{2A}R antagonist decreases luciferase activity (n=6-14) while E) A_{2A}R agonist increases it (n=3-11). F) A_{2A}R effects are prevented by the Glucocorticoid Receptor (GR) antagonist, RU 486 (100 nM, n=5-10). Results are presented as Mean ± SEM of n experiments. *P<0.05 compared to control, #P<0.05 compared with dexamethasone induced luciferase activity, calculated using a one-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test.

Adenosine $A_{2A}R$ promote GR nuclear translocation

To further explore the $A_{2A}R$ modulation of GR transcriptional activity and to extend the observed effects to neuronal cells, the nuclear translocation of the GR was evaluated in primary cortical neuronal cultures. Dexamethasone induced a significant enrichment of GR in the nuclear *versus* cytoplasmatic fraction along time, which was maximal after 90 minutes. This effect was totally prevented by $A_{2A}R$ blockade (Figure 4.3).

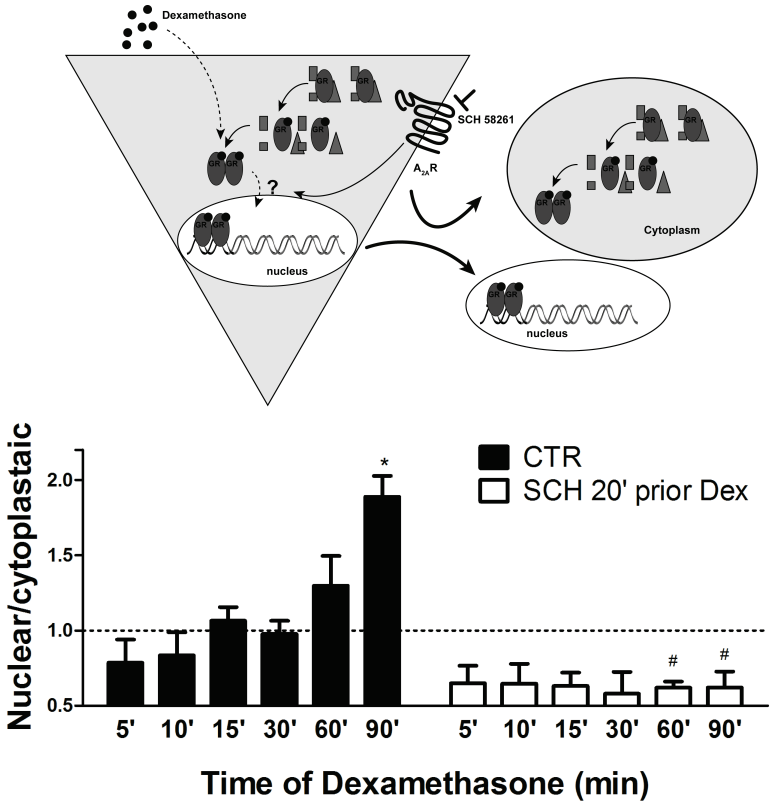

Figure 4.3: Adenosine A_{2A} receptors ($A_{2A}R$) promote dexamethasone induced Glucocorticoid Receptor (GR) translocation to the nucleus.
Left panel illustrates the gradual enrichment of GR in nuclear fraction of neuronal cultures over time of exposure to dexamethasone (100nM, n=2-4). This increase is totally prevented by blocking $A_{2A}R$ with SCH 58261 (50nM, in right panel). Results are presented as Mean ± SEM of n experiments. *P<0.05 compared to control, $^{\#}$P<0.05 compared with dexamethasone calculated using Two-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test.

A_{2A}R blockade rescues dexamethasone effects on synaptic plasticity

Different observations have shown that activation of GR can lead to impairments in memory and synaptic plasticity (Kerr *et al.*, 1994; Krugers *et al.*, 2005; Lee *et al.*, 2014; Wuppen *et al.*, 2010). We then evaluated if A_{2A}R impacted on GR effects in synaptic plasticity. Exposure of hippocampal slices to dexamethasone (100 nM) for 60 min, significantly abolished long-term potentiation (LTP) induced by high-frequency stimulation (55.4±5.2% in control *versus* 12.2±1.5% after 60 min of dexamethasone – Figure 4.4 A, C and D). This effect was prevented by the GR antagonist (RU486, figure 4.4 D). Blockade of A_{2A}R with SCH 58261 (50nM) prevented dexamethasone induced LTP deficits (53.0±5.3%, Figure 4.4 B, C and E), while having no effect in control (non-treated) slices (Figure 4.4 E).

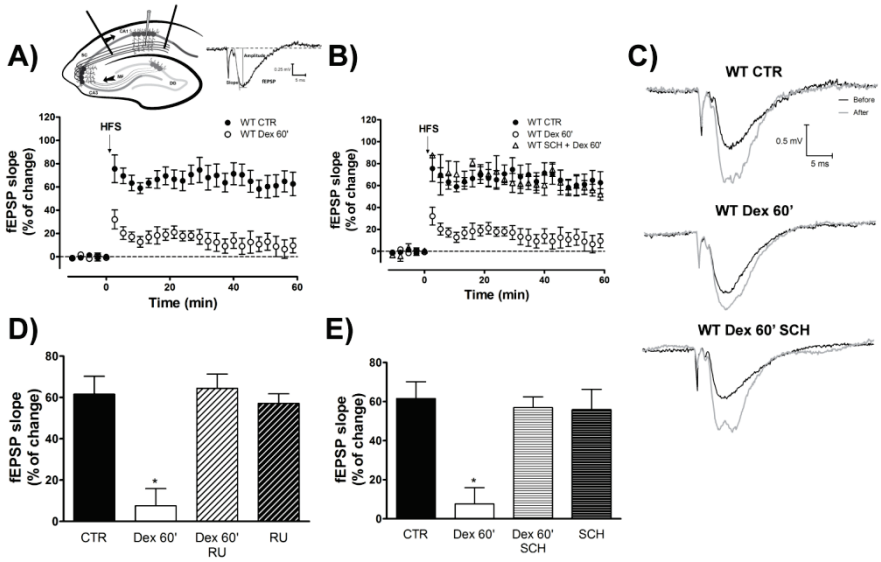

Figure 4.4: Dexamethasone induced deficits in synaptic plasticity are prevented by adenosine A_{2A} receptor (A_{2A}R) blockade.
High frequency stimulation (HFS: 100 HZ, 1s) was used to evaluate synaptic plasticity in hippocampal rat slices. A) and C) Incubation of slices with dexamethasone (100 nM) for 1h decreases LTP magnitude. B) and C) This effect is prevented by SCH58261 (50nM), n=4-8. D) and E) quantification of the Dexamethasone and SCH effect and the dependency on GR activation since it is prevented by RU486 (100 nM), n=3-8. Results are presented as Mean ± SEM of n experiments. *P<0.05 compared to control. #P<0.05 compared with dexamethasone calculated using a one-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test.

Overexpression of A_{2A}R increases susceptibility to dexamethasone

Since GR blockade was able to prevent dexamethasone effect, we then evaluated the effects of A_{2A}R overactivation on susceptibility to dexamethasone. Slices from wild-type (WT) and transgenic rats overexpressing A_{2A}R in the forebrain neurons (Tg(CaMKII-hA_{2A}R)) were incubated for 20 min with dexamethasone. Long-term potentiation (LTP) was induced 90 min later by high-frequency stimulation (HFS) (Krugers et al., 2005). Incubation with dexamethasone had no impact on LTP magnitude in wild-type animals (63.9±9.0% versus 58.0±9.0% Figure 4.5 A, B and E). However, in Tg(CAMKII-hA_{2A}R) animals, exposure to dexamethasone for 20' significantly decreased LTP magnitude (61.8±4.3% versus 30.3±5.5%, P<0.05, Figure 4.5 C, D and E), an effect that was prevented by the GR antagonist RU486 (figure 4.5 F).

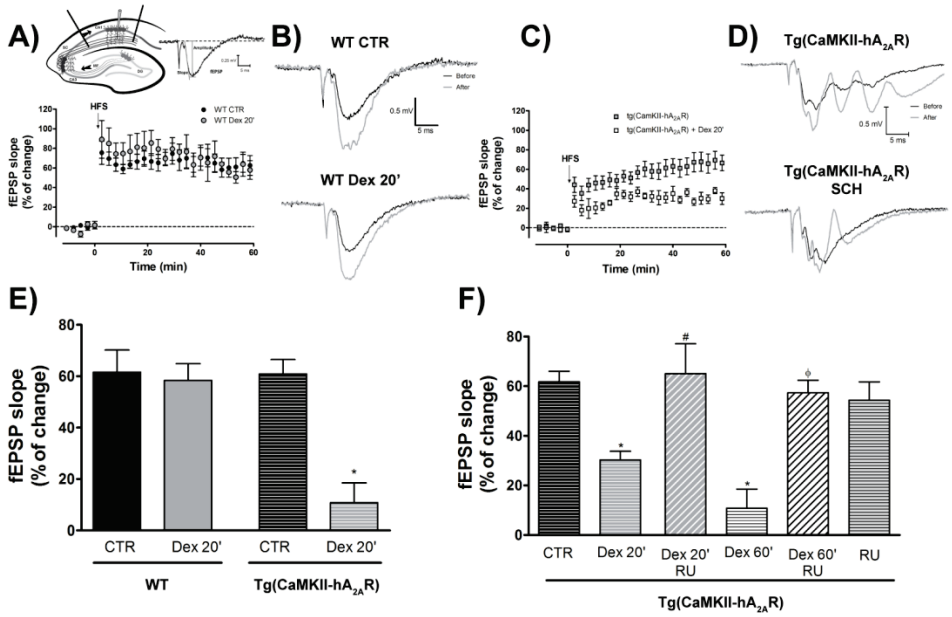

Figure 4.5: Adenosine A_{2A} receptor (A_{2A}R) overexpression increases LTP susceptibility to dexamethasone.

High frequency stimulation (HFS: 100HZ, 1s) was used to evaluate synaptic plasticity in hippocampal slices of WT and tg(CaMKII-hA_{2A}R) animals. A) and B) Incubation of slices with dexamethasone (100nM) for 20 minutes has no effect on LTP magnitude in WT animals (n=3/8), whereas C) and D) in Tg(CAMKII-hA2AR) animals a 20 minute incubation is sufficient induce a significant decrease in LTP magnitude (6-9). E) is the bar plot of the effects of dexamethasone and F) the prevention of the LTP reduction by the GR antagonist RU486 (3-9). Results are presented as Mean ± SEM of n experiments analysed using an unpaired Student t-test for comparisons within WT or Tg(CAMKII-hA2AR) and one-way ANOVA followed by a Bonferroni's multiple comparison *post hoc* test for drug effects. *P<0.05 compared to control, $^{\#}$P<0.05 compared with dexamethasone 20 min, $^{\phi}$P<0.05 compared with dexamethasone 60 min.

A_{2A}R blocking therapy promotes histone H3 acetylation but not DNA methylation of Nr3c1

We next investigated if an in vivo long-term therapy with an A_{2A}R antagonist might influence GR mRNA expression in the hippocampus. The treatment of WT rats for 1 month with the selective A_{2A}R antagonist, KW6002 [3 mg/kg/day, orally, the same dose shown to rescue GR expression in stressed rats; (Batalha *et al.*, 2013)], increased the hippocampal GR mRNA levels (Figure 4.6 A). To assess if epigenetic mechanisms could account for this A_{2A}R-mediated control of the *Nr3c1* gene encoding GR, we measured alterations in DNA methylation in the hippocampus at two different CpG islands within the *Nr3c1* gene (CpG147 and CpG11) in response to KW6002 treatment. We report that A_{2A}R blockade did not alter DNA methylation at either CpG island (Figure 4.6 B). Since increases in histone H3 acetylation are associated with increased transcription (McFarland *et al.*, 2012), we next measured alterations in histone H3 acetylation (H3K9K15ac2; AcH3). We found a significant increase of the association of AcH3 with the *Nr3c1* gene upon A_{2A}R blockade in the hippocampus, an association that seems specific to the *Nr3c1* gene since it did not occur for the control GAPDH gene (Figure 4.6 C). Thus, these AcH3 increases emerge as a possible mechanism underlying the increase of GR mRNA levels following A_{2A}R antagonist treatment (Figure 4.6A).

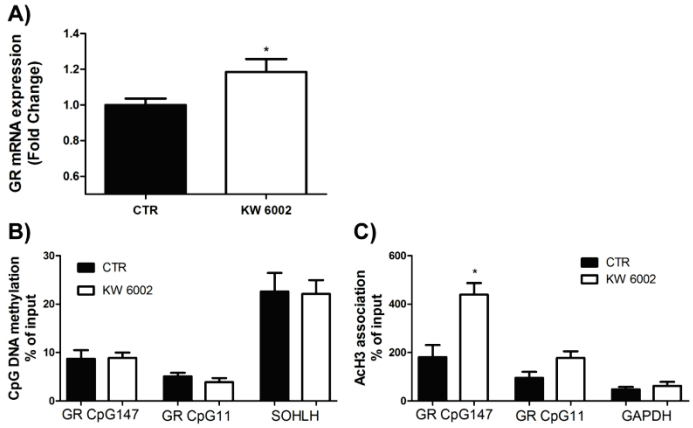

Figure 4.6 - Adenosine A_{2A} receptor blocking therapy promotes histone H3 acetylation
A) Increased mRNA levels encoding GR in rats treated with KW6002. B) Effect of KW6002 treatment on DNA methylation of two CpG islands (CpG147 and CpG11 according to UCSC genome browser) within the Nr3c1 gene in the rat hippocampus and C) effect of KW6002 treatment on histone H3 acetylation of the Nr3c1 gene. Results are the mean ± SEM of 4 to 7 experiments; (*)P<0.05, compared to wt.

Discussion

Our findings demonstrate for the first time that $A_{2A}R$ modulate GR transcriptional activity and nuclear localization, thereby critically affecting GR function and impacting in HPA-axis. Furthermore, the combined evidence that $A_{2A}R$ overexpression increases the susceptibility to GR agonists and that $A_{2A}R$ blockade prevents the deleterious effects of GR activation on synaptic plasticity, leads to the conclusion that $A_{2A}R$ play a critical role in the control of memory dysfunction by directly modulating GR expression and activation. Overall the present results provide the first evidence that $A_{2A}R$ do play a role in stress effects by directly modulating GR receptors, either in their actions or its expression. This novel $A_{2A}R$-GR interaction may have far-reaching implications in multiple pathologies that are alleviated by $A_{2A}R$ antagonists and where corticosteroids play a pivotal role.

Stress hormones and HPA-axis dysfunction has long been recognized as a critical feature underlying brain aging and pathology (Porter and Landfield, 1998). Indeed, altered cortisol levels are observed in post-traumatic stress syndrome or major depression (Gerritsen et al., 2011) and elevated salivary levels of cortisol were found to be correlated with poor cognitive function in a large study of humans aged 50–70 years (Lee et al., 2007). Increased glucocorticoid activity has a predominant impact in the hippocampus, which plays an inhibitory role in regulating the HPA axis (Jacobson and Sapolsky, 1991) and controls mood and memory (Fanselow and Dong, 2010). Thus, chronic exposure to glucocorticoids leads to cell death and hippocampal atrophy (Knoops *et al.*, 2010; Sapolsky and Meaney, 1986) and is associated with memory impairment in the elderly (Lupien et al., 1998). Accordingly, recent evidence supports a pivotal role of stress hormones in neurodegenerative diseases, namely in Alzheimer's disease (AD) (Rothman and Mattson, 2010). This is re-enforced by the following observations: 1) administration of the GR antagonist, RU486, exacerbates AD pathology (Baglietto-Vargas *et al.*, 2013); 2) repeated stress worsens AD-induced deficits (Joshi et al., 2012); 3) elevated cortisol levels are associated with a faster disease progression in AD (Csernansky et al., 2006); 4) systemic administration of

glucocorticoids or stress potentiate memory impairments, hippocampal damage, β-amyloid formation and Tau accumulation in transgenic AD mice (Chadwick *et al.*, 2011; Green *et al.*, 2006; Yao *et al.*, 2011).

Interestingly, in many pathologies where a dysfunction of the HPA-axis is present, aging included, there is also an upsurge of $A_{2A}R$ in the hippocampus (Batalha *et al.*, 2013; Lopes *et al.*, 1999b) and their blockade has proven to be beneficial (Arendash *et al.*, 2006; Batalha *et al.*, 2013). We now show that $A_{2A}R$ neuronal overexpression disturbs HPA-axis function and elevates plasma corticosterone levels. This provides a tentative connection between the adenosine neuromodulation system and the control of GR signaling, in agreement with previous reports that $A_{2A}R$ activation in a model of spinal cord injury mimicked the effects of GR activation in attenuating neuronal damage (Okonkwo *et al.*, 2006). Notably, we directly showed that $A_{2A}R$ modulate GR transcriptional activity, which is prevented by a GR antagonist. Furthermore, we showed that $A_{2A}R$ blockade was sufficient to prevent the gene expression-dependent deleterious impact of a GR mimetic (dexamethasone) on hippocampal synaptic plasticity and that the *in vivo* $A_{2A}R$ blockade affected the association of acetylated histone H3 with the *Nr3c1* gene encoding GR. Altogether, these findings provide the first direct demonstration that $A_{2A}R$ can directly control GR expression and suggest that $A_{2A}R$ are acting through modulation of GR. This tight $A_{2A}R$-GR interaction prompt a new perspective on how HPA-axis dysfunction may emerge, and also supports the therapeutic utility of $A_{2A}R$ antagonists as an important alternative to GR antagonists to reestablish HPA-axis dysfunction present in different clinical conditions (Batalha et al., 2013).

In fact, the therapeutic interest of using selective $A_{2A}R$ antagonists against multiple pathologies is increasing and $A_{2A}R$ antagonists have been recently approved as co-adjuvant therapy for Parkinson's disease (Chen *et al.*, 2013). Various studies also support the ability of caffeine and $A_{2A}R$ blockade to prevent memory impairment in multiple conditions (Cunha and Agostinho, 2010), and recent work revealed that $A_{2A}R$ antagonists can even have pro-cognitive effects (Borota et al., 2014). $A_{2A}R$ antagonism

was also proposed for the treatment of depression and anxiety-like disorders (Cunha et al., 2008b) in agreement with the decreased incidence of depression in individuals consuming caffeine (Lucas *et al.*, 2011). However, the lack of knowledge in regards to the mechanism of action of A_{2A}R antagonists compromised their acceptance for clinical use. The present report shows that A_{2A}R not only regulates HPA-axis function, but also directly modulates GR, which represent key findings for understanding the mechanisms by which A_{2A}R antagonism is effective, in agreement with our previous demonstration that A_{2A}R blockade overcame stress effects by reestablishing the HPA-axis and GR levels in the hippocampus (Batalha et al., 2013). These findings are critical, not only for the treatment of the memory dysfunction associated with psychopathologies, but can also be extended to aging and other circumstances in which the glucocorticoid response is impaired.

One aspect that is not clarified in the present study is the transducing pathways recruited by A_{2A}R to trigger GR/GRE transcriptional activity. This largely stems from the complexicity of A_{2A}R signaling (Fredholm *et al.*, 2007) and its engagement in numerous signalosome protein complexes (Keuerleber *et al.*, 2011). Although A_{2A}R can recruit multiple signaling pathways, the most common in the hippocampus are the c-AMP/PKA/CREB and the PKC and MAPK pathways (Ribeiro and Sebastiao, 2010). A possible hypothesis may be that modulation of CREB, which is known to interact with GR, and regulates gene expression through the activation of a non-canonic "composite" GRE (Diaz-Gallardo et al., 2010). Alternatively, a direct PKA modulation of GR binding to GRE is also possible, as previously described (Rangarajan et al., 1992). Two other broad questions emerged from our study and remain to be addressed, namely the mechanism by which A_{2A}R control the epigenome and the more global consequences related to this A_{2A}R-mediated control of transcriptional activity.

Finally there is an apparent paradox that rises from the present study: the fact that stress and A_{2A}R upregulation decrease GR levels in the hippocampus while simultaneously potentiating GR activation. The former is however reconciled by the fact that GR activation is an important pathway to decrease GR expression and activation effects

(Oakley and Cidlowski, 1993; Ramamoorthy and Cidlowski, 2013; Surjit *et al.*, 2011) particularly upon chronic exposure to glucocorticoids (Herman and Spencer, 1998), as in chronic stress. Therefore, A_{2A}R by increasing GR nuclear location and mediated transcription, are not only increasing the susceptibility to stress but also, through the same pathway, contributing to the downregulation of GR. Even if the levels of GR are lower, they become more prone to activation. If this is due to a higher affinity to DNA or to a faster kinetics is not yet known, however the data regarding epigenetic regulation seem to indicate that A_{2A}R might directly affect DNA availability to GR binding. Additionally, a redistribution of GR receptors in hippocampus was also observed after exposure to corticosteroids (Herman and Spencer, 1998) which may also account for a modified susceptibility upon higher circulating corticosterone levels.

Although the specific mechanisms for A_{2A}R/GR interaction are not yet clarified, the present results show that this has implications in the stress-mediated responses. A_{2A}R overexpression disrupts HPA-axis function and increases dexamethasone susceptibility of hippocampal slices. Conversely, their blockade prevented GR deleterious effects on synaptic plasticity. This suggests A_{2A}R as good therapeutic targets for the treatment of pathological conditions that impact on memory and synaptic plasticity. Moreover, it provides mechanistic data on how A_{2A}R blockade is effective, which is by suppressing GR mediated effects and reestablishing HPA-axis function.

In summary, our results provide the first evidence for an interaction between GR and A_{2A}R, revealing that A_{2A}R can modulate GR transcriptional activity and nuclear location. We also show that this impacts on GR mediated effects on synaptic plasticity and that, by modulating A_{2A}R, GR deleterious effects can be prevented. These observations strengthen the rationale for A_{2A}R blockade as therapeutic application, providing a novel transversal mechanistic possibility for its success.

Supplementary information

Figure S4.1: Construct used to generate tg(CaMKII-hA2AR) rats.

Chapter 5*: General discussion and conclusions*

The first evidence that adenosine A_{2A} receptors ($A_{2A}R$) could be implicated in stress comes from the observation that genetic deletion of $A_{2A}R$ affects anxiety and aggressive behaviour (Ledent et al., 1997). The subsequent report that $A_{2A}R$ antagonists applied prior and during a single episode of acute stress could prevent synaptic loss (Cunha et al., 2006), suggested that $A_{2A}R$ might have a far more important role in the control of stress response that the one considered so far. On the other hand increased hippocampal levels of $A_{2A}R$ were reported in multiple pathologies associated with cognitive decline, and their blockade was revealed to be beneficial (Batalha *et al.*, 2013; Cunha *et al.*, 2006) ref de AD e PD). However, it was still unclear the extent to which $A_{2A}R$ were involved in hippocampal dysfunction. The work developed in these four years and presented in the present dissertation, aimed at clarifying the involvement of $A_{2A}R$ in stress response, to reveal their importance in hippocampal function and to obtain data supporting their potential as therapeutic targets for multiple pathologies.

By taking advantage of a stress model that induces permanent changes in brain function (Sousa et al., 2014), it was possible to show that the stress effects can be reverted by blocking $A_{2A}R$. The administration of a selective $A_{2A}R$ antagonist (KW6002) reverted the long-lasting consequences of stress on spatial memory, synaptic plasticity and neuronal morphology in the hippocampus. These findings strongly suggest that $A_{2A}R$ overactivation may be the cause rather than the consequence of the herein reported hippocampal deficits resulting from chronic stress. This was later confirmed by overexpressing $A_{2A}R$ in the forebrain and evaluating the consequences for memory performance. Neuronal selective overexpression of $A_{2A}R$ in the forebrain neurons triggered hippocampal dysfunction, inducing alterations similar to what is observed upon aging: increased long term potentiation and decreased memory performance. The results have also shown that is a shift in $A_{2A}R$ signaling, whenever over-activated, that induces hippocampal dysfunction. The signaling mediated by $A_{2A}R$, either in stressed animals or upon overexpression, follows the same pattern as the one observed in aging and is reestablished together with the reestablishment of hippocampal function in stressed animals, when treated with the $A_{2A}R$ antagonist. These findings unequivocally

support the use of $A_{2A}R$ antagonists as novel therapeutic approach, not only to stress related pathologies but also to other in which hippocampal dysfunction and adenosine deregulation are present.

The reversion of stress-induced deficits was not restricted to the hippocampus. $A_{2A}R$ blockade was also able to reestablish the normal function of the stress response system. This indicated that $A_{2A}R$ might have a broader role in modulating stress effects. Additionally, $A_{2A}R$ overexpression under CaMKII promoter impaired the stress response system. These observations constitute the first evidence that $A_{2A}R$ can control hormonal circadian oscillation. However, if this is due to the reestablishment/impairment of hippocampal function, or instead, due to a direct effect of $A_{2A}R$, still needs to be clarified.

Since $A_{2A}R$ were shown to have an important effect in controlling the stress response, it was crucial to understand if there was a direct regulation of the glucocorticoid receptor (GR) effects. The data now presented reveal that $A_{2A}R$ activation increases GR transcriptional activity and nuclear location, thus affecting GR effects. More importantly, it was shown that $A_{2A}R$ can increase the susceptibility to GR agonists, and their blockade can prevent its effects, thus supporting the instrumental role of $A_{2A}R$ in triggering hippocampal dysfunction. Moreover, this novel interaction may explain the beneficial effects of the $A_{2A}R$ antagonists and their role in pathology. The $A_{2A}R$/GR loop may actually be the trigger for hippocampal dysfunction (Fig 5.1) and constitute the underlying mechanism for the effectiveness of $A_{2A}R$ blockade. In fact, increased plasmatic levels of stress hormones are observed in association with increased hippocampal $A_{2A}R$ levels, in aging, stress models or Alzheimer disease.

Finally, the results now presented not only strongly support the use of $A_{2A}R$ antagonists therapeutically, since they are effective in reverting already established deficits, but also provide original data on a novel transversal mechanism underlying pathology. It is important to note that until now, there were no scientific data explaining how $A_{2A}R$ blockade was beneficial and how $A_{2A}R$ were involved in pathology. Here a novel mechanism is revealed, not exclusive from stress, on how $A_{2A}R$, by modulating GR

actions, can induce damage (increased susceptibility to dexamethasone by $A_{2A}R$ overexpression) and how $A_{2A}R$ blockade can prevent GR effects (dexamethasone effect in synaptic plasticity is totally prevented by blocking $A_{2A}R$).

In summary, the present work unraveled a novel mechanism by which $A_{2A}R$ by modulating the stress receptors GR, can be instrumental in the development of hippocampal dysfunction. This knowledge is crucial for the therapeutic use of $A_{2A}R$ antagonists, but can also have important consequences for other biological circumstances in which an $A_{2A}R$ / GR crosstalk is involved.

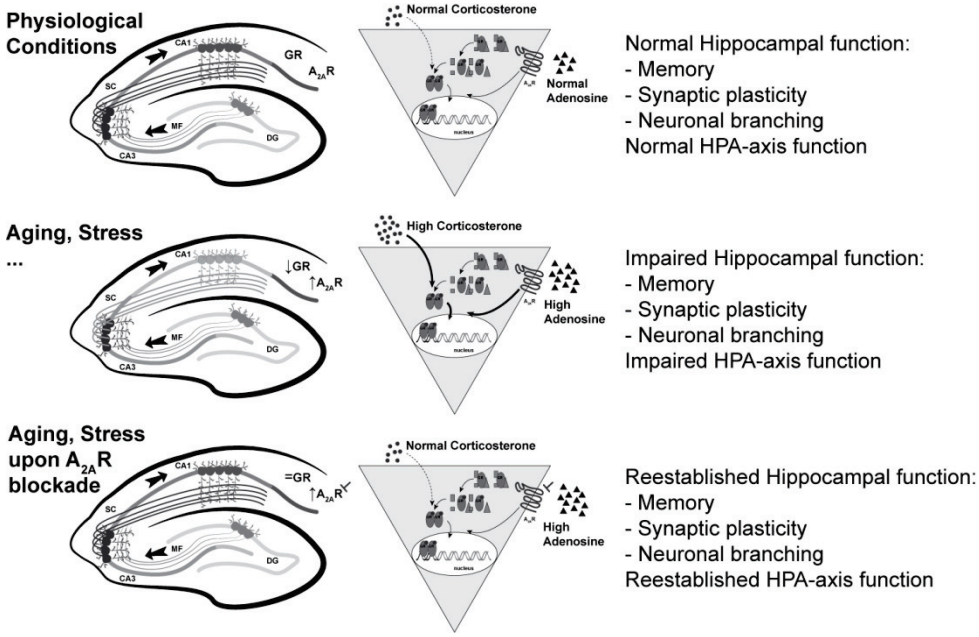

Figure 4.6 – Schematic representation of the interaction between $A_{2A}R$ and GR in pathological conditions and its outcome for hippocampal function.
Upon stress or aging there is an impairment of the HPA-axis and as a consequence, the levels of corticosterone are increased. This is accompanied by an increase is adenosine levels and upregulation of $A_{2A}R$, as well as decreased GR levels in the hippocampus. Is such situations, $A_{2A}R$ reinforce GR actions contributing to hippocampal dysfunction namely dendritic retraction, memory and synaptic plasticity impairments. In situations where $A_{2A}R$ are blocked, the hippocampal function is reestablished, resulting in restored HPA-axis function, memory performance and synaptic plasticity .

Future perspectives

Research aims at reaching to conclusions. However, most of the times it opens more doors that those that were closed and launches new questions.

These results indicate that $A_{2A}R$ overexpression/overactivation can indeed be the trigger to neuronal dysfunction and unravel a novel mechanism by which this might be occurring. However it is still to be proven if the $A_{2A}R$ deleterious effects are mediated by promoting GR actions. In my opinion, it is crucial to understand whether the effects of $A_{2A}R$ overexpression could be prevented if GR effects were blocked. Recent work has found beneficial effects of a GR antagonist in a triple transgenic model to Alzheimer disease (Baglietto-Vargas et al., 2013), with improvements in memory performance and decreased amyloid beta levels, similar to what had been previously observed with $A_{2A}R$ antagonism in other models (Arendash *et al.*, 2006; Espinosa *et al.*, 2013). However, it is not yet clear if:

1) It is the hippocampal dysfunction, driven by $A_{2A}R$ overexpression that impairs HPA-axis function? or;
2) It is the $A_{2A}R$ overexpression that impairs HPA-axis function and the consequent increased corticosterone levels that drive hippocampal dysfunction?

But then again, will science ever be able to answer what was born first: the egg or the chicken? It is nonetheless an indisputable fact that hippocampal $A_{2A}R$ overexpression and HPA-axis dysfunction are observed in association in aging, stress related pathologies and AD.

The increase in $A_{2A}R$ was accompanied by alterations in their signaling and impairments in hippocampal function. Moreover, the reestablishment of hippocampal function by $A_{2A}R$ blockade was observed together with a reestablishment of normal $A_{2A}R$ signaling. This strongly supports that a shift in $A_{2A}R$ signaling is driving the deleterious effect of $A_{2A}R$. However there are no data on the exact mechanism by which this might be happening. It is crucial to understand if the molecular entity of $A_{2A}R$ is different upon overexpression. The coupling of $A_{2A}R$ to different signaling pathways

can be due to post-transcriptional modifications, different membrane locations or different protein/protein interactions. Understanding this, will be a key finding to better target $A_{2A}R$ and to understand how their increase is driving pathology. In addition, if we take these evidences together with the modulatory action of $A_{2A}R$ upon GR, there is still a question that needs to be answered: is the $A_{2A}R$ modulation of GR effects the same in control conditions or upon overexpression; and whether there are specific $A_{2A}R$ that modulate GR. This can have important implications for other systems where $A_{2A}R$ interfere with GR and would constitute an enormous therapeutic advantage, since the molecular target would be exclusive of pathological situations reducing unwanted side effects.

The role of $A_{2A}R$ in controlling GR actions has important consequences for the nervous system. However GR have much broader actions in different systems. GR can mediate important immunosuppressive actions, regulate metabolism and affect cell division and differentiation (Kadmiel and Cidlowski, 2013). Adenosine is present in all cells and is produced particularly in situations of high metabolic demand or oxygen deprivation and has also important roles in inflammation with immunosuppressive actions being mediated by $A_{2A}R$ activation (Hasko and Cronstein, 2013). Surprisingly, there are no data on how adenosine might modulate GR actions in other contexts. The expansion of this interaction to the immune response, cell proliferation, tumor response and other cellular functions that imply GR or corticosteroids use in therapeutics, could have an enormous clinical impact.

Finally, the divergence between $A_{2A}R$ activation and $A_{2A}R$ blockade in different disease contexts and physiological systems has been discussed. $A_{2A}R$ have been shown to mediate immunosuppressive actions in the periphery (Hasko and Cronstein, 2013) and to have paradoxical pro-inflammatory and anti-inflammatory actions in the central nervous system (Dai and Zhou, 2011). Once again these clues suggest the existence of different signaling pathways, different targets and different effects mediated by $A_{2A}R$.

Acknowledgments

Não poderia terminar sem antes agradecer a todos aqueles que permitiram a realização desta tese, no laboratório e fora dele, em Portugal ou no mundo.

À minha orientadora Luísa, obrigado por me ensinares a dar os primeiros passos no mundo da ciência, por me dares liberdade para pensar e crescer, por me incentivares a ir mais longe e a procurar sempre uma resposta. Obrigado pela boa disposição e companheirismo, pela diversão. Por todo o conhecimento que transmitiste e que permitiu que este trabalho se realizasse.

Obrigado aos Professores Ana Sebastião e Joaquim Alexandre Ribeiro por me terem recebido no seu laboratório quando iniciei esta caminhada.

Thank you, to my advisors abroad. David, for your contribution ever since I met you here in Lisbon, even before working with you. Your ideas are always fresh and you always have a new and different perspective to give us. Ghaz, thank you for your friendship, help and all the knowledge you gave me. It is a shame that US is so far away from Europe, it was a pleasure to work with you.

Obrigado a todos os colegas que me acompanharam ao longo destes anos, nas longas horas de experiência, nos bons e nos maus resultados. À Daniela pelas noites passadas no laboratório e a companhia no microscópio em Braga. À Natália, ao Vasco, à Sofia, à Sandra e à Raquel que me ajudaram sendo um modelo e me passaram muito do conhecimento que hoje tenho: a Raquel na electro, a Natália nos blots e ao Vasco no comportamento animal. Sandra e Sofia um modelo, sempre. Ao Jorge, que foi fundamental nos primeiros passos deste trabalho, mas que, mais que isso, permitiu que eu hoje seja quem sou, ao levar-me à Escola da Paz. E claro ao Sr. João por toda a ajuda com os animais.

Um obrigado especial à Júlia, que de colega passou a amiga, de amiga a irmã, obrigado pelo apoio e a amizade no laboratório e fora dele. Sabes o quanto foste, e és importante.

A todos os "mais pequenos" que deram nova alma e nova vida ao laboratório, que nos mantiveram mais jovens, Mariana, Filipe, Noémia, Catarina, Armando, Sara, e Clara.

À Rita, ao André e ao Diogo que iniciaram comigo esta caminhada de doutoramento com quem partilhei muitos congressos, nunca esquecerei a nossa primeira FENS!

Joana e Diana obrigado!! Sem vocês, o lab era uma seca!! Obrigado pelas discussões pelas experiências, por me aturarem, chata e exigente, por me ajudarem a crescer. Mas acima de tudo obrigado pela amizade e companheirismo, pelas piadas, pela diversão, pela alegria, por fazerem do Lopes lab um sítio onde é divertido trabalhar. Rui, também estás incluído nesta categoria!!

Nas minhas incursões ao estrangeiro há também algumas pessoas a quem não posso deixar de agradecer. Obrigado à Ana Tellechea e à Inês Lima que me ajudaram quando cheguei aos EUA e permitirem que lá encontrasse um pouco de casa portuguesa. Ana, obrigado por todas as conversas e pela amiga que te tornaste e por todos os amigos que me apresentaste. In France, thank you to Antoine and Cyril that helped me in the lab and thought me all about PCR. Sophie I could not forget you, for all the help and friendship, if it was not for you, Lille wouldn't be the same. Carina não me esqueci de ti, obrigado pelo apoio e amizade foste e és espetacular!

E porque os últimos são sempre os primeiros, um obrigado à minha família. À minha mãe e ao meu pai a quem o trabalho roubou tanto tempo. Acho que frequentemente pensaram que a minha casa era o laboratório, na verdade não deixa de ser. À minha irmã que além de tudo o resto foi preciosa em cada artigo, em cada figura com as suas ilustrações espectaculares. Mesmo que por vezes pareça que me esqueço de vós, estão sempre no meu coração.

Mas afinal, ao longo destes anos acabei por ganhar uma nova família, a comunidade em Portugal e no mundo. Em Portugal, àqueles que me permitiram ter uma vida fora do laboratório e ter vontade de viver, Joana, Rita, Ana Filipa. São especiais porque sabem o que mudei desde que vos conheci, o que crescemos, o que lutámos, obrigado por estarem sempre lá! As aventuras foram muitas mas serão ainda mais. Cláudio, Pena, Telmo, Maria, Miguel, obrigado por todos os momentos de alegria, partilha e apoio. À Daniela que me lembrou sempre que vale a pena lutar, vale a pena acreditar e que os imprevistos são, no fundo, oportunidades. To my friends in Belgium, Spain, Germany that together in Albany revealed to me a new dimension of life. Those days were so important in the way I see the world today that also changed drastically the way I foresee the lab and science. To Amra, Sarah, Tobi, Alba, among many, many others. Also from the community, I have to thank to Kerri, from Boston, for your friendship and teaching.

Não menos importantes e porque muitas vezes foram elas que me mantiveram de pé quando tinha vontade de desistir, porque me acompanharam desde o inicio, um grande obrigado às crianças da Escola da Paz. Elas fazem com que valha a pena.

References

Aisa, B., Elizalde, N., Tordera, R., Lasheras, B., Del Rio, J. and Ramirez, M. J. (2009) Effects of neonatal stress on markers of synaptic plasticity in the hippocampus: implications for spatial memory. *Hippocampus* **19,** 1222-1231.

Aisa, B., Tordera, R., Lasheras, B., Del Rio, J. and Ramirez, M. J. (2007) Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology* **32,** 256-266.

Allen, C. and Kendall, J. W. (1967) Maturation of the circadian rhythm of plasma corticosterone in the rat. *Endocrinology* **80,** 926-930.

Amaral, D. G. and Dent, J. A. (1981) Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. *J Comp Neurol* **195,** 51-86.

Anand-Srivastava, M. B., Cantin, M. and Gutkowska, J. (1989) Adenosine regulates the release of adrenocorticotropic hormone (ACTH) from cultured anterior pituitary cells. *Mol Cell Biochem* **89,** 21-28.

Andrasfalvy, B. K., Smith, M. A., Borchardt, T., Sprengel, R. and Magee, J. C. (2003) Impaired regulation of synaptic strength in hippocampal neurons from GluR1-deficient mice. *J Physiol* **552,** 35-45.

Anisman, H., Zaharia, M. D., Meaney, M. J. and Merali, Z. (1998) Do early-life events permanently alter behavioral and hormonal responses to stressors? *Int J Dev Neurosci* **16,** 149-164.

Arch, J. R. and Newsholme, E. A. (1978) The control of the metabolism and the hormonal role of adenosine. *Essays Biochem* **14,** 82-123.

Arendash, G. W. and Cao, C. (2010) Caffeine and coffee as therapeutics against Alzheimer's disease. *J Alzheimers Dis* **20 Suppl 1,** S117-126.

Arendash, G. W., Schleif, W., Rezai-Zadeh, K., Jackson, E. K., Zacharia, L. C., Cracchiolo, J. R., Shippy, D. and Tan, J. (2006) Caffeine protects Alzheimer's

mice against cognitive impairment and reduces brain beta-amyloid production. *Neuroscience* **142,** 941-952.

Assaife-Lopes, N., Sousa, V. C., Pereira, D. B., Ribeiro, J. A. and Sebastiao, A. M. (2013) Regulation of TrkB receptor translocation to lipid rafts by adenosine A receptors and its functional implications for BDNF-induced regulation of synaptic plasticity. *Purinergic Signal*.

Baglietto-Vargas, D., Medeiros, R., Martinez-Coria, H., LaFerla, F. M. and Green, K. N. (2013) Mifepristone alters amyloid precursor protein processing to preclude amyloid beta and also reduces tau pathology. *Biol Psychiatry* **74,** 357-366.

Bakshi, V. P. and Kalin, N. H. (2000) Corticotropin-releasing hormone and animal models of anxiety: gene-environment interactions. *Biol Psychiatry* **48,** 1175-1198.

Banihashemi, L., O'Neill, E. J. and Rinaman, L. (2011) Central neural responses to restraint stress are altered in rats with an early life history of repeated brief maternal separation. *Neuroscience* **192,** 413-428.

Barnes, C. A. (1988) Aging and the physiology of spatial memory. *Neurobiol Aging* **9,** 563-568.

Batalha, V. L., Hamdane, M., Sebastião, A. M., Buée, L., Blum, D. and Lopes, L. V. (2011) Modulation of glucocorticoid receptor (GR) transcriptional activity by adenosine A2A receptors: consequences for stress response. In: *Champalimaud Neuroscience Symposium*: Lisbon.

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

Belanoff, J. K., Gross, K., Yager, A. and Schatzberg, A. F. (2001a) Corticosteroids and cognition. *J Psychiatr Res* **35,** 127-145.

Belanoff, J. K., Kalehzan, M., Sund, B., Fleming Ficek, S. K. and Schatzberg, A. F. (2001b) Cortisol activity and cognitive changes in psychotic major depression. *Am J Psychiatry* **158,** 1612-1616.

Bessa, J. M., Ferreira, D., Melo, I., Marques, F., Cerqueira, J. J., Palha, J. A., Almeida, O. F. and Sousa, N. (2009) The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol Psychiatry* **14,** 764-773, 739.

Bliss, T. V. and Lomo, T. (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol* **232,** 331-356.

Blum, D., Galas, M. C., Pintor, A., Brouillet, E., Ledent, C., Muller, C. E., Bantubungi, K., Galluzzo, M., Gall, D., Cuvelier, L., Rolland, A. S., Popoli, P. and Schiffmann, S. N. (2003) A dual role of adenosine A2A receptors in 3-nitropropionic acid-induced striatal lesions: implications for the neuroprotective potential of A2A antagonists. *J Neurosci* **23,** 5361-5369.

Borota, D., Murray, E., Keceli, G., Chang, A., Watabe, J. M., Ly, M., Toscano, J. P. and Yassa, M. A. (2014) Post-study caffeine administration enhances memory consolidation in humans. *Nat Neurosci* **17,** 201-203.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72,** 248-254.

Burke, S. N. and Barnes, C. A. (2006) Neural plasticity in the ageing brain. *Nat Rev Neurosci* **7,** 30-40.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. and Wittwer, C. T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55,** 611-622.

Caldji, C., Diorio, J. and Meaney, M. J. (2000a) Variations in maternal care in infancy regulate the development of stress reactivity. *Biol Psychiatry* **48,** 1164-1174.

Caldji, C., Francis, D., Sharma, S., Plotsky, P. M. and Meaney, M. J. (2000b) 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,** 219-229.

Canas, P. M., Porciuncula, L. O., Cunha, G. M., Silva, C. G., Machado, N. J., Oliveira, J. M., Oliveira, C. R. and Cunha, R. A. (2009) Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by beta-amyloid peptides via p38 mitogen-activated protein kinase pathway. *J Neurosci* **29,** 14741-14751.

Cantarero, G., Lloyd, A. and Celnik, P. (2013) Reversal of long-term potentiation-like plasticity processes after motor learning disrupts skill retention. *J Neurosci* **33,** 12862-12869.

Chadwick, W., Mitchell, N., Caroll, J., Zhou, Y., Park, S. S., Wang, L., Becker, K. G., Zhang, Y., Lehrmann, E., Wood, W. H., 3rd, Martin, B. and Maudsley, S. (2011) Amitriptyline-mediated cognitive enhancement in aged 3xTg Alzheimer's disease mice is associated with neurogenesis and neurotrophic activity. *PLoS One* **6,** e21660.

Champagne, F. A., Francis, D. D., Mar, A. and Meaney, M. J. (2003) Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol Behav* **79,** 359-371.

Chau, A., Rose, J. C. and Koos, B. J. (1999) Adenosine modulates corticotropin and cortisol release during hypoxia in fetal sheep. *Am J Obstet Gynecol* **180,** 1272-1277.

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

Chen, Y. C., Huang, S. H. and Wang, S. M. (2008) Adenosine-stimulated adrenal steroidogenesis involves the adenosine A2A and A2B receptors and the Janus kinase 2-mitogen-activated protein kinase kinase-extracellular signal-regulated kinase signaling pathway. *Int J Biochem Cell Biol* **40,** 2815-2825.

Cognato, G. P., Agostinho, P. M., Hockemeyer, J., Muller, C. E., Souza, D. O. and Cunha, R. A. (2010) Caffeine and an adenosine A(2A) receptor antagonist prevent memory impairment and synaptotoxicity in adult rats triggered by a convulsive episode in early life. *J Neurochem* **112,** 453-462.

Coogan, A. N., Schutova, B., Husung, S., Furczyk, K., Baune, B. T., Kropp, P., Hassler, F. and Thome, J. (2013) The circadian system in Alzheimer's disease: disturbances, mechanisms, and opportunities. *Biol Psychiatry* **74,** 333-339.

Costenla, A. R., Diogenes, M. J., Canas, P. M., Rodrigues, R. J., Nogueira, C., Maroco, J., Agostinho, P. M., Ribeiro, J. A., Cunha, R. A. and de Mendonca, A. (2011) Enhanced role of adenosine A(2A) receptors in the modulation of LTP in the rat hippocampus upon ageing. *Eur J Neurosci* **34,** 12-21.

Crawley, J. N. (1985) Exploratory behavior models of anxiety in mice. *Neurosci Biobehav Rev* **9,** 37-44.

Cristovao-Ferreira, S., Navarro, G., Brugarolas, M., Perez-Capote, K., Vaz, S. H., Fattorini, G., Conti, F., Lluis, C., Ribeiro, J. A., McCormick, P. J., Casado, V., Franco, R. and Sebastiao, A. M. (2013) A1R-A2AR heteromers coupled to Gs and G i/0 proteins modulate GABA transport into astrocytes. *Purinergic Signal* **9,** 433-449.

Cristóvão-Ferreira, S., Vaz, S. H., Ribeiro, J. A. and Sebastiao, A. M. (2009) Adenosine A2A receptors enhance GABA transport into nerve terminals by restraining PKC inhibition of GAT-1. *J Neurochem* **109,** 336-347.

Csernansky, J. G., Dong, H., Fagan, A. M., Wang, L., Xiong, C., Holtzman, D. M. and Morris, J. C. (2006) Plasma cortisol and progression of dementia in subjects with Alzheimer-type dementia. *Am J Psychiatry* **163,** 2164-2169.

Cunha, G. M., Canas, P. M., Melo, C. S., Hockemeyer, J., Muller, C. E., Oliveira, C. R. and Cunha, R. A. (2008a) Adenosine A2A receptor blockade prevents memory dysfunction caused by beta-amyloid peptides but not by scopolamine or MK-801. *Exp Neurol* **210,** 776-781.

Cunha, G. M., Canas, P. M., Oliveira, C. R. and Cunha, R. A. (2006) Increased density and synapto-protective effect of adenosine A2A receptors upon sub-chronic restraint stress. *Neuroscience* **141,** 1775-1781.

Cunha, R. A. (2001) Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem Int* **38,** 107-125.

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

Cunha, R. A. and Agostinho, P. M. (2010) Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline. *J Alzheimers Dis* **20 Suppl 1,** S95-116.

Cunha, R. A., Constantino, M. C., Sebastiao, A. M. and Ribeiro, J. A. (1995) Modification of A1 and A2a adenosine receptor binding in aged striatum, hippocampus and cortex of the rat. *Neuroreport* **6,** 1583-1588.

Cunha, R. A., Ferre, S., Vaugeois, J. M. and Chen, J. F. (2008b) Potential therapeutic interest of adenosine A2A receptors in psychiatric disorders. *Curr Pharm Des* **14,** 1512-1524.

Cunha, R. A., Johansson, B., van der Ploeg, I., Sebastiao, A. M., Ribeiro, J. A. and Fredholm, B. B. (1994a) Evidence for functionally important adenosine A2a receptors in the rat hippocampus. *Brain Res* **649,** 208-216.

Cunha, R. A., Milusheva, E., Vizi, E. S., Ribeiro, J. A. and Sebastiao, A. M. (1994b) 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,** 207-214.

Cunha, R. A. and Ribeiro, J. A. (2000) Purinergic modulation of [(3)H]GABA release from rat hippocampal nerve terminals. *Neuropharmacology* **39,** 1156-1167.

D'Hooge, R. and De Deyn, P. P. (2001) Applications of the Morris water maze in the study of learning and memory. *Brain Res Brain Res Rev* **36,** 60-90.

Dagyte, G., Van der Zee, E. A., Postema, F., Luiten, P. G., Den Boer, J. A., Trentani, A. and Meerlo, P. (2009) Chronic but not acute foot-shock stress leads to temporary suppression of cell proliferation in rat hippocampus. *Neuroscience* **162,** 904-913.

Dai, S. S. and Zhou, Y. G. (2011) Adenosine 2A receptor: a crucial neuromodulator with bidirectional effect in neuroinflammation and brain injury. *Rev Neurosci* **22,** 231-239.

Dale, N. and Frenguelli, B. G. (2009) Release of adenosine and ATP during ischemia and epilepsy. *Curr Neuropharmacol* **7,** 160-179.

Dall'Igna, O. P., Fett, P., Gomes, M. W., Souza, D. O., Cunha, R. A. and Lara, D. R. (2007) Caffeine and adenosine A(2a) receptor antagonists prevent beta-amyloid (25-35)-induced cognitive deficits in mice. *Exp Neurol* **203,** 241-245.

Daniels, W. M., Pietersen, C. Y., Carstens, M. E. and Stein, D. J. (2004) 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.

de Kloet, E. R., Oitzl, M. S. and Joels, M. (1999) Stress and cognition: are corticosteroids good or bad guys? *Trends in neurosciences* **22,** 422-426.

Dellu, F., Contarino, A., Simon, H., Koob, G. F. and Gold, L. H. (2000) Genetic differences in response to novelty and spatial memory using a two-trial recognition task in mice. *Neurobiol Learn Mem* **73,** 31-48.

Dellu, F., Fauchey, V., Le Moal, M. and Simon, H. (1997) Extension of a new two-trial memory task in the rat: influence of environmental context on recognition processes. *Neurobiol Learn Mem* **67,** 112-120.

Dellu, F., Mayo, W., Cherkaoui, J., Le Moal, M. and Simon, H. (1992) A two-trial memory task with automated recording: study in young and aged rats. *Brain Res* **588,** 132-139.

Diamond, D. M., Park, C. R., Heman, K. L. and Rose, G. M. (1999) Exposing rats to a predator impairs spatial working memory in the radial arm water maze. *Hippocampus* **9,** 542-552.

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

Diaz-Gallardo, M. Y., Cote-Velez, A., Charli, J. L. and Joseph-Bravo, P. (2010) A rapid interference between glucocorticoids and cAMP-activated signalling in hypothalamic neurones prevents binding of phosphorylated cAMP response element binding protein and glucocorticoid receptor at the CRE-Like and composite GRE sites of thyrotrophin-releasing hormone gene promoter. *J Neuroendocrinol* **22,** 282-293.

Diogenes, M. J., Assaife-Lopes, N., Pinto-Duarte, A., Ribeiro, J. A. and Sebastiao, A. M. (2007) Influence of age on BDNF modulation of hippocampal synaptic transmission: interplay with adenosine A2A receptors. *Hippocampus* **17,** 577-585.

Diogenes, M. J., Costenla, A. R., Lopes, L. V., Jeronimo-Santos, A., Sousa, V. C., Fontinha, B. M., Ribeiro, J. A. and Sebastiao, A. M. (2011) Enhancement of LTP in aged rats is dependent on endogenous BDNF. *Neuropsychopharmacology* **36,** 1823-1836.

Diogenes, M. J., Fernandes, C. C., Sebastiao, A. M. and Ribeiro, J. A. (2004) Activation of adenosine A2A receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices. *J Neurosci* **24,** 2905-2913.

Dunwiddie, T. V. and Diao, L. (1994) Extracellular adenosine concentrations in hippocampal brain slices and the tonic inhibitory modulation of evoked excitatory responses. *J Pharmacol Exp Ther* **268,** 537-545.

Espinosa, J., Rocha, A., Nunes, F., Costa, M. S., Schein, V., Kazlauckas, V., Kalinine, E., Souza, D. O., Cunha, R. A. and Porciuncula, L. O. (2013) Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A2A receptors upregulation in the hippocampus of a rat model of sporadic dementia. *J Alzheimers Dis* **34,** 509-518.

Fanselow, M. S. and Dong, H. W. (2010) Are the dorsal and ventral hippocampus functionally distinct structures? *Neuron* **65,** 7-19.

Fedulov, V., Rex, C. S., Simmons, D. A., Palmer, L., Gall, C. M. and Lynch, G. (2007) Evidence that long-term potentiation occurs within individual hippocampal synapses during learning. *J Neurosci* **27,** 8031-8039.

Fernandez, M., Svenningsson, P. and Fredholm, B. B. (1996) Adaptive changes in adenosine receptors following long-term treatment with the adenosine receptor agonist R-phenylisopropyl adenosine. *Life Sci* **58,** 769-776.

File, S. E., Mabbutt, P. S. and Hitchcott, P. K. (1990) Characterisation of the phenomenon of "one-trial tolerance" to the anxiolytic effect of chlordiazepoxide in the elevated plus-maze. *Psychopharmacology (Berl)* **102,** 98-101.

File, S. E., Zangrossi, H., Jr., Sanders, F. L. and Mabbutt, P. S. (1994) Raised corticosterone in the rat after exposure to the elevated plus-maze. *Psychopharmacology (Berl)* **113,** 543-546.

Fitzgerald, L. W. and Dokla, C. P. (1989) Morris water task impairment and hypoactivity following cysteamine-induced reductions of somatostatin-like immunoreactivity. *Brain Res* **505,** 246-250.

Foy, M. R., Stanton, M. E., Levine, S. and Thompson, R. F. (1987) Behavioral stress impairs long-term potentiation in rodent hippocampus. *Behav Neural Biol* **48,** 138-149.

Fredholm, B. B. (1997) Adenosine and neuroprotection. *Int Rev Neurobiol* **40,** 259-280.

Fredholm, B. B., Chern, Y., Franco, R. and Sitkovsky, M. (2007) Aspects of the general biology of adenosine A2A signaling. *Prog Neurobiol* **83,** 263-276.

Gabrieli, J. D. (1992) Neurology of memory. *Curr Opin Neurol Neurosurg* **5,** 83-87.

Gerritsen, L., Comijs, H. C., Deeg, D. J., Penninx, B. W. and Geerlings, M. I. (2011) Salivary cortisol, APOE-epsilon4 allele and cognitive decline in a prospective study of older persons. *Neurobiol Aging* **32,** 1615-1625.

Gibb, R. and Kolb, B. (1998) A method for vibratome sectioning of Golgi-Cox stained whole rat brain. *J Neurosci Methods* **79,** 1-4.

Gimenez-Llort, L., Schiffmann, S. N., Shmidt, T., Canela, L., Camon, L., Wassholm, M., Canals, M., Terasmaa, A., Fernandez-Teruel, A., Tobena, A., Popova, E., Ferre, S., Agnati, L., Ciruela, F., Martinez, E., Scheel-Kruger, J., Lluis, C., Franco, R., Fuxe, K. and Bader, M. (2007) Working memory deficits in transgenic rats overexpressing human adenosine A2A receptors in the brain. *Neurobiol Learn Mem* **87,** 42-56.

Glaser, E. M. and Van der Loos, H. (1981) Analysis of thick brain sections by obverse-reverse computer microscopy: application of a new, high clarity Golgi-Nissl stain. *J Neurosci Methods* **4,** 117-125.

Golgi, C. (1989) On the structure of nerve cells. 1898. *J Microsc* **155,** 3-7.

Green, K. N., Billings, L. M., Roozendaal, B., McGaugh, J. L. and LaFerla, F. M. (2006) Glucocorticoids increase amyloid-beta and tau pathology in a mouse model of Alzheimer's disease. *J Neurosci* **26,** 9047-9056.

Gruen, R. J., Wenberg, K., Elahi, R. and Friedhoff, A. J. (1995) Alterations in GABAA receptor binding in the prefrontal cortex following exposure to chronic stress. *Brain Res* **684,** 112-114.

Gundersen, H. J., Bagger, P., Bendtsen, T. F., Evans, S. M., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., Nyengaard, J. R., Pakkenberg, B. and et al. (1988) The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* **96,** 857-881.

Hall, C. S. (1934) Emotional behavior in the rat: I. Defecation and urination as measures of individual differences in emotionality. *J. Comp. Psychol.* **18,,** 385–403.

Handley, S. L. M., S. (1984) Effects of a-adrenoreceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. *NaunynSchmeideberg's Arch. Pharmacol.* **327,** 1–5.

Hasko, G. and Cronstein, B. (2013) Regulation of inflammation by adenosine. *Front Immunol* **4,** 85.

Hauser, R. A., Hubble, J. P. and Truong, D. D. (2003) Randomized trial of the adenosine A(2A) receptor antagonist istradefylline in advanced PD. *Neurology* **61,** 297-303.

Heim, C. and Nemeroff, C. B. (2001) The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biol Psychiatry* **49,** 1023-1039.

Heim, C., Owens, M. J., Plotsky, P. M. and Nemeroff, C. B. (1997) 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,** 185-192.

Herman, J. P. and Cullinan, W. E. (1997) Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* **20,** 78-84.

Herman, J. P. and Spencer, R. (1998) Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression in vivo. *J Neurosci* **18,** 7462-7473.

Hockemeyer, J., Burbiel, J. C. and Muller, C. E. (2004) Multigram-scale syntheses, stability, and photoreactions of A2A adenosine receptor antagonists with 8-styrylxanthine structure: potential drugs for Parkinson's disease. *J Org Chem* **69,** 3308-3318.

Hogg, S. (1996) A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacol Biochem Behav* **54,** 21-30.

Jacobson, L. and Sapolsky, R. (1991) The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocr Rev* **12,** 118-134.

Janusz, C. A. and Berman, R. F. (1992) The A2-selective adenosine analog, CGS 21680, depresses locomotor activity but does not block amygdala kindled seizures in rats. *Neurosci Lett* **141,** 247-250.

Jarvis, M. F. and Williams, M. (1989) Direct autoradiographic localization of adenosine A2 receptors in the rat brain using the A2-selective agonist, [3H]CGS 21680. *European Journal of Pharmacology* **168,** 243-246.

Joels, M. (2006) Corticosteroid effects in the brain: U-shape it. *Trends Pharmacol Sci* **27,** 244-250.

Joels, M. (2008) Functional actions of corticosteroids in the hippocampus. *European Journal of Pharmacology* **583,** 312-321.

Johnson, E. O., Kamilaris, T. C., Chrousos, G. P. and Gold, P. W. (1992) Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. *Neurosci Biobehav Rev* **16,** 115-130.

Joshi, Y. B., Chu, J. and Pratico, D. (2012) Stress hormone leads to memory deficits and altered tau phosphorylation in a model of Alzheimer's disease. *J Alzheimers Dis* **31,** 167-176.

Kadmiel, M. and Cidlowski, J. A. (2013) Glucocorticoid receptor signaling in health and disease. *Trends Pharmacol Sci* **34,** 518-530.

Kallai, J., Makany, T., Karadi, K. and Jacobs, W. J. (2005) Spatial orientation strategies in Morris-type virtual water task for humans. *Behav Brain Res* **159,** 187-196.

Kalueff, A. V. and Tuohimaa, P. (2004) Experimental modeling of anxiety and depression. *Acta Neurobiol Exp (Wars)* **64,** 439-448.

Kaufman, J., Plotsky, P. M., Nemeroff, C. B. and Charney, D. S. (2000) Effects of early adverse experiences on brain structure and function: clinical implications. *Biol Psychiatry* **48,** 778-790.

Keller, J. N. (2006) Age-related neuropathology, cognitive decline, and Alzheimer's disease. *Ageing Res Rev* **5,** 1-13.

Kerr, D. S., Huggett, A. M. and Abraham, W. C. (1994) Modulation of Hippocampal Long-Term Potentiation and Long-Term Depression by Corticosteroid Receptor Activation. *Psychobiology* **22,** 123-133.

Kesner, R. (2013) Neurobiological foundations of an attribute model of memory. *Comparative Cognition & Behavior Reviews* **8,** 29-59.

Keuerleber, S., Gsandtner, I. and Freissmuth, M. (2011) From cradle to twilight: the carboxyl terminus directs the fate of the A(2A)-adenosine receptor. *Biochim Biophys Acta* **1808,** 1350-1357.

Keuker, J. I., Vollmann-Honsdorf, G. K. and Fuchs, E. (2001) How to use the optical fractionator: an example based on the estimation of neurons in the hippocampal CA1 and CA3 regions of tree shrews. *Brain Res Brain Res Protoc* **7,** 211-221.

Kim, J. J., Foy, M. R. and Thompson, R. F. (1996) Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. *Proc Natl Acad Sci U S A* **93,** 4750-4753.

Kim, J. J., Song, E. Y. and Kosten, T. A. (2006) Stress effects in the hippocampus: synaptic plasticity and memory. *Stress* **9,** 1-11.

Kinney, J. W., Sanchez-Alavez, M., Barr, A. M., Criado, J. R., Crawley, J. N., Behrens, M. M., Henriksen, S. J. and Bartfai, T. (2009) Impairment of memory consolidation by galanin correlates with in vivo inhibition of both LTP and CREB phosphorylation. *Neurobiol Learn Mem* **92,** 429-438.

Kirk, I. P. and Richardson, P. J. (1995) Further characterization of [3H]-CGS 21680 binding sites in the rat striatum and cortex. *Br J Pharmacol* **114,** 537-543.

Knoops, A. J., van der Graaf, Y., Mali, W. P. and Geerlings, M. I. (2010) Age-related changes in hypothalamic-pituitary-adrenal axis activity in patients with manifest arterial disease. *Endocrine* **37,** 231-238.

Konorski, J. Ed (1948) *Conditioned reflexes and neuron organization.* Cambridge biological studies, Univ. Press: Cambridge.

Krugers, H. J., Alfarez, D. N., Karst, H., Parashkouhi, K., van Gemert, N. and Joels, M. (2005) Corticosterone shifts different forms of synaptic potentiation in opposite directions. *Hippocampus* **15,** 697-703.

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

Lado, F. A., Laureta, E. C. and Moshe, S. L. (2002) Seizure-induced hippocampal damage in the mature and immature brain. *Epileptic Disord* **4,** 83-97.

Ledent, C., Vaugeois, J. M., Schiffmann, S. N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J. J., Costentin, J., Heath, J. K., Vassart, G. and Parmentier, M. (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* **388,** 674-678.

Lee, B., Sur, B., Shim, I., Lee, H. and Hahm, D. H. (2014) Baicalin improves chronic corticosterone-induced learning and memory deficits via the enhancement of impaired hippocampal brain-derived neurotrophic factor and cAMP response element-binding protein expression in the rat. *J Nat Med* **68,** 132-143.

Lee, B. K., Glass, T. A., McAtee, M. J., Wand, G. S., Bandeen-Roche, K., Bolla, K. I. and Schwartz, B. S. (2007) Associations of salivary cortisol with cognitive function in the Baltimore memory study. *Arch Gen Psychiatry* **64,** 810-818.

Lehmann, J., Stohr, T. and Feldon, J. (2000) Long-term effects of prenatal stress experiences and postnatal maternal separation on emotionality and attentional processes. *Behavioural brain research* **107,** 133-144.

Li, C. L. and Mc, I. H. (1957) Maintenance of resting membrane potentials in slices of mammalian cerebral cortex and other tissues in vitro. *J Physiol* **139,** 178-190.

Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M. J., Dumont, J. E. and Vassart, G. (1989) Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science* **244,** 569-572.

Linden, J. (2001) Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol* **41,** 775-787.

Lister, R. G. (1987) The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)* **92,** 180–185.

Lopes da Silva, F. H., Witter, M. P., Boeijinga, P. H. and Lohman, A. H. (1990) Anatomic organization and physiology of the limbic cortex. *Physiol Rev* **70,** 453-511.

Lopes, L. V., Cunha, R. A., Kull, B., Fredholm, B. B. and Ribeiro, J. A. (2002) Adenosine A(2A) receptor facilitation of hippocampal synaptic transmission is dependent on tonic A(1) receptor inhibition. *Neuroscience* **112,** 319-329.

Lopes, L. V., Cunha, R. A. and Ribeiro, J. A. (1999a) Cross talk between A(1) and A(2A) adenosine receptors in the hippocampus and cortex of young adult and old rats. *J Neurophysiol* **82,** 3196-3203.

Lopes, L. V., Cunha, R. A. and Ribeiro, J. A. (1999b) Increase in the number, G protein coupling, and efficiency of facilitatory adenosine A2A receptors in the limbic cortex, but not striatum, of aged rats. *Journal of neurochemistry* **73,** 1733-1738.

Lopes, L. V., Marvin-Guy, L. F., Fuerholz, A., Affolter, M., Ramadan, Z., Kussmann, M., Fay, L. B. and Bergonzelli, G. E. (2008) Maternal deprivation affects the neuromuscular protein profile of the rat colon in response to an acute stressor later in life. *J Proteomics* **71,** 80-88.

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

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193,** 265-275.

Lowy, M. T., Gault, L. and Yamamoto, B. K. (1993) Adrenalectomy attenuates stress-induced elevations in extracellular glutamate concentrations in the hippocampus. *J Neurochem* **61,** 1957-1960.

Lucas, M., Mirzaei, F., Pan, A., Okereke, O. I., Willett, W. C., O'Reilly, E. J., Koenen, K. and Ascherio, A. (2011) Coffee, caffeine, and risk of depression among women. *Arch Intern Med* **171,** 1571-1578.

Lupien, S. J., de Leon, M., de Santi, S., Convit, A., Tarshish, C., Nair, N. P., Thakur, M., McEwen, B. S., Hauger, R. L. and Meaney, M. J. (1998) Cortisol levels during human aging predict hippocampal atrophy and memory deficits. *Nat Neurosci* **1,** 69-73.

Lynch, M. A. (2004) Long-term potentiation and memory. *Physiol Rev* **84,** 87-136.

Mackiewicz, M., Nikonova, E. V., Zimmermann, J. E., Romer, M. A., Cater, J., Galante, R. J. and Pack, A. I. (2006) Age-related changes in adenosine metabolic enzymes in sleep/wake regulatory areas of the brain. *Neurobiol Aging* **27,** 351-360.

Madeira, M. D., Pereira, A., Cadete-Leite, A. and Paula-Barbosa, M. M. (1990) Estimates of volumes and pyramidal cell numbers in the prelimbic subarea of the prefrontal cortex in experimental hypothyroid rats. *J Anat* **171,** 41-56.

Magarinos, A. M., McEwen, B. S., Flugge, G. and Fuchs, E. (1996) Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. *J Neurosci* **16,** 3534-3540.

Maia, L. and de Mendonca, A. (2002) Does caffeine intake protect from Alzheimer's disease? *Eur J Neurol* **9,** 377-382.

Marchetti, C. and Marie, H. (2011) Hippocampal synaptic plasticity in Alzheimer's disease: what have we learned so far from transgenic models? *Rev Neurosci* **22,** 373-402.

Marco, E. M., Macri, S. and Laviola, G. (2011) Critical age windows for neurodevelopmental psychiatric disorders: evidence from animal models. *Neurotox Res* **19,** 286-307.

Marques, S., Batalha, V. L., Lopes, L. V. and Outeiro, T. F. (2011) Modulating Alzheimer's disease through caffeine: a putative link to epigenetics. *J Alzheimers Dis* **24 Suppl 2,** 161-171.

Martin, S., Henley, J. M., Holman, D., Zhou, M., Wiegert, O., van Spronsen, M., Joels, M., Hoogenraad, C. C. and Krugers, H. J. (2009) Corticosterone alters AMPAR mobility and facilitates bidirectional synaptic plasticity. *PLoS One* **4,** e4714.

Mayford, M., Bach, M. E. and Kandel, E. (1996) CaMKII function in the nervous system explored from a genetic perspective. *Cold Spring Harb Symp Quant Biol* **61,** 219-224.

McEwen, B. S. (1999a) Stress and hippocampal plasticity. *Annu Rev Neurosci* **22,** 105-122.

McEwen, B. S. (1999b) Stress and the aging hippocampus. *Front Neuroendocrinol* **20,** 49-70.

McEwen, B. S. (2007) Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiol Rev* **87,** 873-904.

McEwen, B. S. (2012) The ever-changing brain: cellular and molecular mechanisms for the effects of stressful experiences. *Dev Neurobiol* **72,** 878-890.

McEwen, B. S., Weiss, J. M. and Schwartz, L. S. (1968) Selective retention of corticosterone by limbic structures in rat brain. *Nature* **220,** 911-912.

McFarland, K. N., Das, S., Sun, T. T., Leyfer, D., Xia, E., Sangrey, G. R., Kuhn, A., Luthi-Carter, R., Clark, T. W., Sadri-Vakili, G. and Cha, J. H. (2012) Genome-wide histone acetylation is altered in a transgenic mouse model of Huntington's disease. *PLoS One* **7,** e41423.

McKinney, W. T. (1984) Animal models of depression: an overview. *Psychiatr Dev* **2,** 77-96.

Meerlo, P., Roman, V., Farkas, E., Keijser, J. N., Nyakas, C. and Luiten, P. G. (2004) Ageing-related decline in adenosine A1 receptor binding in the rat brain: an autoradiographic study. *J Neurosci Res* **78,** 742-748.

Meller, E., Shen, C., Nikolao, T. A., Jensen, C., Tsimberg, Y., Chen, J. and Gruen, R. J. (2003) Region-specific effects of acute and repeated restraint stress on the phosphorylation of mitogen-activated protein kinases. *Brain Res* **979,** 57-64.

Miller, D. B. and O'Callaghan, J. P. (2005) Aging, stress and the hippocampus. *Ageing Res Rev* **4,** 123-140.

Milner, B., Squire, L. R. and Kandel, E. R. (1998) Cognitive neuroscience and the study of memory. *Neuron* **20,** 445-468.

Mizoguchi, K., Ikeda, R., Shoji, H., Tanaka, Y., Maruyama, W. and Tabira, T. (2009) Aging attenuates glucocorticoid negative feedback in rat brain. *Neuroscience* **159,** 259-270.

Montgomery, K. C. (1958) The relation between fear induced by novel stimulation and exploratory behavior. *J. Comp. Physiol. Psychol.* **48,** 254–260.

Morris, R. G. (2003) Long-term potentiation and memory. *Philos Trans R Soc Lond B Biol Sci* **358,** 643-647.

Morris, R. G., Anderson, E., Lynch, G. S. and Baudry, M. (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature* **319,** 774-776.

Morris, R. G., Garrud, P., Rawlins, J. N. and O'Keefe, J. (1982) Place navigation impaired in rats with hippocampal lesions. *Nature* **297,** 681-683.

Morris, R. G. M. (1981) Spatial Localization Does Not Require the Presence of Local Cues. *Learning and Motivation* **12(2)**.

Muller, T. (2013) Suitability of the adenosine antagonist istradefylline for the treatment of Parkinson's disease: pharmacokinetic and clinical considerations. *Expert Opin Drug Metab Toxicol* **9,** 1015-1024.

Newport, D. J., Stowe, Z. N. and Nemeroff, C. B. (2002) Parental depression: animal models of an adverse life event. *Am J Psychiatry* **159,** 1265-1283.

Nishi, M., Horii-Hayashi, N., Sasagawa, T. and Matsunaga, W. (2013) Effects of early life stress on brain activity: implications from maternal separation model in rodents. *Gen Comp Endocrinol* **181,** 306-309.

Nishizaki, T., Nagai, K., Nomura, T., Tada, H., Kanno, T., Tozaki, H., Li, X. X., Kondoh, T., Kodama, N., Takahashi, E., Sakai, N., Tanaka, K. and Saito, N. (2002) A new neuromodulatory pathway with a glial contribution mediated via A(2a) adenosine receptors. *Glia* **39,** 133-147.

O'Kane, E. M. and Stone, T. W. (1998) Interaction between adenosine A1 and A2 receptor-mediated responses in the rat hippocampus in vitro. *European Journal of Pharmacology* **362,** 17-25.

O'Keefe, J. and Conway, D. H. (1978) Hippocampal place units in the freely moving rat: why they fire where they fire. *Exp Brain Res* **31,** 573-590.

O'Keefe, J. and Dostrovsky, J. (1971) The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res* **34,** 171-175.

Oakley, R. H. and Cidlowski, J. A. (1993) Homologous down regulation of the glucocorticoid receptor: the molecular machinery. *Crit Rev Eukaryot Gene Expr* **3,** 63-88.

Okonkwo, D. O., Reece, T. B., Laurent, J. J., Hawkins, A. S., Ellman, P. I., Linden, J., Kron, I. L., Tribble, C. G., Stone, J. R. and Kern, J. A. (2006) A comparison of adenosine A2A agonism and methylprednisolone in attenuating neuronal damage and improving functional outcome after experimental traumatic spinal cord injury in rabbits. *J Neurosurg Spine* **4,** 64-70.

Okuda, S., Roozendaal, B. and McGaugh, J. L. (2004) Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proc Natl Acad Sci U S A* **101,** 853-858.

Palacios, S. D., Pak, K., Rivkin, A. Z., Kayali, A. G., Austen, D., Aletsee, C., Melhus, A., Webster, N. J. and Ryan, A. F. (2004) Role of p38 mitogen-activated protein kinase in middle ear mucosa hyperplasia during bacterial otitis media. *Infect Immun* **72,** 4662-4667.

Palomero-Gallagher, N. and Zilles, K. Eds (2004) *The rat isocortex. In: Paxinos, G.: The Rat Nervous System.* Academic Press: San Diego.

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

Park, P., Volianskis, A., Sanderson, T. M., Bortolotto, Z. A., Jane, D. E., Zhuo, M., Kaang, B. K. and Collingridge, G. L. (2014) NMDA receptor-dependent long-term potentiation comprises a family of temporally overlapping forms of synaptic plasticity that are induced by different patterns of stimulation. *Philos Trans R Soc Lond B Biol Sci* **369,** 20130131.

Paxinos, G. and Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*. Academic Press.

Pellow, S., Chopin, P., File, S. E. and Briley, M. (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* **14,** 149-167.

Piccolino, M. (1998) Animal electricity and the birth of electrophysiology: the legacy of Luigi Galvani. *Brain Res Bull* **46,** 381-407.

Pickering, C., Gustafsson, L., Cebere, A., Nylander, I. and Liljequist, S. (2006) Repeated maternal separation of male Wistar rats alters glutamate receptor expression in the hippocampus but not the prefrontal cortex. *Brain Res* **1099,** 101-108.

Popova, E. A., Krivokharchenko, A. S. and Vil'ianovich, L. I. (2002) [In vitro development of murine embryos using different types of microinjections]. *Ontogenez* **33,** 107-110.

Porter, N. M. and Landfield, P. W. (1998) Stress hormones and brain aging: adding injury to insult? *Nat Neurosci* **1,** 3-4.

Prut, L. and Belzung, C. (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur J Pharmacol* **463,** 3-33.

Pugliese, A. M., Traini, C., Cipriani, S., Gianfriddo, M., Mello, T., Giovannini, M. G., Galli, A. and Pedata, F. (2009) The adenosine A2A receptor antagonist ZM241385 enhances neuronal survival after oxygen-glucose deprivation in rat CA1 hippocampal slices. *Br J Pharmacol* **157,** 818-830.

Quinn, W. G., Harris, W. A. and Benzer, S. (1974) Conditioned behavior in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **71,** 708-712.

Ramamoorthy, S. and Cidlowski, J. A. (2013) Ligand-induced repression of the glucocorticoid receptor gene is mediated by an NCoR1 repression complex formed by long-range chromatin interactions with intragenic glucocorticoid response elements. *Mol Cell Biol* **33,** 1711-1722.

Rangarajan, P. N., Umesono, K. and Evans, R. M. (1992) Modulation of glucocorticoid receptor function by protein kinase A. *Mol Endocrinol* **6,** 1451-1457.

Rebola, N., Carta, M., Lanore, F., Blanchet, C. and Mulle, C. (2011a) NMDA receptor-dependent metaplasticity at hippocampal mossy fiber synapses. *Nat Neurosci* **14,** 691-693.

Rebola, N., Rodrigues, R. J., Lopes, L. V., Richardson, P. J., Oliveira, C. R. and Cunha, R. A. (2005) Adenosine A1 and A2A receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. *Neuroscience* **133,** 79-83.

Rebola, N., Sebastiao, A. M., de Mendonca, A., Oliveira, C. R., Ribeiro, J. A. and Cunha, R. A. (2003) Enhanced adenosine A2A receptor facilitation of synaptic transmission in the hippocampus of aged rats. *Journal of neurophysiology* **90,** 1295-1303.

Rebola, N., Simoes, A. P., Canas, P. M., Tome, A. R., Andrade, G. M., Barry, C. E., Agostinho, P. M., Lynch, M. A. and Cunha, R. A. (2011b) Adenosine A2A receptors control neuroinflammation and consequent hippocampal neuronal dysfunction. *J Neurochem* **117,** 100-111.

Reitz, C., Brayne, C. and Mayeux, R. (2011) Epidemiology of Alzheimer disease. *Nat Rev Neurol* **7,** 137-152.

Reppert, S. M., Weaver, D. R., Stehle, J. H. and Rivkees, S. A. (1991) Molecular cloning and characterization of a rat A1-adenosine receptor that is widely expressed in brain and spinal cord. *Mol Endocrinol* **5,** 1037-1048.

Reul, J. M. and de Kloet, E. R. (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* **117,** 2505-2511.

Ribeiro, J. A. and Sebastiao, A. M. (2010) Modulation and metamodulation of synapses by adenosine. *Acta Physiol (Oxf)* **199,** 161-169.

Ribeiro, J. A., Sebastiao, A. M. and de Mendonca, A. (2003) Participation of adenosine receptors in neuroprotection. *Drug News Perspect* **16,** 80-86.

Rivera-Oliver, M. and Diaz-Rios, M. (2014) Using caffeine and other adenosine receptor antagonists and agonists as therapeutic tools against neurodegenerative diseases: A review. *Life Sci*.

Roceri, M., Hendriks, W., Racagni, G., Ellenbroek, B. A. and Riva, M. A. (2002) Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. *Mol Psychiatry* **7,** 609-616.

Rodgers, R. J. and Dalvi, A. (1997) Anxiety, defence and the elevated plus-maze. *Neurosci Biobehav Rev* **21,** 801-810.

Rodgers, R. J., Haller, J., Holmes, A., Halasz, J., Walton, T. J. and Brain, P. F. (1999) Corticosterone response to the plus-maze: high correlation with risk assessment in rats and mice. *Physiol Behav* **68,** 47-53.

Rosenzweig, E. S., Redish, A. D., McNaughton, B. L. and Barnes, C. A. (2003) Hippocampal map realignment and spatial learning. *Nat Neurosci* **6,** 609-615.

Rothman, S. M. and Mattson, M. P. (2010) Adverse stress, hippocampal networks, and Alzheimer's disease. *Neuromolecular Med* **12,** 56-70.

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

Sapolsky, R. M. (1985) A mechanism for glucocorticoid toxicity in the hippocampus: increased neuronal vulnerability to metabolic insults. *J Neurosci* **5,** 1228-1232.

Sapolsky, R. M., Krey, L. C. and McEwen, B. S. (1984) Stress down-regulates corticosterone receptors in a site-specific manner in the brain. *Endocrinology* **114,** 287-292.

Sapolsky, R. M. and Meaney, M. J. (1986) Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. *Brain Res* **396,** 64-76.

Sapolsky, R. M., Meaney, M. J. and McEwen, B. S. (1985) The development of the glucocorticoid receptor system in the rat limbic brain. III. Negative-feedback regulation. *Brain Res* **350,** 169-173.

Scaccianoce, S., Navarra, D., Di Sciullo, A., Angelucci, L. and Endroczi, E. (1989) Adenosine and pituitary-adrenocortical axis activity in the rat. *Neuroendocrinology* **50,** 464-468.

Schiffmann, S. N., Libert, F., Vassart, G. and Vanderhaeghen, J. J. (1991) Distribution of adenosine A2 receptor mRNA in the human brain. *Neurosci Lett* **130,** 177-181.

Schiffmann, S. N. and Vanderhaeghen, J. J. (1993a) Adenosine A2 receptors regulate the gene expression of striatopallidal and striatonigral neurons. *J Neurosci* **13,** 1080-1087.

Schiffmann, S. N. and Vanderhaeghen, J. J. (1993b) Age-related loss of mRNA encoding adenosine A2 receptor in the rat striatum. *Neurosci Lett* **158,** 121-124.

Schmittgen, T. D. and Livak, K. J. (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* **3,** 1101-1108.

Sebastiao, A. M. and Ribeiro, J. A. (2009) Adenosine receptors and the central nervous system. *Handb Exp Pharmacol***,** 471-534.

Sebastiao, A. M., Stone, T. W. and Ribeiro, J. A. (1990) The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: antagonism by 1,3,8-substituted xanthines. *Br J Pharmacol* **101,** 453-459.

Sewards, T. V. and Sewards, M. A. (2003) Input and output stations of the entorhinal cortex: superficial vs. deep layers or lateral vs. medial divisions? *Brain Res Brain Res Rev* **42,** 243-251.

Skrede, K. K. and Westgaard, R. H. (1971) The transverse hippocampal slice: a well-defined cortical structure maintained in vitro. *Brain Res* **35,** 589-593.

Smith, M. A., Makino, S., Kvetnansky, R. and Post, R. M. (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci* **15,** 1768-1777.

Sousa, N., Cerqueira, J. J. and Almeida, O. F. (2008) Corticosteroid receptors and neuroplasticity. *Brain Res Rev* **57,** 561-570.

Sousa, N., Lukoyanov, N. V., Madeira, M. D., Almeida, O. F. and Paula-Barbosa, M. M. (2000) Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. *Neuroscience* **97,** 253-266.

Sousa, V. C., Vital, J., Costenla, A. R., Batalha, V. L., Sebastiao, A. M., Ribeiro, J. A. and Lopes, L. V. (2014) Maternal separation impairs long term-potentiation in CA1-CA3 synapses and hippocampal-dependent memory in old rats. *Neurobiol Aging*.

Squire, L. R. (1992) Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. *Psychol Rev* **99,** 195-231.

Squire, L. R. (2004) Memory systems of the brain: a brief history and current perspective. *Neurobiol Learn Mem* **82,** 171-177.

Stone, C. P. (1932) *Wildness and savageness in rats of dif-*

ferent strains. University of Chicago Press: Chicago.

Stone, E. A., Slucky, A. V., Platt, J. E. and Trullas, R. (1985) Reduction of the cyclic adenosine 3',5'-monophosphate response to catecholamines in rat brain slices after repeated restraint stress. *J Pharmacol Exp Ther* **233,** 382-388.

Sullivan, R., Wilson, D. A., Feldon, J., Yee, B. K., Meyer, U., Richter-Levin, G., Avi, A., Michael, T., Gruss, M., Bock, J., Helmeke, C. and Braun, K. (2006) The International Society for Developmental Psychobiology annual meeting symposium: Impact of early life experiences on brain and behavioral development. *Dev Psychobiol* **48,** 583-602.

Surjit, M., Ganti, K. P., Mukherji, A., Ye, T., Hua, G., Metzger, D., Li, M. and Chambon, P. (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* **145,** 224-241.

Sweatt, D. Ed (2003) *Mechanisms of Memory*. Elsevier: USA.

Tebano, M. T., Martire, A., Potenza, R. L., Gro, C., Pepponi, R., Armida, M., Domenici, M. R., Schwarzschild, M. A., Chen, J. F. and Popoli, P. (2008) Adenosine A(2A) receptors are required for normal BDNF levels and BDNF-induced potentiation of synaptic transmission in the mouse hippocampus. *J Neurochem* **104,** 279-286.

Terry, A. V., Jr. (2009) Spatial Navigation (Water Maze) Tasks.

Teyler, T. J. and DiScenna, P. (1985) The role of hippocampus in memory: a hypothesis. *Neurosci Biobehav Rev* **9,** 377-389.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76,** 4350-4354.

Tsigos, C. and Chrousos, G. P. (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J Psychosom Res* **53,** 865-871.

Valadas, J. S., Batalha, V. L., Ferreira, D. G., Gomes, R., Coelho, J. E., Sebastiao, A. M., Diogenes, M. J. and Lopes, L. V. (2012) Neuroprotection afforded by adenosine A2A receptor blockade is modulated by corticotrophin-releasing factor (CRF) in glutamate injured cortical neurons. *J Neurochem* **123,** 1030-1040.

Varani, K., Vincenzi, F., Tosi, A., Gessi, S., Casetta, I., Granieri, G., Fazio, P., Leung, E., MacLennan, S., Granieri, E. and Borea, P. A. (2010) A2A adenosine receptor overexpression and functionality, as well as TNF-alpha levels, correlate with motor symptoms in Parkinson's disease. *FASEB J* **24,** 587-598.

Von Lubitz, D. K., Lin, R. C. and Jacobson, K. A. (1995) Cerebral ischemia in gerbils: effects of acute and chronic treatment with adenosine A2A receptor agonist and antagonist. *Eur J Pharmacol* **287,** 295-302.

Walf, A. A. and Frye, C. A. (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* **2,** 322-328.

Warrington, E. K. (1979) Neuropsychological evidence for multiple memory systems. *Ciba Found Symp***,** 153-166.

Willner, P. (1997) Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology (Berl)* **134,** 319-329.

Willner, P., Sanger, D. and Oglesby, M. (1997) The behavioural pharmacology of anxiety and depression. *Behav Pharmacol* **8,** 475-476.

Wuppen, K., Oesterle, D., Lewicka, S., Kopitz, J. and Plaschke, K. (2010) A subchronic application period of glucocorticoids leads to rat cognitive dysfunction whereas physostigmine induces a mild neuroprotection. *J Neural Transm* **117,** 1055-1065.

Xu, L., Anwyl, R. and Rowan, M. J. (1997) Behavioural stress facilitates the induction of long-term depression in the hippocampus. *Nature* **387,** 497-500.

Yang, M., Soohoo, D., Soelaiman, S., Kalla, R., Zablocki, J., Chu, N., Leung, K., Yao, L., Diamond, I., Belardinelli, L. and Shryock, J. C. (2007) Characterization of the potency, selectivity, and pharmacokinetic profile for six adenosine A2A receptor antagonists. *Naunyn Schmiedebergs Arch Pharmacol* **375,** 133-144.

Yao, Y. Y., Wu, Q. S., Li, W. Z. and Li, W. P. (2011) Dexamethasone potentiated Abeta-induced learning and memory impairment in rats. *Neurol Res* **33,** 371-380.

Yau, J. L. and Seckl, J. R. (2012) Local amplification of glucocorticoids in the aging brain and impaired spatial memory. *Front Aging Neurosci* **4,** 24.

Zalachoras, I., Houtman, R. and Meijer, O. C. (2013) Understanding stress-effects in the brain via transcriptional signal transduction pathways. *Neuroscience* **242,** 97-109.

Appendix *(Reprint of published papers)*

Molecular Psychiatry (2012), 1–12

www.nature.com/mp

ORIGINAL ARTICLE

Adenosine A_{2A} receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation

VL Batalha[1], JM Pego[2,3], BM Fontinha[1], AR Costenla[1], JS Valadas[1], Y Baqi[4], H Radjainia[4], CE Müller[4], AM Sebastião[1] and LV Lopes[1]

[1]*Institute of Pharmacology and Neurosciences, Neurosciences Unit, Instituto de Medicina Molecular, Faculty of Medicine, University of Lisbon, Lisbon, Portugal;* [2]*Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal;* [3]*ICVS/3B's—PT Government Associate Laboratory, Braga/Guimarães, Portugal and* [4]*PharmaCenter Bonn, Pharmazeutische Chemie I, Pharmazeutisches Institut, University of Bonn, Bonn, Germany*

Maternal separation (MS) is an early life stress model that induces permanent changes in the central nervous system, impairing hippocampal long-term potentiation (LTP) and spatial working memory. There are compelling evidences for a role of hippocampal adenosine A_{2A} receptors in stress-induced modifications related to cognition, thus opening a potential window for therapeutic intervention. Here, we submitted rats to MS and evaluated the long-lasting molecular, electrophysiological and behavioral impairments in adulthood. We then assessed the therapeutic potential of KW6002, a blocker of A_{2A} receptors, in stress-impaired animals. We report that the blockade of A_{2A} receptors was efficient in reverting the behavior, electrophysiological and morphological impairments induced by MS. In addition, this effect is associated with restoration of the hypothalamic-pituitary-adrenal axis (HPA-axis) activity, as both the plasma corticosterone levels and hippocampal glucocorticoid receptor expression pattern returned to physiological-like status after the treatment. These results reveal the involvement of A_{2A} receptors in the stress-associated impairments and directly in the stress response system by showing that the dysfunction of the HPA-axis as well as the long-lasting synaptic and behavioral effects of MS can be reverted by targeting adenosine A_{2A} receptors. These findings provide a novel evidence for the use of adenosine A_{2A} receptor antagonists as potential therapy against psychopathologies.
Molecular Psychiatry advance online publication, 28 February 2012; doi:10.1038/mp.2012.8

Keywords: adenosine A_{2A} receptors; corticosterone; hippocampus; HPA-axis; maternal separation; stress

Introduction

Exposure to stress has deleterious effects on brain structure and function, which could be manifested either immediately after stress,[1] as a long-term vulnerability to cognitive deficits[2] or even as an increased susceptibility to neuropsychiatric disorders, where stress has a major role.[3,4]

Mother–infant interaction is a key factor for brain maturation and disease susceptibility which in humans can manifest in cognitive and behavioral disorders later in life.[5,6] In rats, the daily separation of the litter from their mothers for 180 min each day during postnatal days (PNDs) 2–14 will result in an alteration of maternal behavior, namely with a significant reduction in licking/grooming duration.[7] During this period, the hippocampus, which is critically involved in long-term memory formation[8] and is also a primary target for stress hormones in the central nervous system,[1,9] goes through great development. The majority of hippocampal granule neurons develop and extend their axons between PND 1 and 21[10] and the peak period of neurogenesis and mossy fiber outgrowth overlaps with the stress hyporesponsive period (PND 4–14) in neonatal rats.[11] This will induce changes that persist throughout adult life at the level of gene expression, neurochemistry, electrophysiology proprieties and morphology[12,13] with behavioral and neuroendocrine signs of cognitive deficits and over-activation of the hypothalamic-pituitary-adrenal axis (HPA-axis) as adults.[7,14–16]

Adenosine receptors in the hippocampus are important modulators of synaptic transmission and neuronal excitability. Glutamatergic synaptic transmission in physiological conditions is controlled

Correspondence: Dr LV Lopes, Neurosciences Unit, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Av. Professor Egas Moniz, Lisbon 1649-028, Portugal.
E-mail: lvlopes@fm.ul.pt
Received 29 September 2011; revised 3 January 2012; accepted 17 January 2012

negatively by the dominant adenosine A_1 receptors, and positively to a lesser extent by A_{2A} receptors.[17] Interestingly, this pattern appears to be modified in the aged hippocampus, with a marked increase in the expression of A_{2A} receptors and a decrease in the expression of A_1 receptors.[18,19] These changes are accompanied by a strong direct facilitatory effect of A_{2A} receptors on the release of glutamate.[20] This is also observed in other situations associated to neuronal dysfunction, such as epilepsy, acute stress or animal models of Alzheimer's disease,[21] which suggests a deleterious contribution of A_{2A} receptors to these conditions. The blockade of A_{2A} receptors was proven beneficial against synaptic loss associated with acute stress in the hippocampus.[22] Interestingly, cognitive impairments also occur when excessive levels of corticosteroids are attained due to disease, or due to hypersecretion in response to a stressor.[23,24]

However, it is still unknown the extent to which A_{2A} receptors are involved in the long-term effects of early life stress. Here, we submitted rats to maternal separation (MS) and evaluated the long-lasting molecular, electrophysiological and behavioral impairments at adult age. We then assessed the therapeutic potential of blocking endogenous activation of A_{2A} receptors, by administering a selective antagonist, KW6002 (istradefylline), orally for 1 month to stress-impaired animals. We report that the blockade of A_{2A} receptors was efficient in reverting the long-lasting behavior, morphological and electrophysiological impairments induced by MS. We also show that this effect is associated with the re-establishment of the HPA-axis activity, as both the plasma corticosterone levels and hippocampal glucocorticoid receptor expression pattern returned to physiological-like status after the treatment.

Materials and methods

Animals

Pregnant Wistar rats were purchased (Harlan, Barcelona, Spain) in mid-gestation and were due in our animal facility. All animals were handled according to European Community guidelines and Portuguese law on animal care (1005/92). The animals that were killed by decapitation were anesthetized under halothane atmosphere.

MS protocol

The protocol used has been previously validated and described.[25] Wistar dams and their litters were assigned either to the control (CTR—non-separated) or to the MS groups as described before.[26] To exclude artifacts from genetic background, at PND 2, all the litters were collected together, gender assessed and the pups were randomly distributed to foster dams (gender proportion maintained). MS pups were removed from their cages as a group from PND 2 to 14, for 180 min, daily, at 9 am, and placed in an isolation cage in an adjacent room kept at $32.0 \pm 0.5\,^{\circ}C$. At the end of the separation period, pups were returned to their home-cage and rolled in the soiled home cage bedding before reuniting with the mother. CTR pups were not handled and were maintained in their home-cages until weaning. At day 21 the pup's gender was confirmed, males weaned and housed in groups of 5–8 animals per cage until use at adult age (8–14 weeks; according to diagram in Supplementary Figure S1).

Oral administration of the drug

KW6002 (istradefylline), a selective adenosine A_{2A} receptor antagonist[27] was orally administered diluted in the drinking water, being continuously available. The weight of the animals and the volume intake were assessed twice a week and the concentration of the solution was adjusted so that the drug intake was maintained at $3\,mg\,kg^{-1}$ per day. Animals were divided in four groups: CTR or MS, drinking vehicle (0.025% methylcellulose) and CTR KW or MS KW drinking KW6002 ($3\,mg\,kg^{-1}$ per day, 0.025% methylcellulose). The treatment started at 4–6 weeks old, and was prolonged for 1 month until sacrifice. The KW6002 administration was kept throughout the behavioral assessments.

Corticosterone quantification

Blood was extracted from the tail, in animals previously handled to minimize stress and without anesthesia, at two different time points, 8 am (nadir) and 8 pm (zenith). The plasma was isolated by centrifugation at 2000 g, 4 °C for 15 min and corticosterone quantified by radioimmunoassay using the rat corticosterone ^3H kit from MP Biomedicals, UK according to the manufacturer's protocol.

Behavioral assessments

CTR, MS, CTR KW, MS KW were first handled for 5 days before testing the behavior assays, that were performed in the following sequence: open-field (OF), Elevated plus maze (EPM) and Morris water maze (MWM):[28] Rats were given spatial acquisition training consisting of four trials/day for 4 consecutive days, as performed before.[29] On the 5th day a probe test was given in which the platform was removed and animals were allowed to swim freely for 60 s while recording the percentage of time spent on each quadrant. The latency to found the platform during acquisition and the percentage of time in the platform quadrant in probe test were used to evaluate hipppocampal-dependent memory. EPM: The maze is shaped like a plus sign and consists of two 'open' and two 'closed' arms, arranged perpendicularly, and elevated 50 cm above the floor. Each animal was placed at the center of the equipment, facing an open arm. Each test lasted 5 min and all testing sessions were performed between 10:00 am and 17:00 pm in a sound-attenuated room. The maze was cleaned with a 70% ethanol solution between each animal. The total time spent in the open arms and the total arms entries (number of entries in open + closed arms) were used as anxiety and locomotor measures.[30] Open field:

The animals were placed at the center of the arena (66×66 cm) and allowed to explore for 5 min. Changes in mean speed and path length of the subjects were continuously monitored by an automated tracking system (Smart 2.5, PanLab, Barcelona, Spain). The maze was cleaned with a 70% ethanol solution between each animal.

Histological procedures

The day after the last testing session, five rats from each experimental group were perfused transcardially with phosphate-buffered saline, under deep pentobarbital anesthesia. Brains were removed and split into two hemispheres, and processed either for stereology, or for Golgi-Cox staining according to the procedures previously described.[31,32] Briefly, for stereology the left hemispheres were included in glycolmethacrylate (Tecnovit 7100; Heraeus Kulzer, Werheim, Germany) and every other microtome-cut section (30 μm) was then collected on a gelatinized slide, stained with Giemsa, and mounted with Entellan New (Merck, Darmstadt, Germany). The shrinkage factor was calculated according to Madeira *et al*.[33] For 3D neuronal reconstructions, hemispheres were removed and immersed in Golgi-Cox solution (a 1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate[34]) for 14 days; hemispheres were then transferred to a 30% sucrose solution (3 days), before being cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed in Kodak Rapid Fix (prepared as manufacturer instructions), dehydrated through a graded series of ethanols, and cleared in xylene before being mounted and coverslipped. Slides were coded before morphometric analysis in both sets.

Region and layer boundaries

We analyzed the following regions of the hipocampal formation: the dentate gyrus (including polymorphic, granule cell layer and molecular layer), CA1 (strata oriens, pyramidale, radiatum and lacunosum-moleculare) and CA3 (strata oriens, pyramidale, lucidum and radiatum). The above-mentioned regions were outlined according to the atlas of Paxinos and Watson,[35] based on noticeable cytoarchitectural differences.[36]

Stereological procedures

Volume estimations were performed using StereoInvestigator software (MicroBrightField, Williston, VT, USA) and a camera (DXC390; Sony, Tokyo, Japan) attached to a motorized microscope (Axioplan 2; Zeiss, Oberkochen, Germany). Cavalieri's principle[37] was used to assess the volume of each region. Briefly, every 10th section was used and its cross-sectional area was estimated by point counting at a final magnification of $\times 112$. For this, we randomly superimposed onto each area a test point grid in which the interpoint distance, at tissue level, was as follows: (1) 150 μm for the three layers of the dentate gyrus, (2) 250 μm for the three layers of CA1 and CA3. The volume of the region of interest was calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

Dendritic tree analysis

Three-D reconstructions of representative Golgi-impregnated neurons from CA1 were made. The criteria used to select neurons for reconstruction were as follows: (i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) dendrites without significant truncation of branches; (iii) relative isolation from neighboring impregnated neurons to avoid interference with the analysis; (iv) no morphological changes attributable to incomplete dendritic impregnation of Golgi–Cox stain. Golgi-impregnated pyramidal-like neurons of the CA1 region were readily identified by their characteristic pyramidal or piriform soma, spine-sparse primary dendrites and spine-dense secondary dendrites (Figure 2e for representative reconstructions). For each selected neuron, all branches of the dendritic tree and the location of all dendritic spines were reconstructed at $\times 600$ magnification, using a motorized microscope (Carl Zeiss Axioplan 2, Hamburg, Germany, with oil-objectives), attached to a camera (DXC-390, Sony, Tokyo, Japan) and Neurolucida software (Microbrightfield). Three-D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). In each hemisphere, 10 CA1 pyramidal neurons were reconstructed; as a result in this study we have analyzed 200 neurons. Several aspects of dendritic morphology were examined. To assess overall changes, total dendritic length, number of ramifications and number of dendrites were compared between groups. Sholl analysis was performed to assess changes in the ramification pattern.

Electrophysiological recordings

After decapitation the brain was rapidly removed and the hippocampi were dissected free in ice-cold Krebs solution composed of (mM): NaCl 124; KCl 3; NaH2PO4 1.25; NaHCO3 26; MgSO4 1; CaCl2 2; and glucose 10, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. 400 μM slices were obtained with a McEwen tissue shopper and field excitatory post-synaptic potentials (fEPSPs) were recorded as previously[29] in *stratum radiatum* of the CA1 area. Input–output (I/O) curves and long-term potentiation (LTP, 100 Hz, 1 s, 100 pulses induced at 0.5 mV/ms; $<50\%$ max) were recorded as previously. The second hippocampus was rapidly frozen in liquid nitrogen for further analysis.

Tissue processing

Samples were homogenized either in radio immunoprecipitation-assay buffer (50 mM Tris, 1 mM EDTA,

150 mM NaCl, 0.1% SDS, 1% NP 40, pH 8;[38] or in 0.32 M sucrose solution with 50 mM Tris at pH 7.6[19] supplemented with protease inhibitors (ROCHE, Mannheim, Germany). The first were centrifuged at 14 000 *g* for 15 min, and the second at 1000 *g* for 10 min, at 4 °C. The supernatant was collected, corresponding to whole-tissue lysate and whole-tissue homogenate, respectively. For membrane isolation the whole-tissue homogenate was centrifuged at 14 000 *g* for 12 min, at 4 °C, the pellet is the membrane fraction. Protein was quantified using the BioRad Protein or DC Protein based on procedures previously described.[39,40]

Saturation-binding assays

The radioligand-binding experiments were performed as described[41] with membrane fractions. Briefly, [^{3}H]ZM 241385 binding (0–10 nM) was for 1 h with 20–35 μg of protein/well for striatum membranes and [^{3}H]DPCPX (0–10 nM) binding was for 2 h with 40–60 μg protein/well of hippocampal, 60–100 μg protein/well of cortex and 20–40 μg protein/well of striatum membranes. Specific binding was determined subtracting the non-specific binding, measured in the presence of 2 μM of XAC and normalized for protein concentration. Radioactivity was determined after 12 h with an efficiency of 55–60% for 2 min. All binding assays were performed in triplicate.

Immunoblotting

Lysates or homogenates were denatured with 5 × sample buffer (350 mM Tris pH 6.8, 30% glycerol, 10% SDS, 600 mM DTT and 0.012% Bromophenol blue, pH 6.8) and heated either at 95 °C for 5 min or at 60–70 °C for 30 min, respectively, and further processed as before.[29] A_{2A}R and $GABA_A$R antibodies (Upstate/Millipore, Temecula, CA, USA; 05–717 and 05–474) were at 1:2000, GR, MR (Santa Cruz Biotechnology, Heidelberg, Germany; sc-1004 and sc-11412) at 1:1000 and 1:200, NMDAR2B (Cell Signaling, Danvers, MA, USA; D15B3) at 1:1000 and glutamate receptor one (GluR1) (Millipore, 05–855) at 1:6000. Optical density was determined with Image-J software and normalized to the respective β-actin or α-tubulin band density.

Drugs

A_{2A}R-selective antagonist, 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo(4,3-e)(1,2,4)triazolo(1,5-c)pyrimidin-5-amine (SCH58261) and the non-selective adenosine receptor antagonist 8-(4-((2-minoethyl) amino)carbonylmethyloxyphenyl)xanthine (XAC) were purchased from Tocris Cookson, UK. These solutions were diluted in the assay solution from 5 mM stock aliquots made in DMSO stored at −20 °C. A_{2A}R-selective antagonist, (*E*)-8-(2-(3,4-dimethoxyphenyl)-vinyl)-1,3-diethyl-7-methyl-3,7-dihydropurine-2,6-dione (KW6002, istradefylline) was synthesized according to a published procedure.[42] The purity of the product was determined by HPLC analysis coupled to electrospray ionization mass spectrometry and was >98%. Adenosine deaminase (from calf intestine 10 mg/2 ml, EC 3.5.4.4) was from ROCHE; A_1R-selective antagonist, (propyl-^{3}H)8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX, specific activity 100 Ci per mmol) was from Amersham, Buckinghamshire UK, and A_{2A}R-selective antagonist, 4-(2-(7-Amino-2-(2-furyl)(1,2,4)-triazolo(2,3-a) (1,3,5)triazin-5-ylamino)ethyl)phenol, ([^{3}H]ZM 241385, specific activity 27.4 Ci per mmol) was from ARC, St Louis, MO, USA. All these drugs were diluted directly in the incubation solution each day. HRP-coupled secondary antibodies were from Santa Cruz Biotechnology. All other reagents used were of the highest purity available either from Merck or Sigma Aldrich, Madrid, Spain.

Statistics

Values presented are mean ± s.e.m. of *n* experiments. To test the significance of the differences between CTR and MS groups, an unpaired Student's *t* test was used. When comparing CTR, MS, CTR KW and MS KW groups a one-way ANOVA was used, followed by a Bonferroni's Multiple Comparison *post hoc* test. For the Sholl analysis of reconstructed neurons a repeated measures analysis was used. For the saturation binding curves an *F*-test 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. For the analysis of the MWM acquisition curve and corticosterone circadian oscilation, the statistical differences were evaluated using two-way ANOVA repeated measures test. Values of $P<0.05$ were considered to be statistically significant.

Results

MS induces long-lasting regional effects in the brain

MS induces numerous changes in the brain particularly in the hippocampus.[43,44] The balance between mineralocorticoid and glucocorticoid receptors (MR and GR, respectively) can determine the impact of stress in different brain areas.[45] We quantified the levels of GR and MR in the hippocampus, cortex and striatum of adult animals previously subjected to MS (Figure 1a). MS has lead to a long-lasting decrease of the GR levels that was more evident in the hippocampus (0.74 ± 0.04 of CTR, $n=8$, $P<0.05$) than in cortex (0.86 ± 0.03 of CTR, $n=4$, $P<0.05$) or striatum (0.90 ± 0.03 of CTR, $n=4$; $P<0.05$). MR levels were not modified in any of the brain areas analyzed (Figure 1b). Concomitant changes on the levels of adenosine receptors were observed. Comparing with CTR, MS animals presented a 1.49 ± 0.04-fold increase ($n=9$; $P<0.05$) in the levels of A_{2A}R that was restricted to the hippocampus (Figure 1c). A hippocampal-specific decrease in the A_1R levels was also observed; B_{max} values were of 1202 ± 33 fmol per mg protein ($n=4$) for CTR animals and 1073 ± 23 fmol per mg protein for MS animals, ($n=4$; $P<0.05$; Figure 1d).

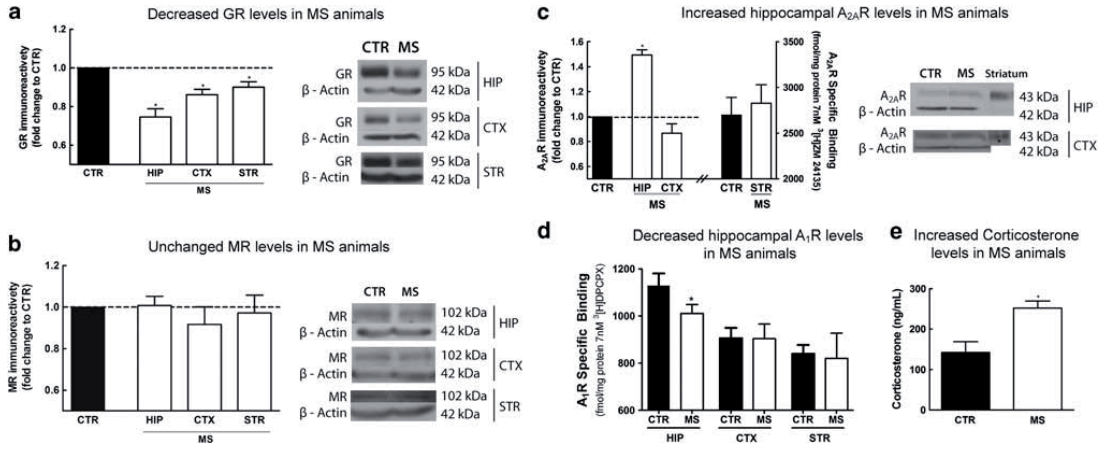

Figure 1 Region-specific effects of MS. MS induced region-specific changes in, GR (**a**) MR (**b**) $A_{2A}R$ (**c**) and A_1R (**d**) and an increase in plasmatic corticosterone levels (**e**). Protein levels of GR, MR (in all brain areas) and $A_{2A}R$ (in hippocampus and cortex) were evaluated by western blotting. Specific immunoreactivity was normalized to that of β-Actin or α-tubulin. For $A_{2A}R$ immunoreactivity 5 μg of striatum were used as positive control. Results are the mean ± s.e.m. of 3–9 experiments; $^*P<0.05$, comparing with CTR, calculated using an unpaired Student *t*-test. A_1R levels in all areas and $A_{2A}R$ levels in striatum were measured by saturation-binding curves with the A_1 or A_{2A} receptor selective antagonist [^{3}H]DPCPX or [^{3}H]ZM 24135, respectively. [^{3}H]DPCPX or [^{3}H]ZM 24135 (7 nM) were incubated with 20–100 μg of membranes in a final volume of 300 μl for 2 h/1 h at room temperature. The ordinates represent the specific binding obtained upon subtraction of the non-specific binding, determined in the presence of 2 μM of XAC, from total binding. Values are the mean ± s.e.m. of 4–5 experiments performed in triplicate. $^*P<0.05$ calculated using an *F*-test compared with CTR. Corticosterone levels in the morning period (8 am) were measured by radioimmunoassay using the rat corticosterone [^{3}H] kit. Results are mean ± s.e.m. of nine experiments; $^*P<0.05$ obtained using a unpaired Student *t*-test.

MS animals also presented a sustained increase in plasmatic corticosterone levels (Figure 1e).

Adenosine A_{2A} receptors are involved in synaptic changes induced by MS

To evaluate the impact of stress in synaptic transmission and plasticity, fEPSPs were measured in the CA1 area of the dorsal hippocampus. Basal synaptic transmission was accessed by performing I/O curves, whereas synaptic plasticity was evaluated by LTP induced by high frequency stimulation (100 Hz, 1 s).

The I/O curve was not modified by MS (Figure 2a, $n=3$). However, LTP magnitude (Figure 2b) was reduced in MS animals to 34.4 ± 2.7% from 50.7 ± 3.4% of potentiation obtained in CTR ($n=7$, $P<0.05$).

To evaluate whether the increase in A_{2A} adenosine receptors was involved in the impairments observed in synaptic plasticity, LTP was induced in the presence of SCH58261 (50 nM), a selective $A_{2A}R$ antagonist. The *ex vivo* blockade of $A_{2A}R$ reverted the LTP deficits induced by MS without affecting LTP magnitude in CTR animals (53.1 ± 3.7% and 57.3 ± 1.7% of potentiation, respectively, $n=4$–7, $P>0.05$; Figure 2c).

Moreover, the *in vivo* administration of the A_{2A}-selective antagonist KW6002 for 1 month to adult MS animals was also able to revert the LTP deficits observed (47.6 ± 3.9% of potentiation in MS KW animals, $n=4$, $P>0.05$ versus CTR; Figure 2d).

In order to better characterize the plastic changes observed in the electrophysiological studies, a 3D morphological analysis of dendritic arborizations of CA1 pyramidal neurons was performed. Data revealed a significant treatment effect in the total length of apical dendrites of pyramidal neurons ($F=7.371$, $P<0.001$), and in the total number of apical dendrite ramifications ($F=9.272$, $P<0.001$); *post-hoc* analysis showed that MS induced a significant decrease in the total length of apical dendrites when compared with CTR ($P<0.05$) (Figure 2e). Similarly, MS pyramidal neurons had significantly less ramifications in apical dendrites when compared with CTR ($P<0.05$). Both parameters were restored by KW6002 treatment ($P>0.05$ versus CTR). There were no significant differences in the structure or number of basal dendrites. Sholl analysis showed coherent changes in the pattern of ramification ($F=5.691$, $P<0.001$). The changes in apical dendrite arborizations observed in MS animals when compared with CTR ($P<0.05$) were reverted by KW6002 (Supplementary Figure S2).

To further evaluate the consequences to the overall morphology we undertook an estimation of hippocampal formation volumes. Our data revealed that neither MS nor KW6002 treatment significantly affected volumetric estimates (Supplementary Table S1).

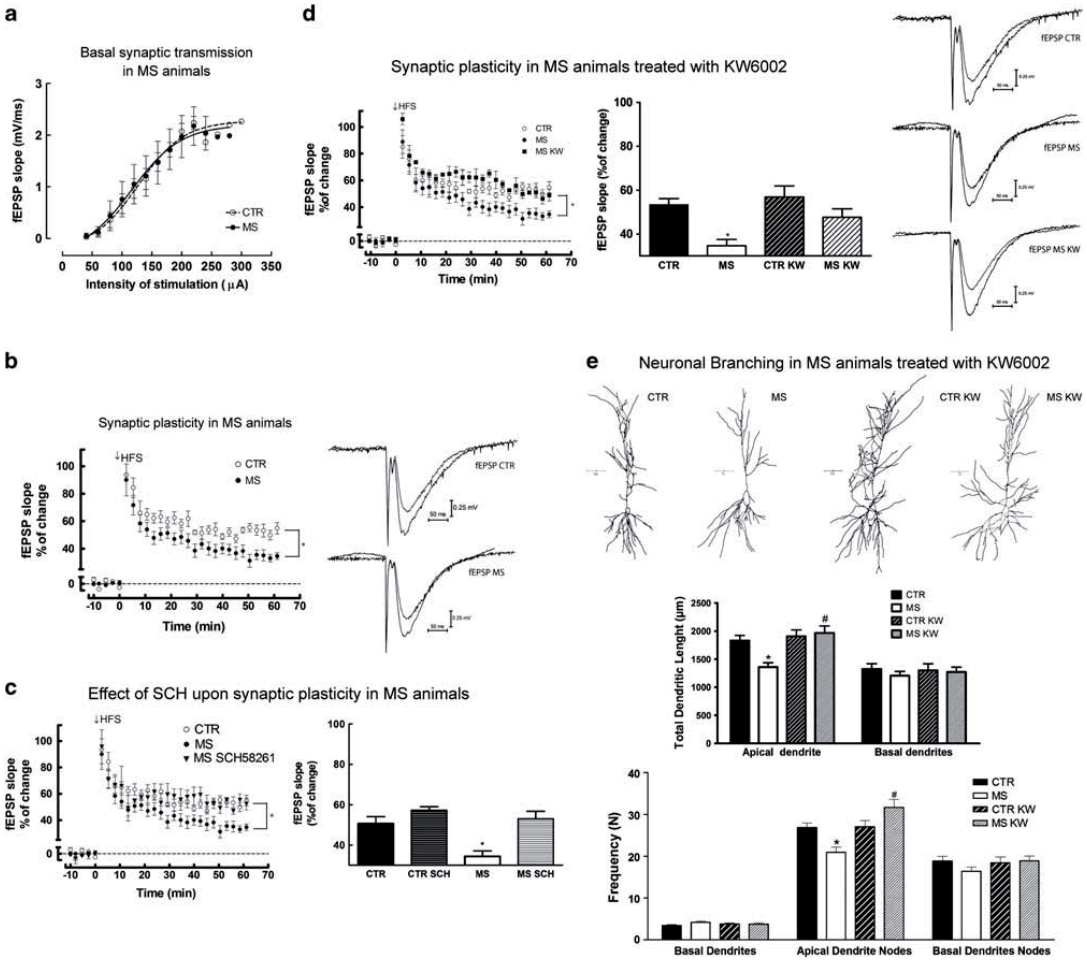

Figure 2 Involvement of adenosine A_{2A} receptors in the synaptic changes induced by MS. (**a**) I/O curves performed to evaluate synaptic transmission in CTR and MS animals and (**b**) LTP (high frequency stimulation, 100 Hz,1 s), used to evaluate synaptic plasticity. Representative recordings of the fEPSPs obtained both for CTR and MS animals before LTP induction and in the last 10 min are presented. The effect of SCH58261 (50 nM) application for 30 min before LTP induction and throughout the protocol is shown in (**c**). The outcome of KW6002 treatment upon LTP is in (**d**) with representative recordings of the fEPSPs obtained for CTR, MS and MS + KW6002 animals, before LTP induction and 1 h after LTP. Bar graphs are obtained by making the average of the last five timepoints of each experiment. Results are the mean ± s.e.m. of 3 (**a**) or 4–7 experiments $^{*}P<0.05$, comparing with CTR. (**e**) Administration of KW6002 reverses dendritic atrophy induced by MS in CA1 pyramidal neurons. Upper panel depicts representative schematics of 3D reconstructions of CTR, MS, CTR KW and MS KW CA1 neurons. $^{*}P<0.05$, comparing with CTR, $^{\#}P<0.05$, comparing with MS, calculated using a one-way ANOVA followed by a Bonferroni's Multiple Comparison Test.

Oral administration of a selective A_{2A} receptor antagonist reverts the stress-induced anxious behavior and learning-deficits

We then evaluate the extent to which $A_{2A}R$ are involved in the stress-induced behavior alterations, by the administration of the $A_{2A}R$-selective antagonist, KW6002, to adult MS animals.

Anxious behavior and hippocampal-dependent memory were evaluated by the EPM and the MWM paradigms, respectively. In the EPM, MS animals presented a higher anxious-related behavior (spent less time in the open arms, 11.4 ± 2.0% versus 28.9 ± 5.3% in CTR, $n = 8$–11, $P < 0.05$), validating the MS stress induction. The hyperanxious behavior in MS animals was reverted upon treatment with KW6002 (time in open arms: 27.3 ± 4.4%, $n = 8$, $P > 0.05$ versus CTR; Figure 3a). KW6002 treatment by itself had no effect in the anxious behavior of CTR

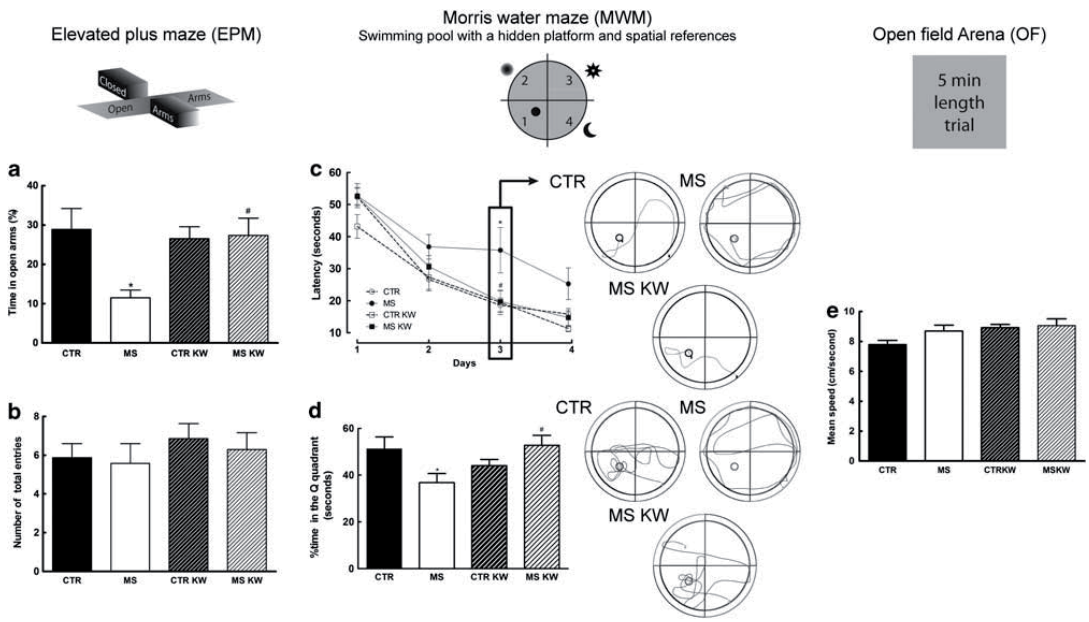

Figure 3 Administration of KW6002 reverts the stress-induced anxious behavior and learning deficits. Anxious behavior (**a**, **b**) and locomotor activity (**e**) were evaluated by the elevated-plus-maze-test and open-field, respectively. Hippocampal-dependent memory performance was assessed by the MWM test, in which acquisition (**c**) and retention (**d**) were evaluated. Results are the mean ± s.e.m. of 6–9 animals; $^{*}P<0.05$, comparing with CTR, $^{\#}P<0.05$ comparing with MS, calculated using two-way ANOVA repeated measures (**a**) or one-way ANOVA followed by a Bonferroni's Multiple Comparison Test.

animals (26.5 ± 3%, $n=7$, $P>0.05$), neither had an impact in locomotor performance in EPM (Figure 3b). On the MWM, the learning ability (Figure 3c) of MS animals was impaired, so that at day three MS performed worse than CTR animals ($F(3,132)=8.56$, $n=6$, $P<0.0001$). These deficits were reverted by blocking $A_{2A}R$ *in vivo* ($n=10$, $P<0.05$). The retention ability of MS animals was also compromised, as in the probe test MS animals spent less time in the platform quadrant than CTR (36.8 ± 3.9%, $n=8$; $P<0.05$ versus 51.3 ± 5.0%, $n=7$; Figure 3d). When treated with the $A_{2A}R$ antagonist the retention ability of MS animals was restored (52.8 ± 4.2% of the time in the platform quadrant; $n=8$, $P>0.05$; Figure 3d). KW6002 by itself had no effect in the performance of CTR animals.

$A_{2A}R$ are highly abundant in striatum exerting important effects in motor control.[46] To evaluate directly locomotor activity, the open-field arena test was used. In accordance with data obtained in the EPM, neither MS nor the KW6002 treatment induced changes in the locomotor performance of the animals, as no alterations were observed in the mean speed on the open-field arena (Figure 3e).

Oral administration of a selective A_{2A} receptor antagonist re-establishes stress-induced modifications on synaptic markers

Given the positive effects of the *in vivo* KW6002 treatment in behavior, in *ex vivo* synaptic plasticity and in neuronal morphology, we next explored the molecular changes that could underlie the observed therapeutic effects. The levels of $A_{2A}R$ and GR were measured in the hippocampus of CTR and MS animals treated with KW6002. Given the role of AMPA, $GABA_A$ and NMDA receptors in synaptic transmission and plasticity, AMPA-GluR1, $GABA_A$-$\beta_{2/3}$ and NMDAR2B subunits levels were also evaluated. AMPA-GluR1 levels in MS animals were significantly decreased comparing with CTR (0.81 ± 0.02, $n=9$, $P<0.05$; Figure 4a). These values were re-established by KW6002 (0.98 ± 0.03, $n=4$, $P>0.05$ versus CTR). $GABA_A$-$\beta_{2/3}$ levels decreased in MS animals (0.78 ± 0.02, $n=8$; $P<0.05$; Figure 4b) and increased to 1.15 ± 0.06 of CTR ($n=6$, $P<0.05$) upon KW6002 treatment. The levels of the NMDAR2B subunit were not altered by MS nor by KW6002 treatment ($n=5$; Figure 4c). Furthermore, the increased levels of $A_{2A}R$ observed in MS animals (1.49 ± 0.04-fold to CTR, $n=9$; $P<0.05$) were maintained in MS KW animals (1.56 ± 0.05-fold to CTR, $n=5$, $P>0.05$; Figure 4d). The KW6002 administration alone increased the levels of $A_{2A}R$ to 1.25 ± 0.09 of CTR ($n=5$, $P<0.01$; Figure 4d), as could be expected from a chronic administration of receptor antagonist. The GR levels (Figure 3e) were not changed by KW6002 in CTR animals ($n=7$, $P>0.05$); however, when KW6002 was administered to MS animals the levels of GR increased to values similar to CTR (1.033 ± 0.04, $n=6$, $P>0.05$).

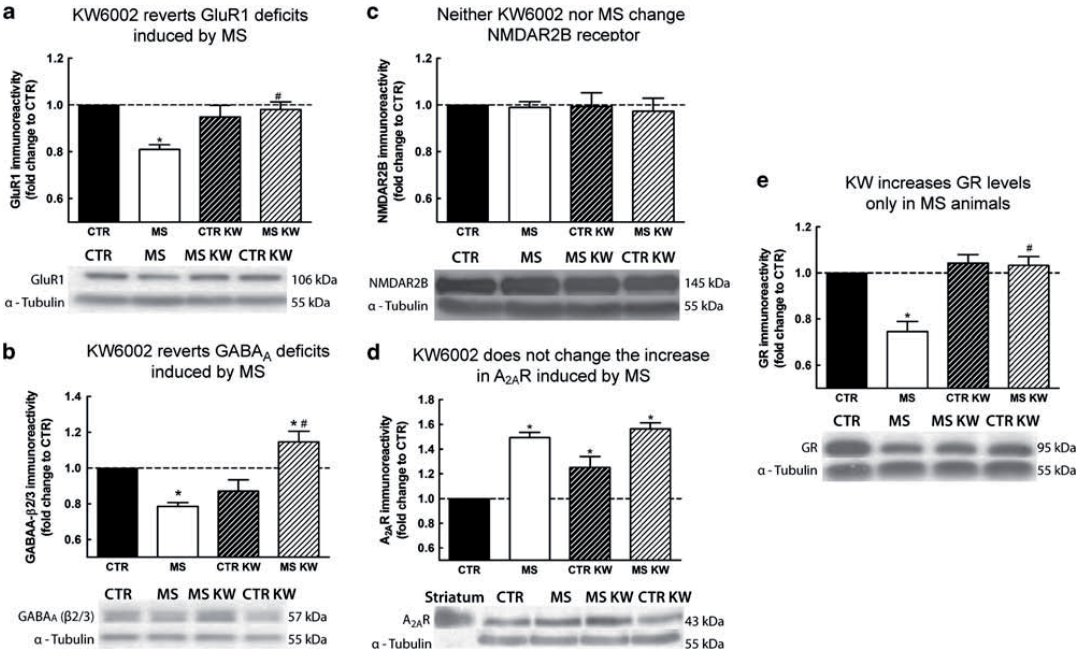

Figure 4 Administration of KW6002 reverts the synaptic changes induced by MS. The effects of the treatment with KW6002 in the levels of GluR1 subunit of AMPA receptors (**a**), β2/3 subunit of $GABA_A$ receptors (**b**), NMDAR2B (**c**), A_{2A}R (**d**), and GR (**e**) were evaluated by western blotting. Specific immunoreactivity normalized to that of α-tubulin. Results are the mean ± s.e.m. of 4–9 experiments; $^*P<0.05$, comparing with CTR and $^{\#}P<0.05$, comparing with MS, calculated using a one-way ANOVA followed by a Bonferroni's Multiple Comparison Test.

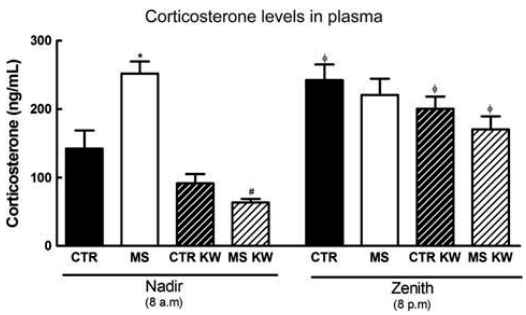

Figure 5 The KW6002 administration re-establishes the corticosterone circadian oscillation. Corticosterone levels in the plasma measured at nadir and zenith. Results are mean of 6–9 animals.$^*P<0.05$, comparing with CTR, $^{\#}P<0.05$ comparing with MS, $^{\phi}P<0.05$, comparing with am values, calculated using a one-way ANOVA followed by a Bonferroni's Multiple Comparison Test.

The A_{2A}R antagonist re-establishes the corticosterone circadian oscillation

As the blockade of A_{2A}R reestablishes the GR levels in the hippocampus, we hypothesized that this could involve a regulation of the HPA-axis function, which is compromised due to the early life stress.[14] HPA-axis activity was evaluated by measuring circadian changes in plasmatic corticosterone levels (Figure 5).

CTR animals had the expected circadian oscillation, with corticosterone levels significantly elevated at 8 pm comparing with those measured at 8 am MS animals present significantly higher corticosterone levels already at 8 am (234 ± 13 ng ml^{-1} versus 142 ± 27 ng ml^{-1}; $P<0.05$, $n=9$) comparing with CTR at the same time of the day, and the absence of a circadian oscillation. Animals treated with KW6002 had a restored circadian variation, with plasmatic corticosterone levels at 8 am similar to CTR (63 ± 5 ng ml^{-1}), ($F(3,24)=9.04$, $P=0.0003$). KW6002 alone did not affect corticosterone levels, neither at zenith nor at nadir.

Discussion

The data now reported reveal that adenosine A_{2A} receptor activation is directly involved in the stress deleterious effects in the brain. We show, for the first time, that the administration of a selective adenosine A_{2A} receptor antagonist reverts the long-lasting consequences of stress on spatial memory, synaptic plasticity and neuronal morphology in the hippocampus. Moreover, our data indicate that these effects are

associated with the re-establishment of the HPA-axis activity.

An imbalance in adenosine receptors has been observed in multiple conditions,[21] particularly with progressive aging,[18,19] which has consequences to their modulatory effects.[19,41] In the aged rat brain, adenosine A_1 receptor density is decreased,[47] particularly in hippocampus and cortex.[18] However, A_{2A} receptor levels are differently affected: they decrease in striatum, but in contrast there is an increase in their expression in cortical and hippocampal areas.[18] As we now show, the changes in adenosine receptor levels induced by MS, follow a close pattern to the one occurring in the aged brain, that is, an increase in A_{2A} and a decrease in A_1 receptor levels. The modifications observed are, however, restricted to the hippocampus, probably due to the changes in GR levels that are more profound in this brain area. Thus, as observed in aging,[19,48] MS induces a decrease in GR levels, increasing the MR/GR ratio, an increase in plasma corticosterone levels and changes in adenosine receptor levels. Thereupon our data reinforce the hypothesis that stress is associated with an early aging in the hippocampal area.[2,49] Different brain regions have a distinct vulnerability to stress due to the differential expression of GR and MR in the brain.[50] In the hippocampus, corticosterone is able to trigger signaling pathways activated by both GR and MR due to their particular high-affinity ratio for GR, which does not occur in other brain areas.[50] This confers to the hippocampus a particular susceptibility to stress effects and consequent deficits. Additional region-specific effects were reported previously, such as alterations in $GABA_A$ receptor levels and MAP kinase activity.[51,52]

We have observed a sustained increase in the plasmatic levels of corticosterone, a feature that is also shared with ageing.[53] Such an increase is usually associated to a downregulation in GR,[11,54,55] as a way to limit their action. This is generally an isolated and reversible effect, reverted whenever the plasma levels of corticosterone return to baseline.[55] However, as we show, MS animals exhibit elevated plasma corticosterone levels throughout life and an associated sustained downregulation of GR in the hippocampus. These receptors regulate memory and synaptic plasticity.[45,56] Accordingly, we found that LTP is impaired in MS animals and this is accompanied by a poorer performance in a spatial memory task, the MWM. The observed changes in synaptic plasticity can be related to the altered levels in $GABA_A$ and AMPA receptors, reported here. Others have described that MS induces a decrease in markers of synaptic plasticity, such as NCAM or synaptophysin,[43] as well as in the levels of NMDAR2B, AMPAGluR1 and GluR2[44] in the hippocampus. Changes now observed in glutamate receptor levels had, however, no impact upon basal synaptic transmission, possibly because they are accompanied by a decrease in $GABA_A$ receptors, which will result in a final compensatory balance in order to maintain homeostasis.

The observed impairments in LTP were overcome by blocking adenosine A_{2A} receptors. These receptors are known to have stimulatory effects on basal synaptic transmission in the hippocampus[17,41] by promoting glutamate release,[57] and were recently shown to potentiate LTP when exogenously activated.[58] The protective effect of administering A_{2A} receptor antagonists *in vivo* has already been described.[21] By contrast, we have observed before that, in particular conditions, the acute treatment of slices with SCH58261 may instead cause a LTP drop. This is particular to overexcitability conditions, such as ageing, in which LTP is enhanced[29,59] due to an age-induced shift in A_{2A} receptor signaling.[19,41] However, in more chronic patho-physiological situations, in which LTP is decreased, the SCH58261 is able to promote its restoration,[60] in accordance with what we now report for stress-induced deficits.

More importantly, the chronic administration of a selective antagonist, KW6002, for 1 month, clearly re-established the MS-driven impairment in LTP. However, the A_{2A} receptor antagonist did not alter LTP in CTR animals, whereas clearly promoting the recovery of the impaired LTP, but only in MS animals. This suggests that, rather than having a direct effect on glutamatergic transmission, A_{2A} receptors may be instead modulating the GR-mediated effects. Indeed, we have recent data showing the ability of A_{2A} receptors to influence GR transcriptional activity and nuclear translocation.[61] Thus, the chronic blockade of A_{2A} receptor may decrease GR transcriptional activity and thereby the overall GR-driven effects.

Genetic deletion of A_{2A} receptors affects anxiety and aggressive behavior,[62] and this constituted the first evidence that A_{2A} receptors could be implicated in stress. The subsequent report that A_{2A} receptor antagonists applied before and during a single episode of acute stress prevented synaptic loss,[22] suggested that A_{2A} receptors overactivation could underlie the genesis of stress-induced changes. Nonetheless, the question whether this overactivation is a consequence of the stress paradigm or a triggering factor to the observed deficits has never been addressed before.

We now explored the possibility that blocking the action of A_{2A} receptors would restore pre-existing stress-associated impairments. The advantage of using KW6002 over other antagonists for A_{2A} receptors is its enhanced bioavailability, permeability to the brain blood barrier, having a longer half-life and high affinity and selectivity towards A_{2A} receptors.[27] Additionally, KW6002 has undergone clinical trials for Parkinson's and therefore its safety has been established.[63] We now report that oral administration of KW6002, for 1 month, to adult animals previously subjected to MS, reestablishes impaired hippocampal-dependent memory, synaptic plasticity and morphology, and reverts the anxious behavior. The learning ability of MS animals was restored by the treatment, as well as the retrieval, evaluated by the time spent in the previous retained platform

quadrant. This is associated with a re-establishment of the hippocampal CA1-induced LTP. The insertion of AMPA receptors containing $GluR_1$ subunit is a constitutive part of LTP induction[64] and is modulated by GR.[65] We found that GluR1-subunit is decreased by MS, which may explain the decreased LTP. Moreover, the LTP re-establishment is accompanied by a concomitant restoration of GluR1 levels upon KW6002 treatment. Accordingly, MS leads to a decrease in apical dendritic length, as described using other stress models,[66] but this structural effect is reverted by the blockade of A_{2A} receptors.

The HPA-axis maintains the physiological circadian oscillation of corticosterone levels, which reach their maximum at zenith (8 pm) and the minimum at nadir (8 am), for rodents.[67] The hippocampus is crucial in the negative feedback required to limit HPA-axis activation, particularly in stressful situations.[1,2] However, this function can be compromised when glucocorticoid levels are persistently high as in chronic stress, aging or in psychopathologies.[48] The observation that the A_{2A} receptor antagonist was able to re-establish the decreased GR levels in the hippocampus lead us to test whether the observed effects were related to a modification of the HPA axis, by measuring the circadian levels of corticosterone in plasma. MS animals present not only higher plasmatic levels of corticosterone, but also an impaired circadian fluctuation. Corticosterone levels were chronically higher in MS animals and did not decrease along the night. This is probably associated to an impaired inhibition of the HPA-axis, which is consequence of a decrease in hippocampal GR levels. Interestingly, by blocking A_{2A} receptors, the basal levels of corticosterone were re-established, so as the circadian rhythm and the GR levels in the hippocampus. Altogether, these data suggest that A_{2A} receptors have a role in the regulation of the HPA-axis, either directly or by regulating hippocampal function. This effect may be due to interference with the release of corticotrophin-releasing hormone, adrenocorticotrophin, which is known to be affected by adenosine,[68] or by modulating glucocorticoids. The beneficial effect resulting from A_{2A} receptor antagonism may derive instead from a re-establishment of hippocampal excitability, which in turn would restore the inhibitory tonus onto the HPA-axis. Overall, the blockade of adenosine A_{2A} receptors by KW6002 has a beneficial effect in overcoming the hippocampal-related deficits induced by MS. Interestingly, this effect of KW6002 *in vivo* does not result from a decrease in $A_{2A}R$ levels, which remain high in MS animals under KW6002. Indeed, it would be unlikely that KW6002 would cause a decrease in $A_{2A}R$ levels as prolonged blockade of receptors usually leads to either no change or upregulation of receptor levels due to compensatory mechanisms following restraining from receptor activation by the endogenous ligand. Remarkably, the findings that blockade of $A_{2A}R$ overcomes the synaptic and memory deficits associated to MS, strongly suggests that A_{2A} receptors overactivation is the cause rather than the consequence of the herein reported changes associated with chronic stress.

In conclusion, our results show, for the first time, that the changes induced by stress are reverted by the *in vivo* blockade of A_{2A} receptors. Moreover, they imply a role of $A_{2A}R$ in the HPA-axis regulation revealing that its blockade is efficient in re-establishing the compromised HPA-axis, which has clinical implications for the treatment of psychopathologies. This provides a potential alternative to the established therapies against stress-related pathologies, by targeting a modulatory system rather than interfering directly with neurotransmitters, and thereby limiting the associated side-effects.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We acknowledge Alexandre de Mendonça, David Blum and Rodrigo Cunha for helpful discussions. VLB is thankful to João Baião and Carla Batalha for technical assistance. VLB has been awarded a PhD fellowship from Fundação para a Ciência e Tecnologia (BD/63041/2009). LVL is funded by Fundação para a Ciência e Tecnologia (PTDC/SAU-NEU/099853/2008) and by EU programme Égide-Pessoa. YB and CEM were funded by the Ministry for Education and Research (BMBF, Grant number 01EW0911) in the frame of ERA-NET NEURON.

References

1 McEwen BS. Stress and hippocampal plasticity. *Annu Rev Neurosci* 1999; **22**: 105–122.
2 McEwen BS. Stress and the aging hippocampus. *Front Neuroendocrinol* 1999; **20**: 49–70.
3 McKinney WT. Animal models of depression: an overview. *Psychiatr Dev* 1984; **2**: 77–96.
4 Willner P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology (Berl)* 1997; **134**: 319–329.
5 Sullivan R, Wilson DA, Feldon J, Yee BK, Meyer U, Richter-Levin G *et al*. The International Society for Developmental Psychobiology annual meeting symposium: impact of early life experiences on brain and behavioral development. *Dev Psychobiol* 2006; **48**: 583–602.
6 Heim C, Nemeroff CB. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biol Psychiatry* 2001; **49**: 1023–1039.
7 Ladd CO, Huot RL, Thrivikraman KV, Nemeroff CB, Meaney MJ, Plotsky PM. Long-term behavioral and neuroendocrine adaptations to adverse early experience. *Prog Brain Res* 2000; **122**: 81–103.
8 Morris RG. Long-term potentiation and memory. *Philos Trans R Soc Lond B Biol Sci* 2003; **358**: 643–647.
9 de Kloet ER, Oitzl MS, Joels M. Stress and cognition: are corticosteroids good or bad guys? *Trends Neurosci* 1999; **22**: 422–426.
10 Amaral DG, Dent JA. Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. *J Comp Neurol* 1981; **195**: 51–86.

11 Sapolsky RM, Meaney MJ. Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. *Brain Res* 1986; **396**: 64–76.
12 Kaufman J, Plotsky PM, Nemeroff CB, Charney DS. Effects of early adverse experiences on brain structure and function: clinical implications. *Biol Psychiatry* 2000; **48**: 778–790.
13 Bakshi VP, Kalin NH. Corticotropin-releasing hormone and animal models of anxiety: gene-environment interactions. *Biol Psychiatry* 2000; **48**: 1175–1198.
14 Aisa B, Tordera R, Lasheras B, Del Rio J, Ramirez MJ. Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology* 2007; **32**: 256–266.
15 Anisman H, Zaharia MD, Meaney MJ, Merali Z. Do early-life events permanently alter behavioral and hormonal responses to stressors? *Int J Dev Neurosci* 1998; **16**: 149–164.
16 Lehmann J, Stohr T, Feldon J. Long-term effects of prenatal stress experiences and postnatal maternal separation on emotionality and attentional processes. *Behav Brain Res* 2000; **107**: 133–144.
17 Cunha RA, Johansson B, van der Ploeg I, Sebastiao AM, Ribeiro JA, Fredholm BB. Evidence for functionally important adenosine A2a receptors in the rat hippocampus. *Brain Res* 1994; **649**: 208–216.
18 Cunha RA, Constantino MC, Sebastiao AM, Ribeiro JA. Modification of A1 and A2a adenosine receptor binding in aged striatum, hippocampus and cortex of the rat. *Neuroreport* 1995; **6**: 1583–1588.
19 Lopes LV, Cunha RA, Ribeiro JA. Increase in the number, G protein coupling, and efficiency of facilitatory adenosine A2A receptors in the limbic cortex, but not striatum, of aged rats. *J Neurochem* 1999; **73**: 1733–1738.
20 Rebola N, Sebastiao AM, de Mendonca A, Oliveira CR, Ribeiro JA, Cunha RA. Enhanced adenosine A2A receptor facilitation of synaptic transmission in the hippocampus of aged rats. *J Neurophysiol* 2003; **90**: 1295–1303.
21 Cunha RA. Neuroprotection by adenosine in the brain: from A(1) receptor activation to A (2A) receptor blockade. *Purinergic Signal* 2005; **1**: 111–134.
22 Cunha GM, Canas PM, Oliveira CR, Cunha RA. Increased density and synapto-protective effect of adenosine A2A receptors upon sub-chronic restraint stress. *Neuroscience* 2006; **141**: 1775–1781.
23 Belanoff JK, Gross K, Yager A, Schatzberg AF. Corticosteroids and cognition. *J Psychiatr Res* 2001; **35**: 127–145.
24 Belanoff JK, Kalehzan M, Sund B, Fleming Ficek SK, Schatzberg AF. Cortisol activity and cognitive changes in psychotic major depression. *Am J Psychiatry* 2001; **158**: 1612–1616.
25 Daniels WM, Pietersen CY, Carstens ME, 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* 2004; **19**: 3–14.
26 Lopes LV, Marvin-Guy LF, Fuerholz A, Affolter M, Ramadan Z, Kussmann M *et al*. Maternal deprivation affects the neuromuscular protein profile of the rat colon in response to an acute stressor later in life. *J Proteomics* 2008; **71**: 80–88.
27 Yang M, Soohoo D, Soelaiman S, Kalla R, Zablocki J, Chu N *et al*. Characterization of the potency, selectivity, and pharmacokinetic profile for six adenosine A2A receptor antagonists. *Naunyn Schmiedebergs Arch Pharmacol* 2007; **375**: 133–144.
28 Morris RG, Garrud P, Rawlins JN, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. *Nature* 1982; **297**: 681–683.
29 Diogenes MJ, Costenla AR, Lopes LV, Jeronimo-Santos A, Sousa VC, Fontinha BM *et al*. Enhancement of LTP in aged rats is dependent on endogenous BDNF. *Neuropsychopharmacology* 2011; **36**: 1823–1836.
30 Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 1985; **14**: 149–167.
31 Gibb R, Kolb B. A method for vibratome sectioning of Golgi-Cox stained whole rat brain. *J Neurosci Methods* 1998; **79**: 1–4.
32 Keuker JI, Vollmann-Honsdorf GK, Fuchs E. How to use the optical fractionator: an example based on the estimation of neurons in the hippocampal CA1 and CA3 regions of tree shrews. *Brain Res Brain Res Protoc* 2001; **7**: 211–221.
33 Madeira MD, Pereira A, Cadete-Leite A, Paula-Barbosa MM. Estimates of volumes and pyramidal cell numbers in the prelimbic subarea of the prefrontal cortex in experimental hypothyroid rats. *J Anat* 1990; **171**: 41–56.
34 Glaser EM, Van der Loos H. Analysis of thick brain sections by obverse-reverse computer microscopy: application of a new, high clarity Golgi-Nissl stain. *J Neurosci Methods* 1981; **4**: 117–125.
35 Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. 3rd edn. Academic Press: Waltham, MA, USA, 1998.
36 Palomero-Gallagher N, Zilles K. The rat isocortex. In: Paxinos G (ed). *The Rat Nervous System*. Academic Press: San Diego, CA, USA, 2004; 728–760.
37 Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N *et al*. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* 1988; **96**: 857–881.
38 Palacios SD, Pak K, Rivkin AZ, Kayali AG, Austen D, Aletsee C *et al*. Role of p38 mitogen-activated protein kinase in middle ear mucosa hyperplasia during bacterial otitis media. *Infect Immun* 2004; **72**: 4662–4667.
39 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
40 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
41 Lopes LV, Cunha RA, 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* 1999; **82**: 3196–3203.
42 Hockemeyer J, Burbiel JC, Muller CE. Multigram-scale syntheses, stability, and photoreactions of A2A adenosine receptor antagonists with 8-styrylxanthine structure: potential drugs for Parkinson's disease. *J Org Chem* 2004; **69**: 3308–3318.
43 Aisa B, Elizalde N, Tordera R, Lasheras B, Del Rio J, Ramirez MJ. Effects of neonatal stress on markers of synaptic plasticity in the hippocampus: implications for spatial memory. *Hippocampus* 2009; **19**: 1222–1231.
44 Pickering C, Gustafsson L, Cebere A, Nylander I, Liljequist S. Repeated maternal separation of male Wistar rats alters glutamate receptor expression in the hippocampus but not the prefrontal cortex. *Brain Res* 2006; **1099**: 101–108.
45 Joels M. Corticosteroid effects in the brain: U-shape it. *Trends Pharmacol Sci* 2006; **27**: 244–250.
46 Janusz CA, Berman RF. The A2-selective adenosine analog, CGS 21680, depresses locomotor activity but does not block amygdala kindled seizures in rats. *Neurosci Lett* 1992; **141**: 247–250.
47 Meerlo P, Roman V, Farkas E, Keijser JN, Nyakas C, Luiten PG. Ageing-related decline in adenosine A1 receptor binding in the rat brain: an autoradiographic study. *J Neurosci Res* 2004; **78**: 742–748.
48 Pardon MC, 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* 2008; **32**: 1103–1120.
49 Miller DB, O'Callaghan JP. Aging, stress and the hippocampus. *Ageing Res Rev* 2005; **4**: 123–140.
50 Reul JM, de Kloet ER. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* 1985; **117**: 2505–2511.
51 Gruen RJ, Wenberg K, Elahi R, Friedhoff AJ. Alterations in GABAA receptor binding in the prefrontal cortex following exposure to chronic stress. *Brain Res* 1995; **684**: 112–114.
52 Meller E, Shen C, Nikolao TA, Jensen C, Tsimberg Y, Chen J *et al*. Region-specific effects of acute and repeated restraint stress on the phosphorylation of mitogen-activated protein kinases. *Brain Res* 2003; **979**: 57–64.
53 Mizoguchi K, Ikeda R, Shoji H, Tanaka Y, Maruyama W, Tabira T. Aging attenuates glucocorticoid negative feedback in rat brain. *Neuroscience* 2009; **159**: 259–270.
54 Sapolsky RM, Meaney MJ, McEwen BS. The development of the glucocorticoid receptor system in the rat limbic brain. III. Negative-feedback regulation. *Brain Res* 1985; **350**: 169–173.

55 Sapolsky RM, Krey LC, McEwen BS. Stress down-regulates corticosterone receptors in a site-specific manner in the brain. *Endocrinology* 1984; **114**: 287–292.
56 Sousa N, Cerqueira JJ, Almeida OF. Corticosteroid receptors and neuroplasticity. *Brain Res Rev* 2008; **57**: 561–570.
57 Lopes LV, Cunha RA, Kull B, Fredholm BB, Ribeiro JA. Adenosine A(2A) receptor facilitation of hippocampal synaptic transmission is dependent on tonic A(1) receptor inhibition. *Neuroscience* 2002; **112**: 319–329.
58 Dias RB, Ribeiro JA, Sebastiao AM. Enhancement of AMPA currents and GluR1 membrane expression through PKA-coupled adenosine A(2A) receptors. *Hippocampus* 2012; **22**: 276–291.
59 Costenla AR, Diogenes MJ, Canas PM, Rodrigues RJ, Nogueira C, Maroco J *et al.* Enhanced role of adenosine A(2A) receptors in the modulation of LTP in the rat hippocampus upon ageing. *Eur J Neurosci* 2011; **34**: 12–21.
60 Rebola N, Carta M, Lanore F, Blanchet C, Mulle C. NMDA receptor-dependent metaplasticity at hippocampal mossy fiber synapses. *Nat Neurosci* 2011; **14**: 691–693.
61 Batalha VL, Hamdane M, Sebastião AM, Buée L, Blum D, Lopes LV. Modulation of glucocorticoid receptor (GR) transcriptional activity by adenosine A2A receptors: consequences for stress response. *Champalimaud Neurosci Symp* 2011; http://symposium.neuro.fchampalimaud.org/files/CNP_symp_Abstracts_2.pdf.
62 Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ *et al.* Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* 1997; **388**: 674–678.
63 Hauser RA, Hubble JP, Truong DD. Randomized trial of the adenosine A(2A) receptor antagonist istradefylline in advanced PD. *Neurology* 2003; **61**: 297–303.
64 Andrasfalvy BK, Smith MA, Borchardt T, Sprengel R, Magee JC. Impaired regulation of synaptic strength in hippocampal neurons from GluR1-deficient mice. *J Physiol* 2003; **552**(Part 1): 35–45.
65 Martin S, Henley JM, Holman D, Zhou M, Wiegert O, van Spronsen M *et al.* Corticosterone alters AMPAR mobility and facilitates bidirectional synaptic plasticity. *PLoS One* 2009; **4**: e4714.
66 Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA *et al.* The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol Psychiatry* 2009; **14**: 764–773, 739.
67 Allen C, Kendall JW. Maturation of the circadian rhythm of plasma corticosterone in the rat. *Endocrinology* 1967; **80**: 926–930.
68 Anand-Srivastava MB, Cantin M, Gutkowska J. Adenosine regulates the release of adrenocorticotropic hormone (ACTH) from cultured anterior pituitary cells. *Mol Cell Biochem* 1989; **89**: 21–28.

Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)

Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2012 | 123 | 1030–1040 doi: 10.1111/jnc.12050

ORIGINAL ARTICLE

Neuroprotection afforded by adenosine A_{2A} receptor blockade is modulated by corticotrophin-releasing factor (CRF) in glutamate injured cortical neurons

Jorge S. Valadas,*,† Vânia L. Batalha,*,† Diana G. Ferreira,*,† Rui Gomes,†,‡ Joana E. Coelho,*,† Ana M. Sebastião,*,† Maria José Diógenes*,† and Luísa V. Lopes*,†

*Institute of Pharmacology and Neurosciences, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

†Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

‡Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal

Abstract

In situations of hypoxia, glutamate excitotoxicity induces neuronal death. The release of extracellular adenosine is also triggered and is accompanied by an increase of the stress mediator, corticotrophin-releasing factor (CRF). Adenosine A_{2A} receptors contribute to glutamate excitoxicity and their blockade is effective in stress-induced neuronal deficits, but the involvement of CRF on this effect was never explored. We now evaluated the interaction between A_{2A} and CRF receptors (CRFR) function, upon glutamate insult. Primary rat cortical neuronal cultures (9 days *in vitro*) expressing both CRF_1R and CRF_2R were challenged with glutamate (20–1000 μM, 24 h). CRF_1R was found to co-localize with neuronal markers and CRF_2R to be present in both neuronal and glial cells. The effects of the CRF and A_{2A} receptors ligands on cell viability were measured using propidium iodide and Syto-13 fluorescence staining. Glutamate decreased cell viability in a concentration-dependent manner. Urocortin (10 pM), an agonist of CRF receptors, increased cell survival in the presence of glutamate. This neuroprotective effect was abolished by blocking either CRF_1R or CRF_2R with antalarmin (10 nM) or anti-Sauvagine-30 (10 nM), respectively. The blockade of A_{2A} receptors with a selective antagonist SCH 58261 (50 nM) improved cell viability against the glutamate insult. This effect was dependent on CRF_2R, but not on CRF_1R activation. Overall, these data show a protective role of CRF in cortical neurons, against glutamate-induced death. The neuroprotection achieved by A_{2A} receptors blockade requires CRF_2R activation. This interaction between the adenosine and CRF receptors can explain the beneficial effects of using A_{2A} receptor antagonists against stress-induced noxious effects.

Keywords: adenosine A_{2A} receptors, corticotrophin-releasing factor (CRF), CRF_1R, CRF_2R, hippocampus, neuroprotection. *J. Neurochem.* (2012) **123**, 1030–1040.

Adenosine is a neuronal modulator that binds to different G-protein coupled receptors (Fredholm *et al.* 2001). Among them, the adenosine A_{2A} receptors are attractive pharmacological targets because of their contribution to neuronal excitability, by increasing the release of glutamate (Lopes *et al.* 2002). In situations, where the release of glutamate is exacerbated, neuronal death either by apoptotic or necrotic processes can be detected (Nicotera *et al.* 1999).

Received May 19, 2012; revised manuscript received July 21, 2012; accepted October 7, 2012.

Address correspondence and reprint requests to Luísa V. Lopes, Neurosciences Unit, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Av. Professor Egas Moniz, 1649-028 Lisbon, Portugal. E-mail: lvlopes@fm.ul.pt

Abbreviations used: AMPA, L-α-amino-3-hydroxy-5-methylisoxazole-4-propionate; Ant, antalarmin; a-Sau, anti-Sauvagine-30; Ast, astressin; Aβ, amyloid-β peptide; CRF_1R, CRF receptor of subtype 1; CRF_2R, CRF receptor of subtype 2; CRF, corticotrophin-releasing factor (formerly known by CRH for corticotrophin-releasing hormone); CTR, control; GFAP, glial fibrillary acidic protein; Glu, L-glutamic acid; HPA axis, hypothalamic–pituitary–adrenal axis; MAP2, microtubule-associated protein 2; NMDA, N-methyl-D-aspartic acid; PBS, phosphate buffer saline; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; Urc, urocortin.

In noxious brain conditions, such as accumulation of amyloid-β peptide (Aβ), hypoxic events or upon aging, there is an increase of both extracellular adenosine and adenosine A_{2A} receptors levels in the brain (Latini and Pedata 2001; Rebola *et al.* 2005a; Cunha *et al.* 2006). In such situations, the blockade of A_{2A} receptors can prevent the evoked neurotoxicity (Monopoli *et al.* 1998; Chen *et al.* 1999; Dall'Igna *et al.* 2003; Canas *et al.* 2009). Moreover, memory impairments caused by Aβ are prevented by A_{2A} receptors antagonists (Canas *et al.* 2009). However, the mechanism by which the blockade of A_{2A} receptors is effective in reverting these stressful effects remains unknown.

Stress response, in mammals, is dependent on the activation of the hypothalamic–pituitary–adrenal (HPA) axis. In the sequence of a stress stimulus, such as neurotoxicity induced by glutamate, the corticotrophin-releasing factor (CRF) is released from the hypothalamus, leading to HPA axis activation (Vale *et al.* 1981). CRF, a 41 amino acid peptide, has also important effects in extrahypothalamic sites, namely in thalamus, amygdala, hippocampus, frontal cerebral cortex, striatum, and cerebellum (Fischman and Moldow 1982; Swanson *et al.* 1983). In the hippocampus, CRF is released from inhibitory interneurons (Chen *et al.* 2001), binds to CRF_1R abundant in dendritic spines of pyramidal neurons (Chen *et al.* 2004a, b), and modulates neuronal function and cognition (Radulovic *et al.* 1999). CRF_1R is expressed in forebrain glutamatergic and γ-aminobutyric acid-containing (GABAergic) neurons as well as in midbrain dopaminergic neurons. CRF also binds to CRF_2R (Chen *et al.* 1993; Lovenberg *et al.* 1995). These are predominantly G_s-coupled proteins that use cAMP as intracellular signaling molecule, but they also signal through Gi/o and Gq proteins, with minor involvement (Chen *et al.* 1986; Grammatopoulos *et al.* 2001).

CRF receptors are expressed in several brain regions that include hypothalamic and extrahypothalamic areas (Chalmers *et al.* 1995; Bittencourt *et al.* 1999). The actions of CRF in extrahypothalamic areas are still poorly explored. Although CRF_1R receptor expression is very high in neocortical, cerebellar, and sensory relay structures, CRF_2R receptor expression is generally confined to subcortical structures. The highest levels of CRF_2R receptor mRNA in brain are evident within the lateral septal nucleus, the ventromedial hypothalamic nucleus and the choroid plexus. CRF_2R -expressing cells are also evident, albeit in much lower density in the hippocampal formation and anterior and lateral hypothalmic areas. This heterogeneous distribution of CRF_1R and CRF_2R receptor mRNA suggests distinctive functional roles for each receptor in CRF-related systems (Chalmers *et al.* 1995).

Noteworthy, CRF has a similar effect to that achieved by A_{2A} receptor blockade, by counteracting classic neuronal insults as excitatory amino acids, hypoxia or amyloid-β_{25-35} peptide in cortical neurons (Fox *et al.* 1993; Pedersen *et al.* 2001), but neither the mechanism or subtype of receptor involved are known.

In this study, we investigated the relationship between the neuroprotective effects of adenosine A_{2A} receptors blockade and activation of the two subtypes of CRF receptors. This was assessed using a glutamate insult, a major stress condition that reproduces the excitotoxic events following hypoxia/ischemia or during stroke. We found that, in cultured cortical neurons, CRF avoids cell death induced by glutamate, an effect dependent on the simultaneous activation of both CRF receptors, CRF_1R and CRF_2R. Blockade of A_{2A} receptors is neuroprotective and requires activation of CRF_2R. This interaction between the adenosine and CRF receptors can explain the beneficial effects of using A_{2A} receptor antagonists against stress-induced noxious effects.

Methods

Pharmacological agents

Urocortin and Antalarmin were purchased from Sigma (Madrid, Spain). 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzene propanoic acid (CGS 21680), 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c] pyrimidin-5-amine (SCH 58261), L-Glutamic Acid, Anti-sauvagin-30 and Astressin were purchased from Tocris (UK). $A\beta_{25-35}$ peptide was from Bachem (Bubendorf, Switzerland). These drugs were diluted in the assay solution from stock aliquots made in water or dimethyl sulfoxide stored at −20°C. All other reagents used were of the highest purity available and suitable for cell culture.

Primary rat cortical cultures

Cortical neurons were cultured from 18 days Sprague Dawley rat (Harlan, Barcelona, Spain) embryos according to Pedersen *et al.* 2002. Briefly, pregnant rats were handled according to the Portuguese law on animal care and European Union guidelines (86/609/EEC), and decapitated under deep anesthesia with Halothane. The embryos were collected in Hanks' Balanced Salt Solution and rapidly decapitated. Meninges and white mater were removed and whole cortices were incubated for 15 min in Hanks' Balanced Salt Solution (Calcium 1 mM and Magnesium 1 mM) and 0.025% trypsin. Cells were centrifuged three times and washed with Hanks' Balanced Salt Solution (with Calcium 1 mM and Magnesium 1 mM, 10% fetal bovine serum) and finally re-suspended in Neurobasal Medium. After counted, cells were plated on poly-L-lysine-coated coverslips in 24-well plates at density of 8×10^4 cells/well. Neurons were grown for 9 days at 37°C in a 5% CO2 humidified atmosphere in Neurobasal medium with 2% B-27 supplement, glutamate 25 μM, glutamine 0.5 mM, and 2 U/mL Penicillin/Streptomycin, in the absence of any positive selection for neurons. Medium was totally replaced at day 4 (without glutamate) and 60 min before drug treatment (without glutamate and B-27 supplement). Pure neuronal cultures were obtained by addition at day 3 in culture of 2 μM cytosine arabinoside (Ara-C) and used at day 9.

RT–PCR

Quantitative real time RT-PCR (qPCR) was performed using RNA extracts from pure neuronal cultures. Briefly, neuronal cultures were washed with phosphate buffer saline (PBS) scraped and collected in 0.5 mL Eppendorf vials containing lysis buffer for RNA extraction.

Total RNA was extracted using the RNAspin Mini RNA isolation kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. RNA quantification was determined with nanoDrop 2000 software (Thermo scientific, Wilmington, DE, USA). Reverse transcription was carried out with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Life technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, using both 50 ng/μL random hexamers and 0.5 μg/μL oligo(dT)$_{12\text{-}18}$ in a final volume of 20 μL. Negative controls were made without reverse transcriptase and confirmed the absence of signal. qPCR was carried out with SYBR green PCR Master mix (Applied Biosystems, Warrington, UK), using 2 μL of cDNA in a final volume of 25 μL (4.6 ng/μL of total cDNA), containing 0.3 μM of A2AR primer, 0.2 μM of β-actin primer (reference gene), or 0.4 μM of CRF2 primer. qPCR was performed with a Rotor-Gene 6000 Real Time Rotary Analyzer (Corbett Research, Cambridge, UK) for 45 cycles of 95°C for 20 s, 58°C for 5 s, and a final step of 10 s at 72°C.

The primers used in qPCR include: forward 5′-AACGGCAT CAAGTACAACACGAC-3′ and reverse 5′-CGATTCGGTAATG CAGGTCATAC-3′for CRF2 (product size 142 bp, Invitrogen), forward 5′-ATTCCACTCCGGTACAATGG-3′ and reverse 5′-AGTTGTTCCAGCCCAGCAT-3′ for A2AR (product size 115 bp, Invitrogen) and forward 5′-AGCCATGTACGTAGCCATCC-3′ and reverse 5′-CTCTCAGCTGTGGTGGTGAA-3′ for β-actin (product size 228 bp, Invitrogen). The qPCR products were analyzed by electrophoresis on a 2% agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

Immunocytochemistry

To characterize the primary cortical neuronal cultures with 9 days *in vitro,* cell medium removed, cells were washed with phosphate buffer saline (PBS: NaCl 137 mM, KCl 2.7 mM, KH_2PO_4 1.8 mM and Na_2HPO_4 10 mM, pH 7.4) and fixed for 10 min at 20–23°C with 4% paraformaldehyde in PBS. After washing with PBS, cells were permeabilized with 0.1% Triton-X in PBS, blocked for 30 min with 0.25% gelatine in PBS, washed with PBS 0.05% Tween-20 and incubated for 1 h at 20–23°C with primary antibodies diluted in PBS 0.1% gelatine (mouse anti-MAP2 1 : 200, Millipore-Billerica, MA, USA-MAB3418, rabbit anti-GFAP 1 : 100, Sigma G9269, mouse CD11b 1 : 250, Serotec-Oxford, UK-MCA275R, Goat anti-CRF_1R 1 : 25, Santa Cruz Biotechnology-Santa Cruz, CA, USA-sc 12381 and rabbit anti-CRF_2R 1 : 25, Novus biologicals-Cambridge, UK-nbp1-00767, mouse anti-A2AR 1 : 50, Santa Cruz sc 32261. After washes (PBS 0.05% Tween-20), cells were incubated with secondary antibodies diluted in PBS 0.1% gelatine (anti-mouse Alexa Fluor 568 and anti-rabbit Alexa Fluor 488, both from Invitrogen). 4′,6-diamidino-2-phenylindole (DAPI, 70 μg/mL; Sigma) was used to label cell nucleus (5 min incubation). Coverslips were mounted with MOWIOL (Sigma), and cells were observed either with an Axiovert 200 fluorescence microscope (Carl Zeiss light microscopy, Gottingen, Germany) or Zeiss confocal LSM 710 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Cell treatment

Glutamic acid (or glutamate) was used as neurotoxic insult, concentrations ranging from 20 to 1000 μM were applied for 24 h in primary cultured neuronal cells with 8 days *in vitro* (Tamura *et al.* 1993). Amyloid-$\beta_{25\text{-}35}$ peptide (Aβ, 25 μM) was used as positive control for apoptosis (Estus *et al.* 1997). CRF and adenosine A_{2A} receptors antagonists were applied 15 min before initiating cell insult, while their agonists were added to cell medium immediately prior to glutamate. A_{2A} receptors ligands were used as in Rebola *et al.* (2005b), while CRF receptors agonist and antagonists' use was based on Pedersen *et al.* (2002), Elliott-Hunt *et al.* (2002), and Gulyas *et al.* (1995) reports. Dimethyl sulfoxide concentration in cell medium was always kept below 0.001%.

Propidium iodide and Syto-13 uptake assay

Cells were washed with KREBS-HEPES (NaCl 117 mM, KCl 3 mM, Glucose 10 mM, $NaHCO_3$ 26 mM, Na_2HPO_4 1.25 mM, HEPES 10 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM), incubated with Syto-13 (4 μM, emits preferentially at 509 nm when excited at 488 nm) and propidium iodide (PI, 5 μg/mL, absorbing preferentially at 535 nm and emitting at 617 nm) for 3 min at room temperature and directly observed on Axiovert 200 fluorescence microscope. Three to four arbitrary photographs from each coverslip were shot and an average of 1400 cells was counted per condition in each experiment. Viable cells presented homogeneous cell body labeled with Syto-13, whereas primary and secondary apoptotic cells showed fragmented or condensed nucleus (labeled with Syto-13 or PI). Necrotic cells appeared as diffuse blots, emitting in propidium iodide range (Canas *et al.* 2009). After cell counting (see Fig. 1c), cell viability is presented as the ratio between the number of living cells and the number of total cells counted.

Western blotting

Cells from primary cultures with 9 days *in vitro* were washed with cold PBS. Using NP-40 lysis buffer pH 8.0 (1% Nonidet P40, 150 mM NaCl, 50 mM Tris-base, 1 mM EDTA, 5 mM dithiothreitol, proteases inhibitors - Complete, EDTA-free Protease Inhibitor cocktail tablets; Roche, Manheim, Germany) cells were mechanically scarped. The resulting solution was centrifuged at 16 000 *g* during 10 min at 4°C and pellet was discarded and the supernatant used for western blot. The protein concentration was determined using a BioRad DC Protein assay Kit (based on Lowry *et al.* 1951) because of the high levels of detergents present in the sample. The appropriate volume of each sample was diluted in water and sample buffer (350 mM Tris pH 6.8, 30% glycerol, 10% sodium dodecyl sulfate, 600 mM dithiothreitol and 0.012% Bromophenol blue). The samples were denatured at 95°C for 5 min.

Based on the protocol of Towbin *et al.* (1979), samples and molecular weight markers were separated by sodium dodecyl sulfate –polyacrylamide gel electrophoresis (10% for resolving and 5% for stacking gels) in denaturing conditions and electro-transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% non-fat dry milk for 1 h, washed with TBS-T 0.1% (Tris Buffer Saline with 0.1% Tween-20 solution, 200 nM Tris, 1.5 M NaCl), and incubated with primary antibody (diluted in TBS-T, 3% Bovine Serum Albumin and 0.1% NaN_3) overnight at 4°C. After washing again with TBS-T for 30 min, the membranes were incubated with horseradish peroxidise (HRP, EC 1.11.1.7) conjugated secondary antibody (in 5% non-fat dry milk) for 1 h at 20–23°C, (primary and secondary antibodies: mouse anti-A_{2A} receptor (Upstate/Millipore 05-717), goat anti-CRF_1R (Santa Cruz Biotechnology sc 12381), rabbit anti-CRF_2R (Novus biologicals

nbp1-00767), rabbit anti-caspase-3 (Santa Cruz Biotechnology sc-7148), rabbit anti-tubulin (abcam, Cambridge, UK, ab4074), goat anti-rabbit-HRP (Santa Cruz Biotechnology sc-2004), goat anti-mouse-HRP (Santa Cruz Biotechnology sc-2005), and donkey anti-goat-HRP (Santa Cruz Biotechnology sc-2020). After 40 min of washing with TBS-T, chemoluminescent detection was performed with ECL-PLUS western blotting detection reagent (GE Healthcare) using X-Ray films (Fujifilm, Dusseldorf, Germany). Optical density was determined with Image-J software (NIH, Bethesda, MD, USA).

Statistics

The values presented are mean ± SEM of n independent experiments, corresponding to different cell cultures. In statistical tests between three or more conditions, a one-way ANOVA was used, followed by a Bonferroni's multiple comparison *post-hoc* test. Values of $p < 0.05$ were considered to be statistically significant.

Results

Characterization of the primary neuronal cell cultures

Primary cortical cultures with 9 days *in vitro* were labeled with anti-MAP2 (microtubule-associated protein 2 – neuronal marker) and anti-GFAP (glial fibrillary acidic protein – mature astrocytic marker) antibodies (Fig. 1a). Approximately, 50% of cells were labeled red (MAP2 expression). The presence of astrocytes was less than 20% (green labeled GFAP positive cells). The remaining cells are microglia that express CD11b, a selective marker (labeled in red, Fig. 1b). The cell culture expresses both CRF_1R and CRF_2R, and adenosine A_{2A} receptors (Fig. 1d). CRF_1R are mainly co-localized with neuronal markers (Fig. 1e see also figure S1 panel A), whereas CRF_2R are present in both neuronal and glial cells (astrocytes, Fig. 1f see also figure S1 panel B). We also detected co-expression of mRNA encoding for A_{2A} receptors and CRF_2R in pure neuronal cultures and further confirmed the co-localization of the respective receptor protein by immunocytochemistry (Fig. 1g–h, see also figure S1 panel C).

Cell viability upon glutamate insult

Cell survival upon glutamate insult was evaluated by simultaneous labeling with propidium iodide and Syto-13. The cultures were treated for 24 h with five different glutamate concentrations (20, 50, 100, 500, and 1000 μM) covering several degrees of cell injury which can induce either apoptosis or necrosis (Lipton and Rosenberg 1994; Bonfoco *et al.* 1995). Although propidium iodide is incorporated through the disrupted membrane of dying or dead cells exclusively, emitting red light, Syto-13 is capable of labeling all cells (dead or living cells, emitting green light). Apoptotic cells present either condensed or fragmented nucleus and are labeled in green or red; necrotic cells appear as characteristic red dots. Cell viability was accessed by the percentage of cells that does not present any apoptotic or necrotic markers (see Fig. 1c).

As shown in Fig. 2, incubation for 24 h with glutamate resulted in a reduction of cell viability, in a concentration-dependent manner, with the lowest viability of 66.5 ± 4.23% of control ($p < 0.001$, $n = 4$) produced by the higher glutamate concentration used (1000 μM). This insult was considerably lower when compared to amyloid-$\beta_{25\text{-}35}$ peptide (Aβ, 25 μM), which increases cell death by apoptosis (Estus *et al.* 1997; Kemppainen *et al.* 2012) that caused a 43.1 ± 2.53% reduction in cell viability. We further confirmed that cell death in our experimental conditions occurs mainly in neurons, as illustrated in Fig. 2b (see also figure S2). The density of MAP2 positive cells is decreased in glutamate treated cultures, and 90% of the remaining neurons (350 out of 400 counted neurons) present clear neuronal atrophy visible by a decrease in the number and length of the neuritis.

As observed in Fig. 3, the apoptotic marker caspase-3 increased with increasing glutamate concentrations, reaching a maximum level at 50 μM (468 ± 20.3% of control, $p < 0.001$, $n = 3$). For higher concentrations of glutamate, caspase-3 levels slightly decreased in relation to the maximum level.

Effect of corticotrophin-releasing factor on glutamate neurotoxicity

Urocortin, a peptide belonging to the CRF family, activates both subtype 1 and 2 of CRF receptors, CRF_1R and CRF_2R (Vaughan *et al.* 1995). Urocortin 10 pM was applied to cell medium immediately before glutamate (20 to 1000 μM range). In a similar model, this urocortin concentration provides a maximum neuroprotective effect against an Aβ stimulus (Pedersen *et al.* 2002). As shown in Fig. 4, urocortin increased cell survival in the presence of 50 μM (from 77.8 ± 0.95% to 88.5 ± 0.97%, $p < 0.05$, $n = 4$) and 100 μM (from 78.3 ± 1.37% to 88.7 ± 1.43%, $p < 0.001$, $n = 8$) glutamate. For lower (20 μM) and higher concentrations of glutamate (500 and 1000 μM), urocortin was not able to improve cell viability. Activation of CRF receptors in the absence of glutamate insult did not alter cell viability by itself (94.0 ± 2.18% of CTR, $p > 0.05$, $n = 4$, Fig. 4). Glutamate 100 μM was used in subsequent experiments because of the higher degree of neuroprotection induced by urocortin in this condition.

To distinguish the subtype of CRF receptors underlying the protection afforded by urocortin, the selective CRF_1R and CRF_2R antagonists, antalarmin 10 nM (Ant), and anti-Sauvagine-30 10 nM (a-Sau), as well as a non-selective antagonist for CRF receptors, astressin 10 nM (Ast), were used. As shown in Fig. 5a, the protection by urocortin was lost by blocking either both receptors simultaneously (with astressin, from 88.7 ± 1.43% to

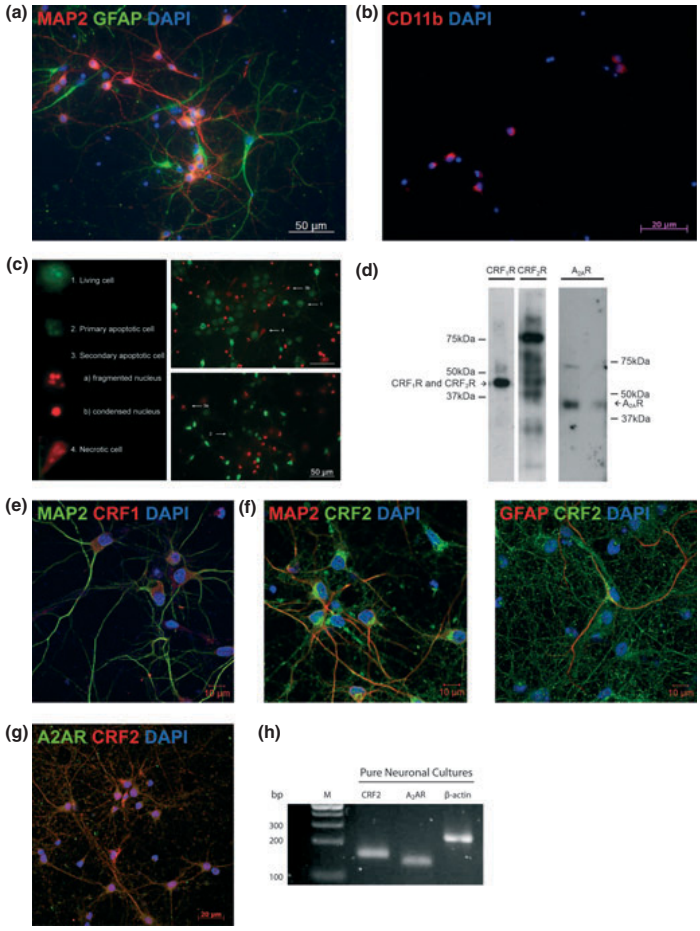

Fig. 1 Characterization of neuronal cultures. (a) The primary cortical cultures at 9 days *in vitro* are composed mostly by neurons (glial contamination is less than 20%). Neurons, in red, are labeled with anti-microtubule-associated protein 2 (MAP2) antibody coupled to a red fluorophore, whereas astrocytes, in green, are probed with anti-glial fibrillary acidic protein (GFAP) antibody coupled to a green fluorophore. Cell nucleus, in blue, is labeled with 4′,6-diamidino-2-phenylindole (DAPI). The photograph is from a control condition of one representative cell culture; (b) Microglial cells are labeled with anti-CD11b antibody, in red. Cell nucleus, in blue, is labeled with DAPI. The photograph is from a control condition of one representative cell culture; (c) Representative images of cultured cells labeled with propidium iodide (PI) and Syto-13. Cells were classified in 4 classes: (1) living cells, which emit green light and presents a homogeneous cell body; (2) primary apoptotic cells, cells labled green with fragmented or condensed nucleus; (3) secondary apoptotic cells that emit red light and presents fragmented (3′) or condensed (3″) cell nucleus; and (4) necrotic cells, observed as diffuse red dots; (d) Western blots of cultured cells probed for the presence of CRF receptor subtype 1 (CRF_1R), CRF receptor subtype 2 (CRF_2R), and Adenosine A_{2A} receptors; (e) Cell culture with triple labeling for CRF_1R, MAP2, and DAPI; (f) Cell culture with triple labeling for CRF_2R, MAP2 and DAPI or CRF_2R, GFAP and DAPI; (g) Cell culture with triple labeling for $A2_AR$ and CRF_2R and DAPI; (h) qPCR products run in an agarose gel showing expression of $A2_AR$ (115 bp) and CRF_2 (142 bp) receptors in pure neuronal cultures, β-actin (228 bp) was used as housekeeping control and RT-minus control yielded no appreciable bands in the expected band size for the primers used. Individual color channels for figures e, f and g are available in suplemental Figure S1.

74.5 ± 1.94%, $p < 0.01$, $n = 4$) or by blocking selectively CRF_1R with antalarmin (from 88.7 ± 1.43% to 74.0 ± 4.46%, $p < 0.01$, $n = 8$) or CRF_2R with anti-Sauvagine-30 (from 88.7 ± 1.43% to 72.0 ± 2.50%, $p < 0.01$, $n = 8$). Both antalarmin and anti-Sauvagine-30 decrease cell viability in cells treated with glutamate (from 78.3 ± 1.37% to 68.1 ± 2.81% and 68.2 ± 4.97%, respectively, $p < 0.05$, $n = 3$). None of these drugs significantly altered cell viability, when applied in the absence of glutamate ($p > 0.05$, $n = 3$, Fig. 5b).

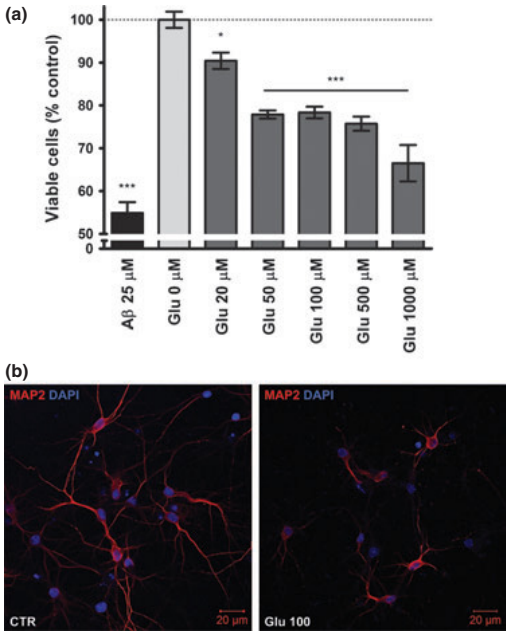

Fig. 2 Glutamate-induced decrease in cell viability is concentration-dependent. Cultures were exposed to glutamate for 24 h. Amyloid-$\beta_{25\text{-}35}$ (Aβ, 25 μM)-induced cell death during a similar period was used as a positive control. Using propidium iodide and Syto-13 labeling cells are distinguished between: living cells, if they are labeled by Syto-13, in green; or dead cells, if propidium iodide crosses the membrane, labeled in red. (a) Glutamate (20 μM to 1000 μM) decreases cell viability in a concentration-dependent. Results are mean ± SEM of four to eleven experiments. $*p < 0.05$ and $***p < 0{,}001$ compared with control, calculated using a one-way ANOVA test plus a Bonferroni's multiple comparison *post-hoc* test. (b) Primary cortical cultures 9 days *in vitro* in control conditions (CTR) and after treatment with glutamate (Glu100 μM) were labeled with the neuronal marker anti-microtubule-associated protein 2 (MAP2) antibody, cell nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI).

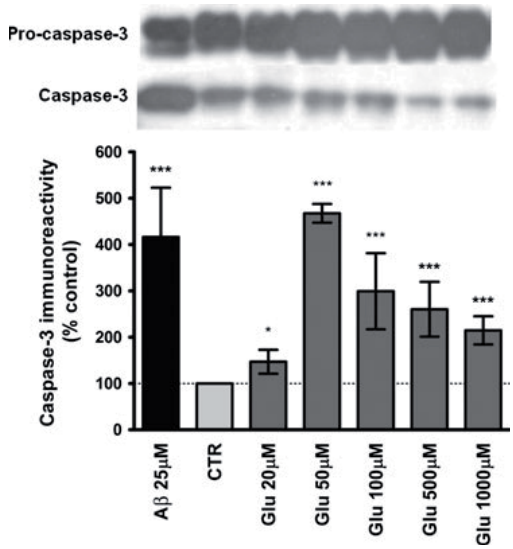

Fig. 3 Glutamate insult (20 to 1000 μM) increases caspase-3 levels in primary cortical cell homogenates. Specific caspase-3 immunoreactivity was normalized to pro-caspase-3 in each condition. $A\beta_{25\text{-}35}$ peptide 25 μM was used as positive control for pro-caspase-3 fragmentation. Each bar is the mean ± SEM of three to four experiments. $*p < 0.05$; $***p < 0.001$ compared with control calculated using a one-way ANOVA test, followed by a Bonferroni's multiple comparison *post-hoc* test. The top panel shows a representative western blot.

Involvement of adenosine A_{2A} receptors on CRF neuroprotection

In view of the involvement of adenosine A_{2A} receptors in excitotoxicity phenomena, we then evaluated the possible interaction between the neuroprotection mediated by A_{2A} receptors blockade and the consequence of activating CRF receptors.

The blockade of A_{2A} receptors by its selective antagonist, SCH 58261 (50 nM), did not change the neuroprotection induced by urocortin ($n = 6$, Fig. 6). However, SCH 58261 (50 nM) alone prevented the cell death induced by glutamate (78.3 ± 1.37% to 88.5 ± 1.60%, $p < 0.01$, $n = 4$). Interestingly, neuroprotection obtained by the CRF receptor agonist, urocortin, was not additive with that achieved by the A_{2A} receptor antagonist, SCH 58261 (Fig. 6) suggesting a common mechanism of action. In contrast, A_{2A} receptor activation with the selective agonist, CGS 21680 (30 nM), did not altered cell death alone (76.4 ± 3.62%, $p > 0.05$, $n = 6$), or in the presence of urocortin (from 88.7 ± 1.43% with urocortin, to 82.4 ± 2.45% with CGS 21680, $n = 8$, Fig. 6).

Furthermore, as presented in Fig. 7, the neuroprotective effect achieved by the blockade of A2AR (in the presence of urocortin) was lost by selective CRF_2R blockade (71.5 ± 2.52%, $p < 0.001$ compared with SCH 58261 plus urocortin, $n = 6$) or the blockade of both CRF receptors simultaneously (70.4 ± 2.34%, $p < 0.001$ compared with SCH 58261 plus urocortin, $n = 4$). Selective blockade of CRF_1R did not affect neuroprotection obtained with SCH 58261 plus urocortin (86.0 ± 1.57%, $n = 6$).

Discussion

The release of adenosine that occurs as a consequence of hypoxic events (Andiné *et al.* 1990) is accompanied by an increase in the levels of the stress regulator, corticotrophin-

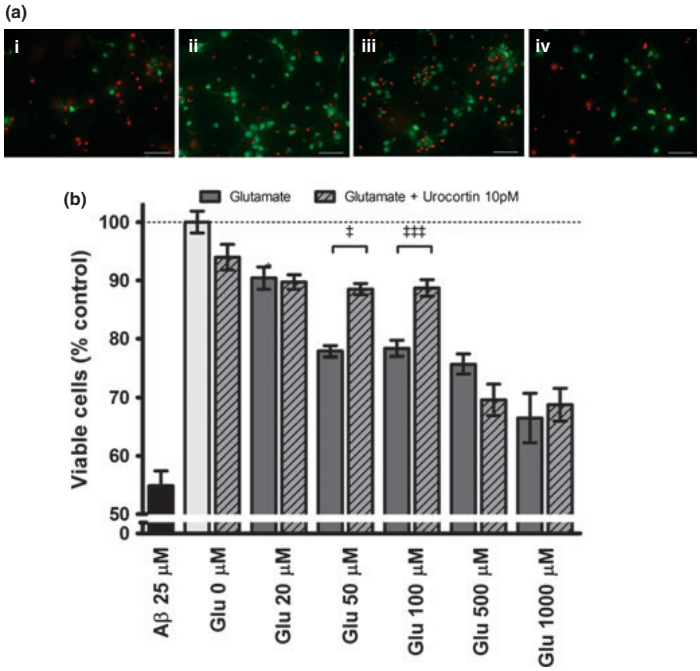

Fig. 4 Corticotrophin-releasing factor (CRF) receptor activation prevents cell death induced by glutamate. (a) Representative images of several conditions: (i) Aβ 25 μM; (ii) CTR; (iii) Glutamate 100 μM; (iv) Glutamate 100 μM + urocortin (CRF agonist; 10 pM). Scale bar: 50 μm. (b) Dark gray bars represent the effects of glutamate alone, gray pattern bars represent the effects of glutamate in the presence of urocortin 10 pM. Aβ was used as a positive control for cell death. Results are mean ± SEM of four to eleven experiments. $^{‡}p < 0.05$, $^{‡‡‡}p < 0.001$, for the two selected conditions, calculated using a one-way ANOVA test plus a Bonferroni's multiple comparison *post-hoc* test.

releasing factor (CRF) in the brain (Chen *et al.* 2004a, b). In addition, the *in vivo* modulation of adenosine A_{2A} receptors is responsible for reversion of stress-induced effects in the hippocampus (Batalha *et al.* 2012), suggesting an involvement of these receptors in the stress response system. This raises the question whether A_{2A} receptors regulate the levels or the function of the main stress mediators, either CRF or glucocorticoids. We now explored the neuroprotective effects of A_{2A} receptors blockade and activation of CRF receptors, under stressful conditions (glutamate insult), to disclose a possible interaction between the mechanisms operated by both receptors.

The major finding of this work is that the protective action of urocortin, a CRF agonist, against a glutamate insult is dependent on both CRF receptor subtypes, CRF_1R and CRF_2R. Moreover, we show for the first time that the neuroprotection achieved by blockade of A_{2A} receptors is effective only if CRF_2R are active.

In hypoxic or ischemic events, the massive release of glutamate induces damage to the surrounding neuronal population, both by necrosis and apoptosis (Nicotera *et al.* 1999). As expected, glutamate increased cell death in a concentration-dependent manner for concentrations higher than 20 μM, which is in accordance with previous reports (Choi *et al.* 1987). Urocortin, a peptide belonging to the CRF family that has equivalent affinity to CRF_1R and CRF_2R, prevented cell death induced by glutamate (50–100 μM). For higher glutamate concentrations, urocortin seems to be inefficient in reverting cell death most probably because of the severe cell necrosis that occurs for such concentrations. On the other hand, for lower glutamate concentrations, the evoked cell death is probably not enough to allow a significant and measurable increase in cell viability induced by urocortin. The concentration of glutamate (100 μM) used in most of the experiments was enough to induce measurable cell death without causing cell detachment because of necrosis, as known to occur for high glutamate concentrations (Ankarcrona *et al.* 1995). On the other hand, this glutamate concentration is able to induce apoptosis, as can be concluded by the increase in apoptotic marker caspase-3.

CRF_1R and CRF_2R are present in neuronal cells (Pedersen *et al.* 2002). CRF_1R but not CRF_2R were shown to be involved in the neuroprotection against oxidative stress, by testing various insults in cortical neurons (Pedersen *et al.* 2002). Urocortin 2, a CRF_2R agonist, is not able to revert cell death caused by radical oxygen species, whereas urocortin, a non-specific CRF receptor agonist is able to protect neuronal cells from an equivalent insult, by activating CRF_1R (Pedersen *et al.* 2002). The different subtypes of CRF receptors have different functions in HPA axis and neuroprotection (Pedersen *et al.* 2002; Rissman *et al.* 2007). To determine which CRF receptor subtype is underlying the urocortin-induced neuroprotection against the glutamate insult, we analyzed the neuroprotective effect mediated by

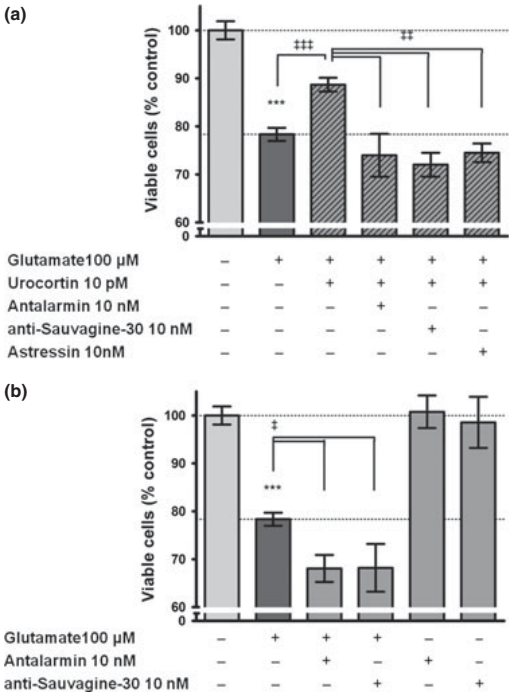

Fig. 5 Both corticotrophin-releasing factor (CRF) receptors are involved in urocortin-induced neuroprotection. (a) Cell viability was assessed in the presence of glutamate 100 μM, the CRF receptor agonist (Urc 10 pM) and antagonists (Ant 10 nM, a-Sau 10 nM, Ast 10 nM). (b) CRF has endogenous neuroprotective effects, as CRF receptors blockade decreases cell viability in presence of glutamate. Cell viability was assessed in the presence of glutamate 100 μM and the CRF receptor antagonists (Ant 10 nM, a-Sau 10 nM). Lines at 100% and 78.3% represent viability values for control and glutamate 100 μM, respectively. Results are mean ± SEM of four to eleven experiments. $^{***}p < 0.001$ compared with control; $^{‡}p < 0.05$, $^{‡‡}p < 0.01$ and $^{‡‡‡}p < 0.001$ when comparing the selected conditions, calculated using a one-way ANOVA test followed by a Bonferroni's multiple comparison *post-hoc* test.

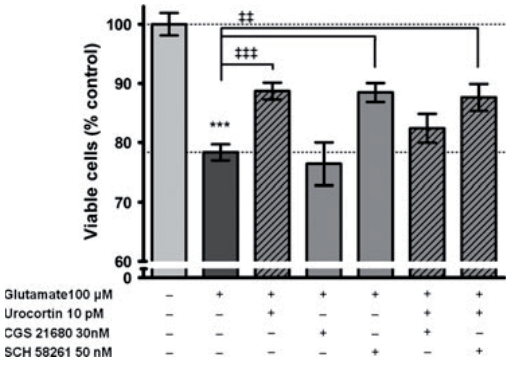

Fig. 6 The blockade of A_{2A} receptors increases cell viability upon a glutamate insult. A_{2A} receptors blockade (SCH 58261) increased cell viability, whereas A_{2A} receptor activation (CGS 21680) did not change cell viability in the presence of glutamate 100 μM. Lines at 100% and 78.3% represent viability values for control and glutamate 100 μM. Results are mean ± SEM of four to eleven experiments. $^{***}p < 0.001$ compared with control; $^{‡}p < 0.05$ and $^{‡‡}p < 0.01$ when comparing the selected conditions calculated using a one-way ANOVA test followed by a Bonferroni's multiple comparison *post-hoc* test.

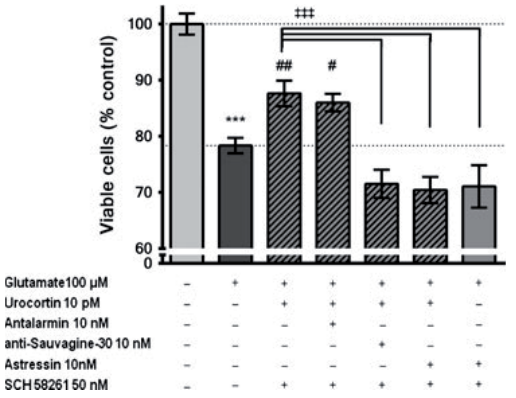

Fig. 7 The neuroprotection induced by A_{2A} receptor blockade requires CRF receptor of subtype 2 (CRF_2R). A_{2A} receptor blockade (by SCH 58261) increases cell viability, only if CRF_2R are not blocked. Lines at 100% and 78.3% represent viability values for control and glutamate 100 μM. Results are mean ± SEM of four to eleven experiments. $^{***}p < 0.001$ compared with control; $^{‡‡‡}p < 0.001$ when comparing the selected conditions; $^{#}p < 0.05$ and $^{##}p < 0.01$ compared with glutamate 100 μM, calculated using a one-way ANOVA test followed by a Bonferroni's multiple comparison *post-hoc* test.

selective agonists and by urocortin in the presence of selective antagonists for each receptor subtype. As we show here, CRF receptor activation by urocortin is neuroprotective against glutamate insults only when both CRF_1R and CRF_2R are active, as the blockade of either of the receptors selectively was sufficient to prevent the neuroprotective effect of urocortin. This data suggest a common mechanism of action of both CRF receptors in neuroprotection against a glutamate insult and that both of them are required to afford neuroprotection.

Adenosine A_{2A} receptors are modulatory targets against neurologic insults, as A_{2A} receptor blockade is known to be neuroprotective (reviewed by de Mendonça *et al.* 2000). Besides the common intracellular signaling pathways (Blank *et al.* 2003; Fredholm *et al.* 2005), CRF receptors also share an ability to interfere with neuroprotection, (Fox

et al. 1993). Therefore, we explored a possible interaction between these two receptors by studying their functional role in the prevention of cell death induced by a glutamate insult. Blockade of A_{2A} receptors by SCH 58261 reverted cell death induced by glutamate (100 μM), as previously observed (Popoli *et al.* 2003). We then tested the combined actions of CRF receptors activation and A_{2A} receptors blockade upon the glutamate insult. Remarkably, the neuroprotection afforded by A_{2A} receptor blockade, was not additive to that provided by urocortin, suggesting a common downstream signaling pathway, shared by both receptor subtypes. Both A_{2A} receptors and CRF receptors are able to alter gene expression. Whereas blockade of A_{2A} receptor is neuroprotective, probably by reducing cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) phosphorylation activity (Fredholm *et al.* 2005), CRF receptor activation leads to an increase in PKA activity (Bayatti *et al.* 2005). Indeed, PKA and PKC phosphorylation leads to insertion of NMDA and AMPA receptors in the cell membrane (Tan *et al.* 1994; Leonard and Hell 1997; Dias *et al.* 2012), and this may exacerbate the excitotoxicity induced by glutamate (Leveille *et al.* 2008). Involvement of p38 in the neuroprotection afforded by A_{2A} receptors blockade has also been reported (Melani *et al.* 2006), whereas a mechanism by which CRF receptors may affect p38 is presently unknown and awaits further investigation.

In the presence of urocortin, the neuroprotective effects of A_{2A} receptor blockade by SCH 58261 were abolished by CRF_2R but not by CRF_1R blockade, suggesting that the A_{2A} receptors role in the control of cell viability is dependent on CRF_2R, but independent of CRF_1R. This least investigated receptor, CRF_2R, has been linked to neuroprotection against glutamate insults in retinal cells (Szabadfi *et al.* 2009). This is the first report regarding neuroprotection mediated by CRF_2R, in the brain. Depending on the type of insult CRF may require different contributions of CRF_1R and/ or CRF_2R to the neuroprotection. Earlier reports in cell cultures have focused on necrosis-inducing insults, in which CRF_1R seem to be the main contributors to the neuroprotection achieved by urocortin. On the contrary, a non- CRF_1R dependent protection by urocortin was only observed when apoptosis-mediated cell death also occurred (see Pedersen *et al.* 2002). The neuroprotective role of urocortin herein described could clearly be ascribed to CRF_2R activation.

Both CRF_2R and A_{2A} receptors were found to be expressed in neurons and to co-localize in the same cells in our mixed cell cultures (Fig. 1g–h). These data suggest that receptor cross talk is the most likely mechanism, but we cannot fully preclude the possibility that A_{2A} receptor activation by glutamate-induced adenosine release could modulate CRF levels. However, the absence of significant changes in cell viability upon direct activation of A_{2A} receptors with the agonist CGS 21680, does not seem to favor the latter possibility.

Overall, the data points to a new role of CRF against glutamate-induced neuronal death either by direct activation of CRF receptors, or modulation of A_{2A} receptor-mediated actions. The observed neuroprotection achieved by A_{2A} receptor blockade requires CRF_2R activation. This interaction between the adenosine and CRF receptors can explain the beneficial effects of using A_{2A} receptor antagonists against stress-induced noxious effects.

Acknowledgements

This work was supported by Fundação para a Ciência e Tecnologia. We are thankful to André Jerónimo-Santos for helpful discussions, to Ana Patricia Simões (CNC, Coimbra) for help in propidium iodide labeling technique, and to João Baião, Carla Batalha, and António Temudo for technical support. The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be seen as a potential conflict of interest. All authors have contributed to and approved the final manuscript. Designed research: AMS, MJD, and LVL. Performed research: JSV, DGF, VLB, and JEC. Analyzed data: JSV, VLB, RG, and LVL. Wrote the manuscript: JSV, VLB, AMS, MJD, and LVL.

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Characterization of the neuronal cultures: Cell culture with double labeling for MAP2 and DAPI with or without glutamate 100 μM.

Figure S2. Effect of glutamate insult on neuronal cultures.

References

Andiné P., Rudolphi K. A., Fredholm B. B. and Hagberg H. (1990) Effect of propentofylline (HWA 285) on extracellular purines and excitatory amino acids in CA1 of rat hippocampus during transient ischaemia. *Br. J. Pharmacol.* **100**, 814–818.

Ankarcrona M., Dypbukt J. M., Bonfoco E., Zhivotovsky B., Orrenius S., Lipton S. A. and Nicotera P. (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15**, 961–973.

Batalha V. L., Pego J. M., Fontinha B. M., Costenla A. R., Valadas J. S., Baqi Y., Radjainia H., Müller C. E., Sebastião A. M. and Lopes L. V. (2012) Adenosine A2A receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation. *Mol. Psychiatry*. doi: 10.1038/mp.2012.8. (Epub ahead of print)

Bayatti N., Hermann H., Lutz B. and Behl C. (2005) Corticotropin-releasing hormone-mediated induction of intracellular signaling pathways and brain-derived neurotrophic factor expression is inhibited by the activation of the endocannabinoid system. *Endocrinology* **146**, 1205–1213.

Bittencourt J. C., Vaughan J., Arias C., Rissman R. and a, Vale, W.W. & Sawchenko, P.E. (1999) Urocortin expression in rat brain: evidence against a pervasive relationship of urocortin-containing

projections with targets bearing type 2 CRF receptors. *J. Comp. Neurol.* **415**, 285–312.

Blank T., Nijholt I., Grammatopoulos D. K., Randeva H. S., Hillhouse E. W. and Spiess J. (2003) Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: role in neuronal excitability and associative learning. *J. Neurosci.* **23**, 700–707.

Bonfoco E., Krainc D., Ankarcrona M., Nicotera P. and Lipton S. A. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl Acad. Sci. USA* **92**, 7162–7166.

Canas P. M., Porciuncula L. O., Cunha G. M., Silva C. G., Machado N. J., Oliveira J. M., Oliveira C. R. and Cunha R. A. (2009) Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by beta-amyloid peptides via p38 mitogen-activated protein kinase pathway. *J. Neurosci.* **29**, 14741–14751.

Chalmers D. T., Lovenberg T. W. and De Souza E. B. (1995) 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**, 6340–6350.

Chen F. M., Bilezikjian L. M., Perrin M. H., Rivier J. and Vale W. (1986) Corticotropin releasing factor receptor-mediated stimulation of adenylate cyclase activity in the rat brain. *Brain Res.* **386**, 49–57.

Chen R., Lewis K. and a, Perrin, M.H. & Vale, W.W. (1993) Expression cloning of a human corticotropin-releasing-factor receptor. *Proc. Natl Acad. Sci. USA* **90**, 8967–8971.

Chen J. F., Huang Z., Ma J., Zhu J., Moratalla R., Standaert D., Moskowitz M. A., Fink J. S. and Schwarzschild M. A. (1999) A (2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J. Neurosci.* **19**, 9192–9200.

Chen Y., Bender R. A., Frotscher M. and Baram T. Z. (2001) Novel and transient populations of corticotropin-releasing hormone-expressing neurons in developing hippocampus suggest unique functional roles: a quantitative spatiotemporal analysis. *J. Neurosci.* **21**, 7171–7181.

Chen Y, Brunson KL, Adelmann G, Bender RA, Frotscher M and Baram TZ. (2004a) Hippocampal corticotropin releasing hormone: pre- and postsynaptic location and release by stress. *Neuroscience* **126**, 533–540.

Chen X. Q., Du J. Z. and Wang Y. S. (2004b) Regulation of hypoxia-induced release of corticotropin-releasing factor in the rat hypothalamus by norepinephrine. *Regul. Pept.* **119**, 221–228.

Choi D. W., Maulucci-Gedde M. and Kriegstein A. R. (1987) Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* **7**, 357–368.

Cunha G. M., Canas P. M., Oliveira C. R. and Cunha R. A. (2006) Increased density and synapto-protective effect of adenosine A2A receptors upon sub-chronic restraint stress. *Neuroscience* **141**, 1775–1781.

Dall'Igna O. P., Porciuncula L. O., Souza D. O., Cunha R. A. and Lara D. R. (2003) Neuroprotection by caffeine and adenosine A2A receptor blockade of beta-amyloid neurotoxicity. *Br. J. Pharmacol.* **138**, 1207–1209.

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

Elliott-Hunt C. R., Kazlauskaite J., Wilde G. J., Grammatopoulos D. K. and Hillhouse E. W. (2002) Potential signalling pathways underlying corticotrophin-releasing hormone-mediated neuroprotection from excitotoxicity in rat hippocampus. *J. Neurochem.* **80**, 416–425.

Estus S., Tucker H. M., van Rooyen C., Wright S., Brigham E. F., Wogulis M. and Rydel R. E. (1997) Aggregated amyloid-beta protein induces cortical neuronal apoptosis and concomitant "apoptotic" pattern of gene induction. *J. Neurosci.* **17**, 7736–7745.

Fischman A. J. and Moldow R. L. (1982) Extrahypothalamic distribution of CRF-like immunoreactivity in the rat brain. *Peptides* **3**, 149–153.

Fox M. W., Anderson R. E. and Meyer F. B. (1993) Neuroprotection by corticotropin releasing factor during hypoxia in rat brain. *Stroke* **24**, 1072–1075.

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

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

Grammatopoulos D. K., Randeva H. S., Levine M. A., Kanellopoulou K. A. and Hillhouse E. W. (2001) Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. *J. Neurochem.* **76**, 509–519.

Gulyas J., Rivier C., Perrin M., Koerber S. C., Sutton S., Corrigan A., Lahrichi S. L. and Craig, a G., Vale, W. & Rivier, J. (1995) Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc. Natl Acad. Sci. USA* **92**, 10575–10579.

Kemppainen S., Rantamäki T., Jerónimo-Santos A. *et al.* (2012) Impaired TrkB receptor signaling contributes to memory impairment in APP/PS1 mice. *Neurobiol. Aging* **33**, 1122–1139.

Latini S. and Pedata F. (2001) Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J. Neurochem.* **79**, 463–484.

Leonard A. S. and Hell J. W. (1997) Cyclic AMP-dependent protein kinase and protein kinase C phosphorylate N-methyl-D-aspartate receptors at different sites. *J. Biol. Chem.* **272**, 12107–12115.

Leveille F., El Gaamouch F., Gouix E., Lecocq M., Lobner D., Nicole O. and Buisson A. (2008) Neuronal viability is controlled by a functional relation between synaptic and extrasynaptic NMDA receptors. *FASEB J.* **22**, 4258–4271.

Lipton S. A. and Rosenberg P. A. (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *N. Engl. J. Med.* **330**, 613–622.

Lopes L. V., Cunha R. A., Kull B., Fredholm B. B. and Ribeiro J. A. (2002) Adenosine A(2A) receptor facilitation of hippocampal synaptic transmission is dependent on tonic A(1) receptor inhibition. *Neuroscience* **112**, 319–329.

Lovenberg T. W., Liaw C. W., Grigoriadis D. E., Clevenger W., Chalmers D. T., De Souza E. B. and Oltersdorf T. (1995) Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. *Proc. Natl Acad. Sci. USA* **92**, 836–840.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin Phenol Reagent, *J. Biol. Chem.* **193**, 265–275.

Melani A., Gianfriddo M., Vannucchi M. G., Cipriani S., Baraldi P. G., Giovannini M. G. and Pedata F. (2006) The selective A2A receptor antagonist SCH 58261 protects from neurological deficit, brain damage and activation of p38 MAPK in rat focal cerebral ischemia. *Brain Res.* **1073–1074**, 470–480.

de Mendonça A., Sebastião A. M. and Ribeiro J. A. (2000) Adenosine: does it have a neuroprotective role after all? *Brain Res. Brain Res. Rev.* **33**, 258–274.

Monopoli A., Lozza G., Forlani A., Mattavelli A. and Ongini E. (1998) Blockade of adenosine A2A receptors by SCH 58261 results in neuroprotective effects in cerebral ischaemia in rats. *NeuroReport* **9**, 3955–3959.

Nicotera P., Leist M. and Manzo L. (1999) Neuronal cell death: a demise with different shapes. *Trends Pharmacol. Sci.* **20**, 46–51.

Pedersen W. A., McCullers D., Culmsee C., Haughey N. J., Herman J. P. and Mattson M. P. (2001) Corticotropin-releasing hormone protects neurons against insults relevant to the pathogenesis of Alzheimer's disease. *Neurobiol. Dis.* **8**, 492–503.

Pedersen W. A., Wan R., Zhang P. and Mattson M. P. (2002) 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**, 404–412.

Popoli P., Frank C., Tebano M. T., Potenza R. L., Pintor A., Domenici M. R., Nazzicone V., Pèzzola A. and Reggio R. (2003) Modulation of glutamate release and excitotoxicity by adenosine A2A receptors. *Neurology* **61**, S69–S71.

Radulovic J., Rühmann A., Liepold T. and Spiess J. (1999) Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: differential roles of CRF receptors 1 and 2. *J. Neurosci.* **19**, 5016–5025.

Rebola N., Porciuncula L. O., Lopes L. V., Oliveira C. R., Soares-da-Silva P. and Cunha R. A. (2005a) Long-term effect of convulsive behavior on the density of adenosine A1 and A 2A receptors in the rat cerebral cortex. *Epilepsia* **46**(Suppl 5), 159–165.

Rebola N., Rodrigues R. J., Oliveira C. R. and Cunha R. A. (2005b) Different roles of adenosine A1, A2A and A3 receptors in controlling kainate-induced toxicity in cortical cultured neurons. *Neurochem. Int.* **47**, 317–325.

Rissman R. A, Lee K.-F., Vale W. and Sawchenko P. E. (2007) Corticotropin-releasing factor receptors differentially regulate stress-induced tau phosphorylation. *J. Neurosci.* **27**, 6552–6562.

Swanson L. W., Sawchenko P. E., Rivier J. and Vale W. W. (1983) Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat barin: an immunohistochemical study. *Neuroendocrinology* **36**, 165–186.

Szabadfi K., Atlasz T., Reglodi D., Kiss P., Dányádi B., Fekete E. M., Zorrilla E. P., Tamás A., Szabó K. and Gábriel R. (2009) Urocortin 2 protects against retinal degeneration following bilateral common carotid artery occlusion in the rat. *Neurosci. Lett.* **455**, 42–45.

Tamura Y., Sato Y., Yokota T., Akaike A., Sasa M. and Takaori S. (1993) Ifenprodil prevents glutamate cytotoxicity via polyamine modulatory sites of N-methyl-D-aspartate receptors in cultured cortical neurons. *J. Pharmacol. Exp. Ther.* **265**, 1017–1025.

Tan S.-E., Wenthold J. and Soderlingl R. (1994) Phosphorylation of AMPA-type glutamate receptors by calcium / protein kinase II and protein kinase C in cultured hippocampal neurons. *J. Neurosci.* **14**, 1123–1129.

Towbin H., Staehelin T. and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA* **76**, 4350–4354.

Vale W., Spiess J., Rivier C. and Rivier J. (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* **213**, 1394–1397.

Vaughan J., Donaldson C., Bittencourt J. *et al.* (1995) Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* **378**, 287–292.

Neurobiology of Aging 35 (2014) 1680–1685

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Brief communication

Maternal separation impairs long term-potentiation in CA1-CA3 synapses and hippocampal-dependent memory in old rats

Vasco C. Sousa [a,b,1], Joana Vital [a], Ana Rita Costenla [a], Vânia L. Batalha [a], Ana M. Sebastião [a,b], Joaquim A. Ribeiro [a,b], Luísa V. Lopes [a,*]

[a] Instituto de Medicina Molecular, Faculty of Medicine, University of Lisbon, Lisbon, Portugal
[b] Institute of Pharmacology and Neurosciences, Faculty of Medicine, University of Lisbon, Lisbon, Portugal

ARTICLE INFO

Article history:
Received 8 October 2013
Received in revised form 9 January 2014
Accepted 21 January 2014
Available online 25 January 2014

Keywords:
Aging
Stress
Hippocampus
Memory
LTP
Maternal separation

ABSTRACT

Exposure to chronic stress during the neonatal period is known to induce permanent long-term changes in the central nervous system and hipothalamic-pituitary-adrenal axis reactivity that are associated with increased levels of depression, anxiety, and cognitive impairments. In rodents, a validated model of early life stress is the maternal separation (MS) paradigm, which has been shown to have long-term consequences for the pups that span to adulthood. We hypothesized that the early life stress-associated effects could be exacerbated with aging, because it is often accompanied by cognitive decline. Using a MS model in which rat pups were separated from their mothers for 3 hours daily, during postnatal days 2–14, we evaluated the long-term functional consequences to aged animals (70-week-old), by measuring synaptic plasticity and cognitive performance. The baseline behavioral deficits of aged control rats were further exacerbated in MS animals, indicating that early-life stress induces sustained changes in anxiety-like behavior and hippocampal-dependent memory that are maintained much later in life. We then investigated whether these differences are linked to impaired function of hippocampal neurons by recording hippocampal long-term potentiation from Schaffer collaterals/CA1 synapses. The magnitude of the hippocampal long-term potentiation induced by high-frequency stimulation was significantly lower in aged MS animals than in age-matched controls. These results substantiate the hypothesis that the neuronal and endocrine alterations induced by early-life stress are long lasting, and are able to exacerbate the mild age-associated deficits.

1. Introduction

Stressful life events play a crucial role in the pathogenesis of debilitating human psychiatric disorders, such as chronic anxiety and depression (Muscatell et al., 2009). Abnormal neonate maternal care is a key environmental stressor with lasting consequences for the developing brain (Francis & Meaney, 1999).

It has been widely observed in rodent models that during early development, abnormal maternal behavior manifested by impaired licking and/or grooming of the pups creates a chronic stressful environment that generates long-term zadverse effects upon stress-mediating pathways, leading to chronic affective disorders later in life (Francis & Meaney, 1999; Joëls & Baram, 2009). Early studies have developed several rodent maternal separation (MS) paradigms, which induce a stressful environment in both dam and pups (Plotsky & Meaney, 1993). Although the adverse effects of the maternal separation protocols appear not to be linked with reduced levels of maternal care, they are likely caused by the absence of the dam and the handling of the animals (Macrí et al., 2004). Thus, the rodent MS model is a valid and useful paradigm to induce higher susceptibility of the hypothalamic-pituitary-adrenal axis to acute stress in adult life, which is linked to several long-term epigenetic and neuronal changes (Kaufman et al., 2000; Meaney, 2001; Zhang & Meaney, 2010).

The hippocampus is a preferential target of action of stress hormones because of its abundant expression of glucocorticoid receptors (McEwen, 1999a; McEwen, 1999b). In addition, hippocampal glucocorticoid receptors form an important part of the retrograde mechanism responsible for inhibiting glucocorticoid release during a response to stress (Sapolsky et al., 1984). Both stress and the exposure to high levels of glucocorticoids in early life have been associated to learning and memory deficits in adulthood (Aisa

* Corresponding author at: Instituto de Medicina Molecular, Av. Prof. Egas Moniz. 1649-028 Lisboa, Portugal. Tel.: +351 21 7985183; fax: +351 21 799 9454.
E-mail address: lvlopes@fm.ul.pt (L.V. Lopes).
[1] Present address: Laboratory of Translational Neuropharmacology, Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden.

0197-4580/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2014.01.024

et al., 2007; Batalha et al., 2013; Champagne et al., 2009; Ivy et al., 2010; Meaney et al., 1988), and to hippocampal atrophy (Sapolsky et al., 1985). This may be largely attributed to the susceptibility of the hippocampus to ongoing neuronal and circuit maturation in the early postnatal stages (Altman & Das, 1965; Bayer, 1980).

Several age-related factors contributing to hippocampal functional decline have been described (Brunson et al., 2005; Burger, 2010; Lupien, et al., 2005). One relevant mechanism is associated with hypersecretion of glucocorticoids (Sapolsky et al., 1983), which was shown to contribute to the loss of neurons in the hippocampus (Sapolsky et al., 1985). Accordingly, we have recently shown that early-life stress leads to hippocampal-dependent memory impairments in adult animals (Batalha et al., 2013), which suggests a premature aging-like phenomenon (Diogenes et al., 2011). However, it is not known whether early-life stress impacts on the hippocampal function and synaptic plasticity in aged rats. In this work, we aimed to show how the negative effects of MS during the critical hippocampal development period are exacerbated in senescence, and how this correlates to hippocampal synaptic plasticity changes.

We found that MS aggravates age-related behavioral deficits, indicating that early-life stress induces sustained changes in anxiety-like behavior and hippocampal function that are maintained throughout life. The magnitude of hippocampal long-term potentiation (LTP) in aged MS animals was significantly lower than in age-matched controls, which suggests that the cognitive deficits resulting from early-life stress may be associated with reduced neuronal plasticity.

2. Experimental procedures

2.1. Animals

Pregnant Wistar rats in mid-gestation were ordered from Harlan Iberica (Barcelona, Spain) and housed in the animal facility with a normal 12 hour light and/or dark cycle, with lights on at 7 AM. While the possibility of transportation-associated prenatal stress cannot be excluded, the observed results indicate statistically significant functional differences despite both control and MS animals having experienced the same prenatal environmental conditions (Batalha et al., 2013). Animal procedures were performed in accordance with the guidelines of the European Communities Council and approved by the Instituto de Medicina Molecular Internal Committee and the Portuguese Animal Ethics Committee (Direcção Geral de Veterinária). Animals were first habituated to handling during 5 days before testing began. According to the humane euthanization procedures indicated by our ethical committee, animals were quickly anesthetized in a cage with halothane atmosphere before decapitation.

2.2. Maternal separation protocol

The protocol used has been previously validated and described (Batalha et al., 2013). Wistar dams and their litters were assigned either to the control (CTR—non-separated) or to the MS group. To exclude artifacts from genetic background, pups from all litters were randomly cross-fostered between the dams at postnatal day (PND) 2, maintaining identical litter sizes and gender distribution. Control and MS litters were briefly handled for a period under 5 minutes, once for cross-fostering and weighing at P2, and for a second weighing at P14. Control litters were otherwise maintained undisturbed in their home-cages until weaning, while MS litters, pups were daily removed from their cages at PND 2–14 for 180 minutes at approximately 9 AM, and placed in an isolated cage in an adjacent room kept at 32.0 ± 0.5 °C. At the end of the separation period, pups were returned to their home-cage. At PND 21 the pup's gender was assessed, they were weaned and only males to be used in subsequent experiments were housed in groups of 5–8 animals per cage until testing at desired age group.

2.3. Experimental design

Experiments were performed in 2 separate cohorts of male animals. The first cohort which consisted of 12 control, and 13 MS rats, from which 6 of each group were tested at PND 40 of age to control for positive stress effects by MS. The remaining were then left and tested in the electrophysiology experiments when animals reached 70 weeks of age. The second cohort consisted of 8 control and 10 MS rats, was used at 70 weeks of age as the "aged" group in the behavioral experiments. From these, 5 from each group were tested at PND40 for effective stress control purposes.

2.4. Corticosterone quantification

At PND 40, blood was extracted from the tail, in animals previously handled to minimize stress and without anesthesia, at 8 AM. The plasma was isolated by centrifugation at 2000*g*, 4 °C, for 15 minutes and corticosterone quantified by radioimmunoassay using the rat corticosterone 3H kit from MP Biomedicals, UK according to the manufacturer's protocol.

2.5. Elevated plus maze

The 4 arms in the maze were each 5 cm × 29 cm, and elevated 50 cm above the floor, 2 arms with no walls (open), and 2 arms with 29 cm high walls (closed), arranged perpendicularly. The test began with each animal at PND 40 or 70-week-old, being placed on the center of the maze, facing the open arm. The test consisted of a single trial of 5 minutes, and an experienced experimenter manually scored the time spent in the open arms, and the total arms entries (as described in Batalha et al., 2013). Between each trial, the maze was cleaned with a 70% ethanol solution.

2.6. Morris water maze

The Morris water maze test was used to measure hippocampal-dependent spatial reference memory, as described before (Diógenes et al., 2011). The maze consisted of a large circular tank (1.8 m in diameter, 0.6 m in height) of water (temperature, 25 ± 1 °C) made opaque with the addition of a small quantity of nontoxic, water-based black paint. An escape platform (10 cm in diameter) was submerged 1 cm below the water. Several visual cues were placed on the walls of the testing room, to be used by the animals as spatial references. An automated tracking system (Smart 2.5, PanLab, Barcelona) monitored all performances in the following parameters: swim pathlength, escape latency, average speed, and percentage of time spent in each quadrant. Rats aged 70-week-old were first given spatial (acquisition) training consisting of 4 trials/d for 5 days, in which the platform was placed at a fixed position in the center of one of the 4 quadrants of the tank (N, S, E, W). The starting position, at which subjects were placed in the tank facing the wall, differed randomly across trials. The intertrial interval was 15 minutes, during which animals were towel-dried and placed in a heated incubator (25 °C) to prevent hypothermia. The maximum trial duration was 60 seconds, after which animals were manually guided to the platform if they failed to locate it. Once animals reached the platform, they were allowed to remain on it for 20 seconds. A probe test in which the platform is removed and animals allowed to swim freely for 60 seconds was given after the last trial on day 5 of the acquisition. The results are expressed in mean ± standard error of the mean.

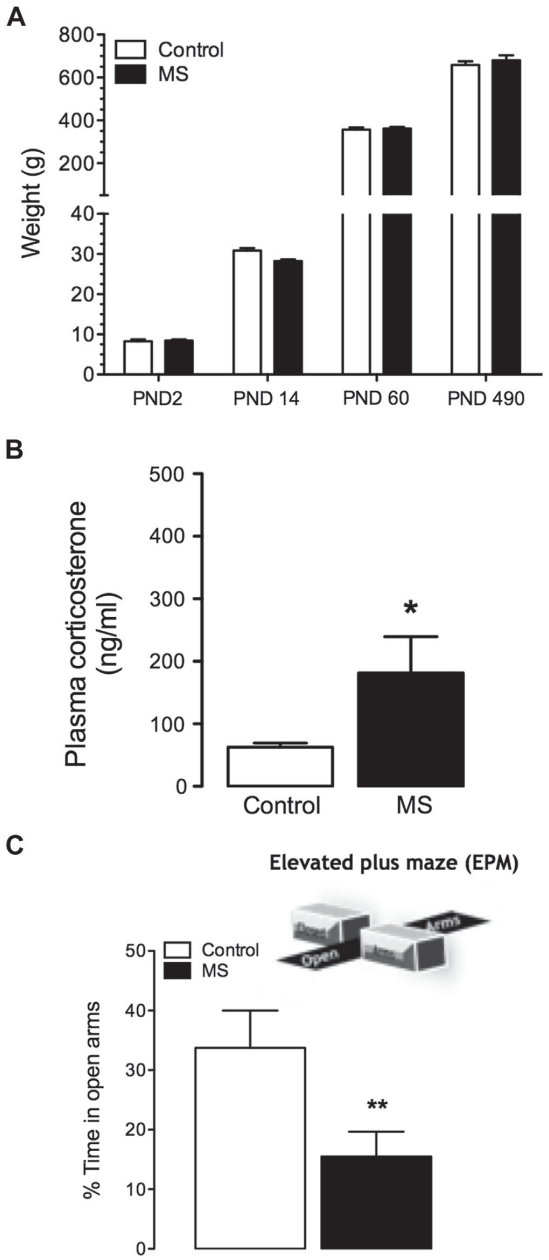

Fig. 1. MS induced a significant increase in plasmatic corticosterone levels and anxious-related behavior at PND 40. (A) Body weight in grams at 4 age points from neonatal to aging in control and MS animals. Compared with controls, animals subjected to MS did not show differences in body weight ($p > 0.05$, Student t test; n = 20 for PNDs 2 and 14, n = 8 for PND 60, and n = 12 for PND 490). (B) Corticosterone levels in the morning period (8 AM) were measured by radioimmunoassay using the rat corticosterone [3H] kit at PND 40. Results are mean ± SEM of 7 experiments in CTR and 4 experiments in MS animals; * $p < 0.05$ obtained using a unpaired Student t test. (C) Percentage of time spent in the open arms of the elevated plus-maze. A significant effect of MS in reducing percentage of time in open arms was observed (n = 5 for CTR and n = 8 for MS). * $p = 0.034$, ** $p = 0.033$ Student t test. Abbreviations: CTR, control; MS, maternal separation; PND, postnatal day.

2.7. Electrophysiological recordings

After decapitation, the brain of 70-week-old animals was rapidly removed and hippocampi were dissected free in ice-cold Krebs solution of the following composition (mM) NaCl 124, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 26, $MgSO_4$ 1, $CaCl_2$ 2, glucose 10, pH 7.4, previously gassed with 95% O_2 and 5% CO_2. The hippocampi were cut in 400 μm slices, perpendicularly to the long axis with a McIlwain tissue chopper and allowed to recover for 1 hour in Krebs solution at room temperature (22 ± 1 °C) before recording. One slice was transferred to a submerged recording chamber (1 mL capacity), where it was continuously superfused at a rate of 3 mL/min with the same gassed solution at 30.5 °C. Field excitatory postsynaptic potentials (fEPSPs) were recorded as previously described (Costenla et al., 2001) in *Stratum radiatum* of the CA1 area through an extracellular microelectrode (2–4 MΩ). Stimulation (rectangular 0.1 ms pulses, once every 20 seconds) was delivered through a bipolar concentric wire electrode placed on the Schaffer collateral-commissural fibers in the *Stratum radiatum* near the CA3-CA1 border. The intensity of the stimulus (80–200 μA) was initially adjusted to obtain a large fEPSP slope with a minimum population spike contamination. Recordings were obtained with an Axoclamp 2B amplifier and digitized (Axon Instruments, Foster City, CA, USA). Individual responses were monitored, and averages of 8 consecutive responses were continuously stored on a personal computer with the LTP program (Anderson & Collingridge, 2001). The protocols were performed after obtaining a stable recording of fEPSP for at least 30 minutes. LTP was induced by High Frequency Stimulation (HFS), consisting of 1 train of 100 pulses at 100 Hz within 1 second (Costenla et al., 2001). LTP magnitude was quantified as the percentage of change in the average fEPSP slope taken from 50–60 minutes after LTP induction in relation to the average fEPSP slope in the 10 minutes that preceded the induction protocol.

2.8. Statistics

The significance of differences between the 2 experimental conditions (corticosterone levels, elevated plus maze [EPM] and LTP recordings) was tested using unpaired Student's t test. Body weight for each age was also analyzed using unpaired Student's t test because of uneven number of subjects for each age. Values of $p < 0.05$ were considered statistically significant. Repeated measures 2-way analysis of variance, followed by Dunnett or Tukey post hoc tests, as indicated, were used to compare performance parameters across time in Morris water maze analyses. The 2-way analysis of variance and post hoc tests were performed using the Predictive Analytics Software 18.0 (SPSS Inc, an IBM Company, Chicago, IL, USA). GraphPad Prism 5.0 (GraphPad Prism Software Inc, San Diego, CA, USA) was used for all other statistical tests. All values are presented as mean ± standard error of the mean of the indicated number of experiments.

3. Results

3.1. Control for effective MS stress establishment

As indicated by the measurements taken at PND 2, 14, 60, 90, and 490 (70-week-old) (Fig. 1A), there were no significant effects of rearing on body weight during development and adulthood ($p > 0.05$; n = 20 in PND 2 and 14; n = 8 in PND 60 and n = 12 in PND 490). To control that the MS protocol was effective across the different cohorts of animals, we quantified the corticosterone levels in plasma of animals in adulthood and we tested them in the EPM, at the same age (PND 40) as described previously (Batalha et al.,

2013) (Fig. 1B and C). The corticosterone levels in plasma were significantly elevated as a result of the maternal separation, compared with control animals, at PND 40 ($p = 0.034$; n = 7 for CTR and n = 4 for MS, Student *t* test).

In the elevated-plus maze, the percentage of time spent in the open arms of the maze (Fig. 1C) reveals a significant effect of rearing ($p = 0.034$; n = 5 for CTR and n = 8 for MS) in anxious behavior at PND 40. The total number of arm entries (not shown), did not change between groups.

3.2. Aged MS rats show deficits on hippocampal-dependent learning and memory, when compared with aged control rats

We then evaluated whether this MS-induced anxious behavior displayed in adulthood was accompanied by cognitive deficits in late life, by testing the animals at 70 weeks of age in the spatial reference memory task of the Morris water maze (Fig. 2).

In aged rats, both CTR (n = 6) and MS (n = 8) groups showed significant learning by improved latency ($F_{4,56} = 15.07$, $p < 0.0001$, Fig. 2D) and reduced pathlength ($F_{4,56} = 15.03$, $p < 0.0001$, data not shown) to reach the platform. We found no major significant differences between groups in the escape latency ($F_{1,56} = 0.23$, $p > 0.05$). However, a memory retention deficit in the aged MS group was statistically significant when animals performed the probe trial: the MS rats (n = 8) spent significantly less time in the platform quadrant than control animals (n = 6; $p = 0.035$, Student *t* test, Fig. 2E).

In the EPM, aged MS animals still presented a higher anxious-related behavior (spent less time in the open arms, 15.5 ± 4.2% in MS (n = 8) vs. 33.7 ± 6.3% in CTR (n = 6), $p = 0.004$, Fig. 2F). The total number of arm entries (not shown), did not change between groups.

3.3. LTP induced by HFS stimulus is impaired in aged MS animals

We then evaluated if the impairments driven by MS in the hippocampal dependent-memory of aged animals, were associated with changes in synaptic plasticity (Fig. 2A). Field excitatory post-synaptic potentials were measured in the CA1 area of the dorsal

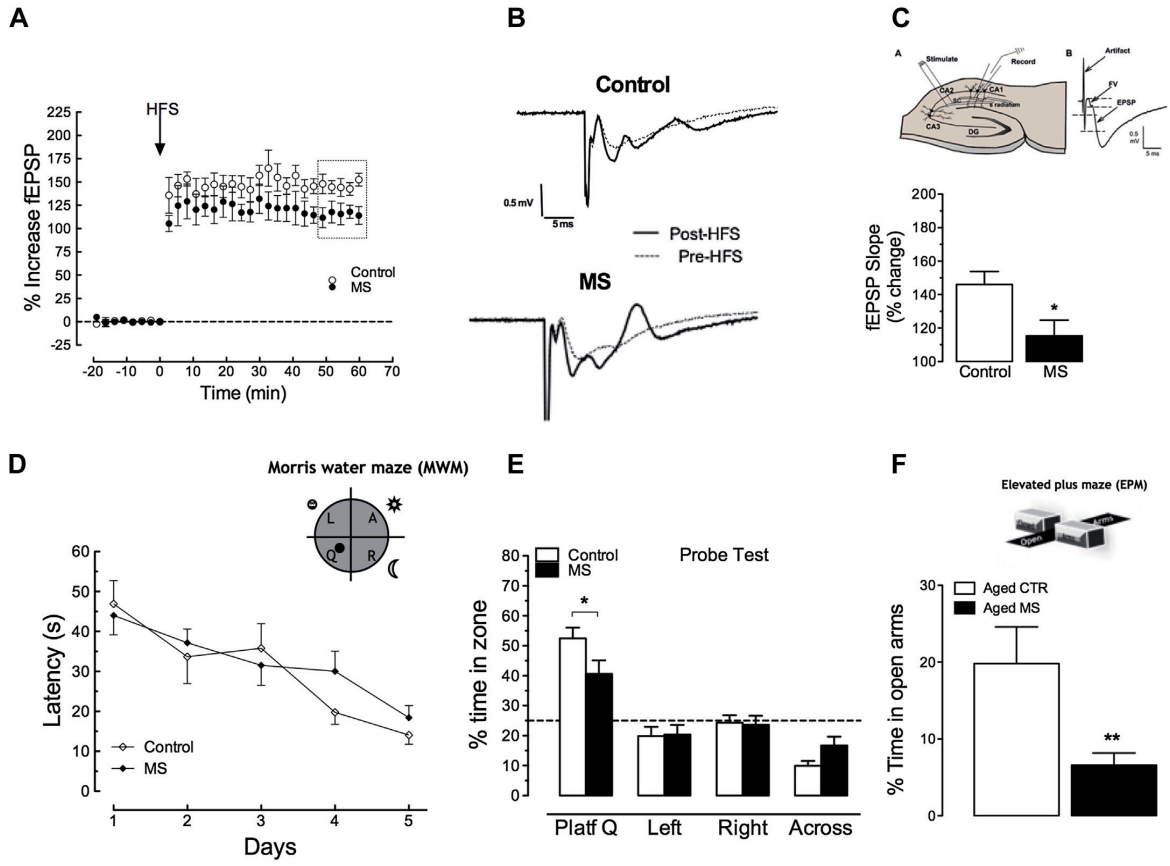

Fig. 2. LTP and spatial memory is aggravated in aged MS animals. High frequency stimulation (HFS)-induced long-term potentiation (LTP) is significantly lower in rats subjected to maternal separation (MS). (A) Averaged time course changes in field excitatory post-synaptic potential (fEPSP) slope induced by HFS in hippocampal slices taken from 70-week-old MS and control rats. (B) Representative recordings of the fEPSPs obtained both for aged CTR and MS animals before LTP induction (pre HFS, dotted line) and in the last 10 minutes (post HFS, solid line) are presented. (C) Percentage of change in magnitude of LTP (fEPSP slope average at 50–60 minutes). * $p = 0.035$, Student *t* test, n = 4/5. The recording setup arrangement is depicted in the upper right panel. Influence of maternal separation (MS) and aging upon spatial memory, as measured by (D) the escape latency and (E) percentage of time spent in the platform quadrant in probe trial, which was conducted 1 hour after the last training trial at day 5, in the absence of escape platform. Note that MS experience affected memory recall in aged animals, as the MS group spent significantly less time in the platform quadrant in the probe trial (* $p = 0.041$, Student *t* test, n = 6 for CTR and n = 8 for MS). (F) Percentage of time spent in the open arms of the elevated plus-maze. A significant effect of MS in reducing percentage of time in open arms was observed in aged rats. (** $p = 0.004$, Student *t* test, n = 6 for CTR and n = 8 for MS). Abbreviation: CTR, control.

hippocampus and LTP magnitude quantified in both control and MS groups from aged animals (70-week-old) (Fig. 2B). The HFS stimulation induced a statistically significant LTP in slices taken from control animals (open circles) of 146.0 ± 7.8%, n = 4 (Fig. 2C). In contrast, in hippocampal slices from MS rats (filled circles) the magnitude of LTP decreased to 115.4 ± 9.3%, n = 5, * $p = 0.035$, Student t test (Fig. 2C).

4. Discussion

In the present work, we show that adult anxious behavior and spatial memory deficits induced by MS persist into old age, where we observed that senescent MS animals have an associated lower magnitude of hippocampal LTP. These results substantiate the hypothesis that the neuronal and endocrine alterations induced by early-life stress are long lasting, and are able to exacerbate the mild age-associated deficits observed in normal aged animals.

Hippocampal synaptic plasticity is widely considered to be the mechanism associated with learning and memory (Bliss & Collingridge, 1993). Stress affects spatial learning and memory in the Morris water maze (de Quervain et al., 1998); accordingly, stress affects hippocampal LTP (Foy et al., 1987), facilitates long-term depression (Kim et al., 1996) and exposure to glucocorticoids reduces hippocampal LTP (McEwen, 1999a). The glucocorticoid cascade hypothesis (Sapolsky et al., 1986) suggests that elevated levels of glucocorticoids cause decreased levels of hippocampal glucocorticoid receptors and neuronal loss. It is known that aged rodents have elevated levels of plasma corticosterone (Landfield et al., 1978). Furthermore, elevated glucocorticoid levels have been linked to cognitive deficits (McEwen, 1999a). However, it remained to be determined the impact of MS on synaptic plasticity in aged animals.

In the present work, we observed that aged (70-week-old) animals subjected to neonatal stress have significantly lower magnitude of hippocampal LTP than the age-matched controls. Because neonatal stress induces long lasting increases in corticosteroids (Batalha et al., 2013) the observed hippocampal dysfunction in aged animals is in agreement with the known susceptibility of the hippocampus to elevated levels of circulating corticosteroids (Jacobson & Sapolsky, 1991).

In humans, altered cortisol levels are observed in individuals with posttraumatic stress syndrome or major depression (Gerritsen et al., 2011). Increased glucocorticoid activity has also been associated with greater hippocampal atrophy and memory impairment in the elderly (Lupien et al., 1998), and with a quicker progression of Alzheimer's disease (AD) (Csernansky et al., 2006). Notably, a large longitudinal study of human subjects aged 50–70 years, found that elevated salivary levels of cortisol were correlated with poor cognitive function across a broad range of domains including language, eye-hand coordination, executive function, verbal learning, memory, and visual memory (Lee et al., 2007). Furthermore, systemic administration of glucocorticoids was shown to exacerbate memory impairments, hippocampal damage, β-amyloid formation, and tau accumulation in rodents (Green et al., 2006; Yao et al., 2011). Conversely, administration of an antagonist of the receptors for corticotropin-releasing hormone immediately after the stress period improved memory performance later in life (Ivy et al., 2010). Interestingly, administration of an adenosine A2A receptor antagonist to adult animals subjected to MS reverted elevated plasma corticosterone levels and restored hippocampal glucocorticoid receptor expression pattern toward values similar to those detected in control rats. Furthermore, the A2A receptor antagonist effectively reverted the behavioral, electrophysiological, and morphologic impairments induced by early-life stress (Batalha et al., 2013). It thus becomes evident that early-life stress induces a hypothalamic-pituitary-adrenal axis dysregulation that continues throughout the life span of the individuals and that, as we now show, impacts in memory dysfunction and synaptic plasticity impairment in aging.

Furthermore, although aged MS animals did not perform worse than age-matched controls in the acquisition phase, they showed significant memory retrieval deficits, as indicated by their significantly poorer performance in the probe test. These observations are in line with existing evidence on the consequences of early-life stress upon aging (Ivy et al., 2010; Lehmann et al., 2002; Oitzl et al., 2000; Solas et al., 2010).

The mechanisms underlying the effects of high corticosterone levels are largely unknown. One relevant effect of glucocorticoid receptor activation that has been linked with memory function (Schaaf et al., 2000) is the suppression of brain-derived neurotrophic factor (BDNF) expression and reduced activation of insulin pathways (Cosi et al., 1993; Solas et al., 2010). Namely, the levels of insulin receptor, phosphorylated insulin receptor, and markers of downstream signaling pathways (pAkt, pGSK3β, pTau, and pERK1 levels) were significantly decreased in MS aged rats. Glucocorticoids are elevated in rodents with experimental diabetes (Magariños and McEwen, 2000) and it has been suggested that cognitive impairment in diabetes may result from glucocorticoid-mediated deficits in neurogenesis and synaptic plasticity (Stranahan et al., 2008). Furthermore, early work demonstrated an absolute requirement for ERK activity in induction of LTP and memory consolidation (Eckel-Mahan et al., 2008). As BDNF signaling activates the ERK1/2 pathway, it could be suggested that the inhibition of ERK cascade in MS aged rats could be related to both a lower BDNF levels and reduced insulin pathway activation. Moreover, we have previously shown an exacerbated tonus of endogenous BDNF leading to a facilitation of LTP (Diógenes et al., 2011). Whether the now reported deficits in synaptic plasticity in the MS aged animals may therefore result from a deficit in endogenous BDNF signaling or/and insulin pathway dysfunction, awaits further research.

The present study provides evidence for an early aging like phenomenon occurring to adult animals subjected to MS. Moreover, it implies that stress induced early in life might increase the susceptibility to cognitive deficits associated with aging. This evidence is thus important for the consideration of the psychosocial environment in early life as a susceptibility factor for age-related affective and cognitive dysfunction.

Disclosure statement

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. The manuscript has been read and approved by all named authors.

Acknowledgements

The authors acknowledge J. Baião for technical assistance in the animal house. Vasco C Sousa and Vânia L Batalha were supported by a grant from Fundação para a Ciência e Tecnologia; Luisa Vaqueiro Lopes is an Investigator FCT, funded by Fundação para a Ciência e Tecnologia and Bial.

References

Aisa, B., Tordera, R., Lasheras, B., Del Río, J., Ramírez, M.J., 2007. Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. Psychoneuroendocrinology 32, 256–266.

Altman, J., Das, G.D., 1965. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J. Comp. Neurol. 124, 319–335.

Anderson, W.W., Collingridge, G.L., 2001. The LTP Program: a data acquisition program for on-line analysis of long-term potentiation and other synaptic events. J. Neurosci. Methods 108, 71–83.
Batalha, V.L., Pego, J.M., Fontinha, B.M., Costenla, A.R., Valadas, J.S., Baqi, Y., Radjainia, H., Muller, C.E., Sebastiao, A.M., Lopes, L.V., 2013. Adenosine A(2A) receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation. Mol. Psychiatry 18, 320–331.
Bayer, S.A., 1980. Development of the hippocampal region in the rat. I. Neurogenesis examined with 3H-thymidine autoradiography. J. Comp. Neurol. 190, 87–114.
Bliss, T.V., Collingridge, G.L., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31–39.
Brunson, K.L., Lin, B., Chen, Y., Colgin, L.L., Yanagihara, T.K., Lynch, G., Baram, T.Z., 2005. Mechanisms of late-onset cognitive decline after early-life stress. Analysis 25, 9328–9338.
Burger C (2010). Region-specific genetic alterations in the aging hippocampus: implications for cognitive aging, Front Aging Neurosci. 2, 1–12.
Champagne, D.L., de Kloet, E.R., Joëls, M., 2009. Fundamental aspects of the impact of glucocorticoids on the (immature) brain. Semin. Fetal Neonatal Med. 14, 136–142.
Cosi, C., Spoerri, P.E., Comelli, M.C., Guidolin, D., Skaper, S.D., 1993. Glucocorticoids depress activity-dependent expression of BDNF mRNA in hippocampal neurones. Neuroreport 4, 527–530.
Costenla, A.R., Lopes, L.V., de Mendonca, A., Ribeiro, J.A., 2001. A functional role for adenosine A3 receptors: modulation of synaptic plasticity in the rat hippocampus. Neurosci. Lett. 302, 53–57.
Csernansky, J.G., Dong, H., Fagan, A.M., Wang, L., Xiong, C., Holtzman, D.M., Morris, J.C., 2006. Plasma cortisol and progression of dementia in subjects with Alzheimer-type dementia. Am. J. Psychiatry 163, 2164–2169.
de Quervain, D.J., Roozendaal, B., McGaugh, J.L., 1998. Stress and glucocorticoids impair retrieval of long-term spatial memory. Nature 394, 787–790.
Diógenes, M.J., Costenla, A.R., Lopes, L.V., Jerónimo-Santos, A., Sousa, V.C., Fontinha, B.M., Ribeiro, J.A., Sebastião, A.M., 2011. Enhancement of LTP in aged rats is dependent on endogenous BDNF. Neuropsychopharmacology 36, 1823–1836.
Eckel-Mahan, K.L., Phan, T., Han, S., Wang, H., Chan, G.C., Scheiner, Z.S., Storm, D.R., 2008. Circadian oscillation of hippocampal MAPK activity and cAMP: implications for memory persistence. Nat. Neurosci. 11, 1074–1082.
Foy, M.R., Stanton, M.E., Levine, S., Thompson, R.F., 1987. Behavioral stress impairs long-term potentiation in rodent hippocampus. Behav. Neural Biol. 48, 138–149.
Francis, D.D., Meaney, M.J., 1999. Maternal care and the development of stress responses. Curr. Opin. Neurobiol. 9, 128–134.
Gerritsen, L., Comijs, H.C., van der Graaf, Y., Knoops, A.J.G., Penninx, B.W.J.H., Geerlings, M.I., 2011. Depression, hypothalamic pituitary adrenal axis, and hippocampal and entorhinal cortex volumes—the SMART Medea study. Biol. Psychiatry 70, 373–380.
Green, K.N., Billings, L.M., Roozendaal, B., McGaugh, J.L., LaFerla, F.M., 2006. Glucocorticoids increase amyloid-beta and tau pathology in a mouse model of Alzheimer's disease. J. Neurosci. 26, 9047–9056.
Ivy, A.S., Rex, C.S., Chen, Y., Dube, C., Maras, P.M., Grigoriadis, D.E., Gall, C.M., Lynch, G., Baram, T.Z., 2010. Hippocampal dysfunction and cognitive impairments provoked by chronic early-life stress involve excessive activation of CRH receptors. J. Neurosci. 30, 13005–13015.
Jacobson, L., Sapolsky, R., 1991. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr. Rev. 12, 118–134.
Joëls, M., Baram, T.Z., 2009. The neuro-symphony of stress. Nat. Rev. Neurosci. 10, 459–466.
Kaufman, J., Plotsky, P.M., Nemeroff, C.B., Charney, D.S., 2000. Effects of early adverse experiences on brain structure and function: clinical implications. Biol. Psychiatry 48, 778–790.
Kim, J.J., Foy, M.R., Thompson, R.F., 1996. Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. Proc. Natl. Acad. Sci. U.S.A 93, 4750–4753.
Landfield, P.W., Waymire, J.C., Lynch, G., 1978. Hippocampal aging and adrenocorticoids: quantitative correlations. Science 202, 1098–1102.
Lee, B.K., Glass, T.A., McAtee, M.J., Wand, G.S., Bandeen-Roche, K., Bolla, K.I., Schwartz, B.S., 2007. Associations of salivary cortisol with cognitive function in the Baltimore memory study. Arch. Gen. Psychiatry 64, 810–818.
Lehmann, J., Pryce, C.R., Jongen-Relo, A.L., Stohr, T., Pothuizen, H.H., Feldon, J., 2002. Comparison of maternal separation and early handling in terms of their neurobehavioral effects in aged rats. Neurobiol. Aging 23, 457–466.
Lupien, S.J., de Leon, M., de Santi, S., Convit, A., Tarshish, C., Nair, N.P., Thakur, M., McEwen, B.S., Hauger, R.L., Meaney, M.J., 1998. Cortisol levels during human aging predict hippocampal atrophy and memory deficits. Nat. Neurosci. 1, 69–73.
Lupien, S.J., Fiocco, A., Wan, N., Maheu, F., Lord, C., Schramek, T., Tu, M.T., 2005. Stress hormones and human memory function across the lifespan. Psychoneuroendocrinology 30, 225–242.
Macrí, S., Mason, G.J., Würbel, H., 2004. Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. Eur. J. Neurosci. 20, 1017–1024.
Magariños, A.M., McEwen, B.S., 2000. Experimental diabetes in rats causes hippocampal dendritic and synaptic reorganization and increased glucocorticoid reactivity to stress. Proc. Natl. Acad. Sci. U.S.A 97, 11056–11061.
McEwen, B.S., 1999a. Stress and hippocampal plasticity. Annu. Rev. Neurosci. 22, 105–122.
McEwen, B.S., 1999b. Stress and the aging hippocampus. Front Neuroendocrinol. 20, 49–70.
Meaney, M.J., 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu. Rev. Neurosci. 24, 1161–1192.
Meaney, M.J., Aitken, D.H., van Berkel, C., Bhatnagar, S., Sapolsky, R.M., 1988. Effect of neonatal handling on age-related impairments associated with the hippocampus. Science 239, 766–768.
Muscatell, K.A., Slavich, G.M., Monroe, S.M., Gotlib, I.H., 2009. Stressful life events, chronic difficulties, and the symptoms of clinical depression. J. Nerv. Ment. Dis. 197, 154–160.
Oitzl, M.S., Workel, J.O., Fluttert, M., Frösch, F., De Kloet, E.R., 2000. Maternal deprivation affects behaviour from youth to senescence: amplification of individual differences in spatial learning and memory in senescent Brown Norway rats. Eur. J. Neurosci. 12, 3771–3780.
Plotsky, P.M., Meaney, M.J., 1993. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res. Mol. Brain Res. 18, 195–200.
Sapolsky, R.M., Krey, L.C., McEwen, B.S., 1983. The adrenocortical stress-response in the aged male rat: impairment of recovery from stress. Exp. Gerontol. 18, 55–64.
Sapolsky, R.M., Krey, L.C., McEwen, B.S., 1984. Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenocortical stress response. Proc. Natl. Acad. Sci. U.S.A 81, 6174–6177.
Sapolsky, R.M., Krey, L.C., McEwen, B.S., 1985. Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. J. Neurosci. 5, 1222–1227.
Sapolsky, R.M., Krey, L.C., McEwen, B.S., 1986. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocr. Rev. 7, 284–301.
Schaaf, M.J., De Kloet, E.R., Vreugdenhil, E., 2000. Corticosterone effects on BDNF expression in the hippocampus. Implications for memory formation. Stress 3, 201–208.
Solas, M., Aisa, B., Mugueta, M.C., Del Río, J., Tordera, R.M., Ramírez, M.J., 2010. Interactions between age, stress and insulin on cognition: implications for Alzheimer's disease. Neuropsychopharmacology 35, 1664–1673.
Stranahan, A.M., Arumugam, T.V., Cutler, R.G., Lee, K., Egan, J.M., Mattson, M.P., 2008. Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. Nat. Neurosci. 11, 309–317.
Yao, Y.Y., Wu, Q.S., Li, W.Z., Li, W.P., 2011. Dexamethasone potentiated Aβ-induced learning and memory impairment in rats. Neurol. Res. 33, 371–380.
Zhang, T.-Y., Meaney, M.J., 2010. Epigenetics and the environmental regulation of the genome and its function. Annu. Rev. Psychol. 61, 439–466. C431–433.

From epidemiology to pathophysiology: what about caffeine in Alzheimer's disease?

Vanessa Flaten*†, Cyril Laurent*†, Joana E. Coelho‡, Ursula Sandau§, Vânia L. Batalha‡, Sylvie Burnouf*†‖, Malika Hamdane*†‡, Sandrine Humez*†, Detlev Boison§, Luísa V. Lopes‡, Luc Buée*†¶ and David Blum*†¶[1]

*Inserm U837, F-59045 Lille, France

†Université Lille-Nord de France, UDSL, Jean-Pierre Aubert Research Centre, Institut de Médecine Prédictive et de Recherche Thérapeutique, F-59045 Lille, France

‡Instituto de Medicina Molecular, Lisbon, Portugal

§Legacy Research Institute, Portland, OR 97232, U.S.A.

‖Max Planck Institute for Biology of Ageing, Joseph-Stelzmann-Strasse 9B, D-50931 Köln, Germany

¶CHRU, F-59037 Lille, France

Abstract

AD (Alzheimer's disease) is the most prevalent form of dementia in the aged population. Definitive diagnosis of AD is based on the presence of senile plaques and neurofibrillary tangles that are identified in post-mortem brain specimens. A third pathological component is inflammation. AD results from multiple genetic and environmental risk factors. Among other factors, epidemiological studies report beneficial effects of caffeine, a non-selective antagonist of adenosine receptors. In the present review, we discuss the impact of caffeine and the adenosinergic system in AD pathology as well as consequences in terms of pathology and therapeutics.

Alzheimer's disease pathology

AD (Alzheimer's disease) is the most frequently encountered form of dementia. In most cases, AD appears as a sporadic multifactorial disease resulting from the interaction of different environmental, epigenetic and genetic factors [1]. Various epidemiological studies have allowed the identification of 'risk factors' and 'protective factors'. High blood pressure, diabetes and obesity are detrimental factors [2–4], whereas physical and intellectual activities, as well as fish consumption have protective effects ([5], and references therein, [6]).

The definitive diagnosis of AD is based on the observation of characteristic brain lesions: senile plaques and neurofibrillary tangles. Senile plaques are characterized by the extracellular accumulation of the Aβ (amyloid β-peptide), whereas neurofibrillary degeneration is due to the pathological accumulation in the neurons of the naturally present tau protein. Aβ derives from a precursor called APP (amyloid precursor protein) through the combined action of two distinct proteolytic enzymatic activities, β- and γ-secretase releasing N- and C-terminal fragments of Aβ respectively [7,8]. Notably, soluble Aβ oligomers are thought to be the most neurotoxic species driving the detrimental impact of amyloid pathology ([9] and references therein). Neurofibrillary degeneration arises from the intraneuronal accumulation of proteinaceous fibrils in PHFs (paired helical filaments) made of hyperphosphorylated tau proteins, forming flame-shaped neurons (for reviews, see [10,11]). Tau is a neuronal protein located within the axonal compartment, essential for the organization, stabilization and dynamics of microtubules [10,11], but tau has additional important neuronal functions at the dendritic and nuclear levels [12–14]. The physiological and pathological functions of tau are also regulated by post-translational modifications, such as phosphorylation. Changes in tau phosphorylation may affect multiple tau functions and facilitate tau aggregation [10,11]. Importantly, spreading of the neurofibrillary lesions in the cortex (first entorhinal cortex, then hippocampus and lastly neocortex), corresponds to the progression of the symptoms [15]. This supports the hypothesis that tau pathology is instrumental in cognitive alterations as stressed by observations that it impairs various forms of synaptic plasticity and cognitive tasks in mouse models [16–21]. Finally, neuroinflammatory processes are considered as a third pathological component in AD as recently pointed out by several genetics studies [13,22]. Although their respective contribution to the amyloid and tau sides of AD remain unclear so far, astrogliosis and neuroinflammatory events, especially those mediated by microglial cells, have an instrumental role in AD. An extensive description of their role in AD is beyond the scope of the present paper, but has been reviewed elsewhere [23,24].

Caffeine and Alzheimer's disease: from epidemiology to pathophysiology

The methylxanthine caffeine (1,3,7-trimethylxanthine) is the world's most popular psychoactive drug. The reason for this

Key words: adenosine receptor A_{2A}, Alzheimer's disease, caffeine, epidemiology.

Abbreviations: Aβ, amyloid β-peptide; APP, amyloid precursor protein; BACE1, β-site APP-cleaving enzyme 1; i.c.v., intracerebroventricular; LTP, long-term potentiation.

[1]**To whom correspondence should be addressed (email david.blum@inserm.fr).**

popularity lies in its psychostimulant properties combined with the absence of substantial negative side effects. Caffeine is contained in coffee, tea, soft drinks and chocolate. Overall, the psychostimulant properties of caffeine are due to its ability to interact with neurotransmission in different regions of the brain, thereby promoting behavioural functions, such as vigilance, attention, mood and arousal as well as improvement of cognition [25].

In humans, cognitive benefits of caffeine have been reported [26]. Brief exposure improves memory and cognitive function in paradigms of scopolamine-induced impairments [27]. Caffeine also improves attention and information processing [28]. In rodents, evidence from the last few years support the cognition-enhancing properties of caffeine in a variety of behavioural tasks that evaluated learning and memory [29]. Different longitudinal studies have investigated the relationship between coffee consumption, cognitive decline or dementia/AD. In the FINE (Finland, Italy and The Netherlands Elderly) Study, among elderly men, drinking three cups of coffee per day was associated with the least 10-year cognitive decline [30]. Furthermore, results from the Three City Study among 65-year-old persons indicated that a consumption over three cups per day was associated with less decline in verbal memory and, to a lesser extent, in visuospatial memory among women [31].

Interestingly, other studies support the hypothesis that caffeine consumption might prevent AD. A retrospective study has shown an inverse correlation between coffee intake and the occurrence of AD later on in life since patients with AD had an average daily caffeine intake of 73.9 ± 97.9 mg during the 20 years that preceded diagnosis of AD, whereas the controls had an average daily caffeine intake of 198.7 ± 135.7 mg during the corresponding 20 years of their lifetimes [32]. In the prospective CSHA (Canadian Study of Health and Aging), daily coffee drinking decreased the risk of AD by 31% during a 5-year follow-up [33]. In line with those findings, another study from the CAIDE (Cardiovascular Risk Factors, Aging and Dementia) population reports that coffee drinkers at midlife had lower risk of dementia and AD later in life compared with those drinking no or only little coffee. The lowest risk (65% decreased) was found in people who drank three to five cups per day [34]. Finally, recent prospective data indicated that high plasma caffeine levels were associated with a reduced risk of dementia or a delayed onset in patients with MCI (mild cognitive impairment) [35]. It is noteworthy that in a recent study from the Honolulu–Asian Aging Study, authors did not find a significant association between caffeine intake and dementia risk [36]. However, interestingly, they reported that, at autopsy, patients in the highest quartile of caffeine intake (>277.5 mg/day) were less likely to have any of the neuropatholgical lesions, such as AD-related lesions, microvascular ischaemic lesions, cortical Lewy bodies, hippocampal sclerosis or generalized atrophy. Therefore the available epidemiological data support the hypothesis that caffeine consumption is able to slow down cognitive decline in the elderly and reduces the risk of developing AD. Of note, although this also looks to be the case in Parkinson's disease [37], caffeine has been recently suggested to exhibit detrimental effects in Huntington's disease [38], suggesting that caffeine is not protective in all neurodegenerative conditions and that its effects depends on underlying instrumental mechanisms.

Several scientific studies support the hypothesis that caffeine is also beneficial in animal models which mimic the amyloid or tau sides of AD. Caffeine mitigates cognitive decline induced by Aβ and reduced the amyloid burden in transgenic mice overexpressing mutant APP (APP_{Sw}) in preventative, but also in therapeutic paradigms. Indeed, APP_{Sw} mice chronically treated from 4 to 9.5 months of age with caffeine (300 mg/l by drinking water) were cognitively improved in several behavioural tasks that evaluated working and spatial memory and exhibited reduction of hippocampal $A\beta_{1-40}$ and $A\beta_{1-42}$ [39]. Importantly, a similar treatment of APP_{Sw} mice at late pathological stages (18–19 months) for 4–5 weeks reversed memory deficits and reduced amyloid deposition as well as soluble Aβ levels in both entorhinal cortex and hippocampus [40]. Such beneficial effects of caffeine against Aβ production were recently confirmed by a different group using an experimental model of sporadic AD based on feeding rabbits with cholesterol-enriched diet that elevates both Aβ levels and tau phosphorylation in the brain [41]. In this study, rabbits fed on the cholesterol-enriched diet were treated with low doses of caffeine (0.5–30 mg/day) through drinking water, corresponding to a maximal 60 mg/day consumption in humans (i.e. approximately one cup of coffee). In this paradigm, caffeine significantly decreased Aβ production in accordance with the results of Arendash et al. [39,40]. Interestingly, reduced production of $A\beta_{1-40}$ and $A\beta_{1-42}$ was also observed in a neuroblastoma model overexpressing mutant APP following treatment with concentrations of caffeine below 10 μM [39], supporting further the notion that caffeine affects mechanisms underlying Aβ production. In accordance, chronic caffeine treatment of APP_{Sw} mice has been associated with decreased PS1 (presenilin 1) and BACE1 (β-site APP-cleaving enzyme 1) protein expression as well as increased IDE (insulin-degrading enzyme) levels, the latter presumably contributing to enhanced Aβ degradation [39,41]. The effect of caffeine on BACE1 expression could relate to its ability to reduce c-Raf1 activity, possibly through PKA (protein kinase A) activation [40]. In addition, caffeine would reduce GSK3 (glycogen synthase kinase 3) expression and/or activity and thereby influencing Aβ production [40]. However, a direct effect of caffeine on γ-secretase activity remains elusive, and mechanisms linking caffeine and Aβ production/clearance deserve further evaluation. It is finally noteworthy that, although the beneficial effects of coffee on cognitive decline and decreased AD risk in humans has been mostly ascribed to caffeine, other coffee constituents may also play an important role towards amyloid pathology. Indeed, two recent studies have shown that non-caffeine components of coffee display neuroprotective effects in *Drosophila melanogaster* and *Caenorhabditis elegans* amyloid models through activation

of the Nrf2 (nuclear factor-erythroid 2-related factor 2) detoxification pathway [42,43]. Interestingly, recent data indicate that caffeine may also have an impact on tau phosphorylation. Indeed, cultured neuronal cells exhibited reduced tau phosphorylation under caffeine treatment [44]. Although caffeine doses used are far above the levels normally obtained following habitual consumption, this indicates a possible relationship to tau. In accordance, we observed recently that chronic caffeine consumption by tau transgenic animals (THY-Tau22 strain) developing neurofibrillary lesions, prevented memory alterations as well as decreased tau phosphorylation in the hippocampus [45].

Caffeine targets and Alzheimer's disease: the role of adenosine receptors

Only at high millimolar concentrations, which are irrelevant for normal consumption, can caffeine act at the level of ryanodine receptors and cyclic nucleotide phosphodiesterases, but it is now well established that, under normal habitual caffeine consumption, the effects exerted in the brain by caffeine depend on its ability to block adenosine A_1 and A_{2A} receptors [25]. Adenosine receptors have a crucial neuromodulatory role and regulate both synaptic transmission and plasticity either by directly modulating synaptic responses or by interfering with other receptors [46].

During aging, we and others have found compelling evidence of cortical and hippocampal upsurge of A_{2A} receptor expression/function. Specifically, in the hippocampus of aged rats, A_{2A} receptor expression is nearly 2-fold higher compared with young rats [47,48]. More importantly, the A_{2A} receptor-dependent activation of glutamate release becomes more pronounced as aging progresses, and shifts from PKC (protein kinase C)-mediated signalling to cAMP-dependent effects [48,49]. This is accompanied by clear behavioural deficits in hippocampus-dependent tasks, such as spatial memory in rats [50]. Accordingly, rats overexpressing hippocampal A_{2A} receptors also exhibit behavioural deficits including spatial memory defects as well as LTP (long-term potentiation) impairments ([51], and L.V. Lopes et al., unpublished work). Interestingly, other detrimental conditions associated to cognitive impairment, such as hypoxia, diabetes or epilepsy share similar A_{2A} receptor overactivation ([48,52], and for a review, see [53]). Recently, we demonstrated decreased adult hippocampal LTP and cognitive/memory impairment in a chronic-stress-induced aging-like model, generated by maternal separation during the early postnatal period, in association with increased hippocampal A_{2A} receptor expression [52]. Strikingly, we observed, in adults, a normalization of synaptic and cognitive dysfunctions following A_{2A} receptor blockade with the selective antagonist KW6002 [52], indicating an instrumental role of A_{2A} receptor dysregulation in the genesis of synaptic dysfunctions underlying cognitive impairment within such aging context.

Importantly, additional convergent data indicate that caffeine protects against the synaptoneurotoxicity induced by Aβ through blockade of A_{2A} receptors. In primary cultures of cerebellar granule cells, low doses of caffeine (1–25 μM), comparable with those achieved following caffeine treatment in animals or moderate coffee consumption in humans [25], are able to counteract $A\beta_{25-35}$ toxicity, an effect mimicked by ZM241385, an A_{2A} receptor antagonist, but not CPT (8-cyclopentyltheophylline), a selective A_1 receptor antagonist [54]. These protective effects were confirmed *in vivo*. Subchronic treatment with caffeine at 30 mg/kg was shown protective against aversive and working memory deficits induced by i.c.v. (intracerebroventricular) injection of $A\beta_{25-35}$ in mice [55] and mimicked by administration of SCH58261, a selective A_{2A} receptor antagonist. A_{2A} receptor blockade, through intraperitoneal injection of SCH58261 and KW6002 or genetic knockout, was also shown to prevent working memory impairment as well as synaptic loss induced by i.c.v. injection of $A\beta_{1-42}$ [56,57]. Working memory improvement observed following A_{2A} receptor blockade was thought to be related to the prevention of synaptotoxicity promoted by Aβ through modulation of p38 MAPK (mitogen-activated protein kinase) and mitochondrial function [57]. Interestingly, it has been demonstrated that memory improvement promoted by A_{2A} receptor blockade following i.c.v. injection of Aβ was not observed in amnestic conditions induced by MK801 or scopolamine [56]. A_{2A} receptor blockade would then mitigate detrimental synaptic effects of Aβ. To date, no studies have been published on the impact of A_{2A} receptor modulation upon tau pathology in AD. However, we demonstrated recently the beneficial impact of A_{2A} receptor deletion in a transgenic mouse model of AD-like tau pathology [45].

Besides its direct action on synapses, the effects of A_{2A} receptor modulation upon AD pathophysiology could be non-neuronal. The role of A_{2A} receptors expressed by astroglial and microglial cells is far from understood (for a review, see [58]). However, a few studies indicate that A_{2A} receptors are up-regulated in both microglial and astroglial cells treated by Aβ [59,60]. A_{2A} receptors may promote activation and proliferation of astroglial cells [61,62], and thereby regulate their ability to release glutamate [63] by controlling glutamate uptake [60], or affect the homoeostasis of the endogenous neuroprotectant adenosine via adenosine kinase [64]. Astroglial A_{2A} receptor up-regulation may thus contribute to the pathophysiology of AD. This idea is in line with the observation of the reinstatement of glutamate uptake in Aβ-treated glial cells following A_{2A} receptor blockade [60]. In addition, it has been shown that A_{2A} receptor stimulation causes microglial activation [59] and potentiates the release of nitric oxide (NO) as well as prostaglandin E_2 release from these cells [65,66]. Different experimental evidence supports the anti-inflammatory effect of A_{2A} receptor blockade in different neuropathological situations [67–69]. Furthermore, A_{2A} receptor blockade mitigates LTP defects as well as microglial activation and IL-1β (interleukin 1β) release following LPS (lipopolysaccharide) administration [69]. Then, blocking microglial A_{2A} receptor could be beneficial in AD. However, as microglia may play a Janus

role in AD [24], this conclusion needs further evaluation. In particular, the role of A_{2A} receptor activation towards the pathophysiology of AD will require further *in vivo* studies with appropriate and reliable cell-type-specific models.

Conclusion

Although recent data indicate that perinatal caffeine may impair interneuron migration and hippocampal network function [70], epidemiological and pre-clinical data, including ours, support the notion that caffeine might be not only a cognitive enhancer, but also a disease modifier in AD. The qualities of caffeine as a safe [71], inexpensive and brain-penetrating agent deserves the translation of these findings into a pilot clinical trial in AD patients. Despite epidemiological data on the effects of caffeine in aged and AD subjects, and data from animal studies, no clinical trials have been performed to date to evaluate the extent by which caffeine can slow down disease progression in patients that have already developed AD. In addition, encouraging preliminary experimental data have been obtained related to the role of A_{2A} receptors in AD. Although more work is still needed to uncover specific A_{2A} receptor functions in AD, all findings to date indicate that antagonists tested in human trials, even when not optimal in terms of efficacy, have reliably been shown to be safe and tolerable ([72], for reviews, see [73,74]). Therefore A_{2A}-based trials will be feasible in the future, if we are able to better delineate the function of A_{2A} receptors in the pathophysiology of AD.

Funding

D.B. and L.B. are supported by France Alzheimer, La Ligue Européenne Contre la Maladie d'Alzheimer (LECMA)/Alzheimer Forschung Initiative, LabEx (excellence laboratory) DISTALZ (Development of Innovative Strategies for a Transdisciplinary approach to ALZheimer's disease), Inserm, Centre National de la Recherche Scientifique (CNRS), Université Lille 2, Région Nord/Pas-de-Calais, Agence Nationale de la Recherche (ADORATAU), ERA-Net (ABeta-ID) and Fonds Unique Interministériel MEDIALZ. V.F. holds a grant from Inserm/Region Nord pas de Calais. C.L. holds a doctoral grant from Lille 2 University. D.B. is supported through grants from the National Institutes of Health [grant number NS065957, MH083973 and NS061844], the U.S. Department of the Army [grant number W81XWH-12-1-0283] and the Legacy Good Samaritan Foundation. L.V.L. is an Intergroupe Francophone de Cancérologie Thoracique (iFCT) Investigator. Her laboratory has support from Fritz-Thyssen Foundation, Bial and Fundação para a Ciência e a Tecnologia (Portugal). D.B. is an Inserm investigator.

References

1 Reitz, C., Brayne, C. and Mayeux, R. (2011) Epidemiology of Alzheimer disease. Nat. Rev. Neurol. **7**, 137–152
2 Pasinetti, G.M. and Eberstein, JA. (2008) Metabolic syndrome and the role of dietary lifestyles in Alzheimer's disease. J. Neurochem. **106**, 1503–1514
3 Ballard, C., Gauthier, S., Corbett, A., Brayne, C., Aarsland, D. and Jones, E. (2011) Alzheimer's disease. Lancet **377**, 1019–1031
4 Leboucher, A., Laurent, C., Fernandez-Gomez, F.J., Burnouf, S., Troquier, L., Eddarkaoui, S., Demeyer, D., Caillierez, R., Zommer, N., Vallez, E. et al. (2013) Detrimental effects of diet-induced obesity on tau pathology are independent of insulin resistance in tau transgenic mice. Diabetes **62**, 1681–1688
5 La Rue, A. (2010) Healthy brain aging: role of cognitive reserve, cognitive stimulation, and cognitive exercises. Clin. Geriatr. Med. **26**, 99–111
6 Belarbi, K., Burnouf, S., Fernandez-Gomez, F.J., Laurent, C., Lestavel, S., Figeac, M., Sultan, A., Troquier, L., Leboucher, A., Caillierez, R. et al. (2011) Beneficial effects of exercise in a transgenic mouse model of Alzheimer's disease-like tau pathology. Neurobiol. Dis. **43**, 486–494
7 Checler, F. (1995) Processing of the β-amyloid precursor protein and its regulation in Alzheimer's disease. J. Neurochem. **65**, 1431–1444
8 De Strooper, B., Vassar, R. and Golde, T. (2010) The secretases: enzymes with therapeutic potential in Alzheimer disease. Nat. Rev. Neurol. **6**, 99–107
9 Brouillette, J., Caillierez, R., Zommer, N., Alves-Pires, C., Benilova, I., Blum, D., De Strooper, B. and Buée, L. (2012) Neurotoxicity and memory deficits induced by soluble low-molecular-weight amyloid-β_{1-42} oligomers are revealed *in vivo* by using a novel animal model. J. Neurosci. **32**, 7852–7861
10 Buée, L., Bussiere, T., Buée-Scherrer, V., Delacourte, A. and Hof, P.R. (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res. Brain Res. Rev. **33**, 95–130
11 Sergeant, N., Bretteville, A., Hamdane, M., Caillet-Boudin, M.L., Grognet, P., Bombois, S., Blum, D., Delacourte, A., Pasquier, F., Vanmechelen, E. et al. (2008) Biochemistry of tau in Alzheimer's disease and related neurological disorders. Expert Rev. Proteomics **5**, 207–224
12 Sultan, A., Nesslany, F., Violet, M., Begard, S., Loyens, A., Talahari, S., Mansuroglu, Z., Marzin, D., Sergeant, N., Humez, S. et al. (2011) Nuclear tau, a key player in neuronal DNA protection. J. Biol. Chem. **286**, 4566–4575
13 Hollingworth, P., Harold, D., Sims, R., Gerrish, A., Lambert, J.C., Carrasquillo, M.M., Abraham, R., Hamshere, M.L., Pahwa, J.S., Moskvina, V. et al. (2011) Common variants at *ABCA7*, *MS4A6A/MS4A4E*, *EPHA1*, *CD33* and *CD2AP* are associated with Alzheimer's disease. Nat. Genet. **43**, 429–435
14 Ittner, L.M., Ke, Y.D., Delerue, F., Bi, M., Gladbach, A., van Eersel, J., Wolfing, H., Chieng, B.C., Christie, M.J., Napier, I.A. et al. (2010) Dendritic function of tau mediates amyloid-β toxicity in Alzheimer's disease mouse models. Cell **142**, 387–397
15 Vingtdeux, V., Sergeant, N. and Buée, L. (2012) Potential contribution of exosomes to the prion-like propagation of lesions in Alzheimer's disease. Front. Physiol. **3**, 229
16 Hoover, B.R., Reed, M.N., Su, J., Penrod, R.D., Kotilinek, L.A., Grant, M.K., Pitstick, R., Carlson, G.A., Lanier, L.M., Yuan, L.L. et al. (2010) Tau mislocalization to dendritic spines mediates synaptic dysfunction independently of neurodegeneration. Neuron **68**, 1067–1081
17 Polydoro, M., Acker, C.M., Duff, K., Castillo, P.E. and Davies, P. (2009) Age-dependent impairment of cognitive and synaptic function in the htau mouse model of tau pathology. J. Neurosci. **29**, 10741–10749
18 Van der Jeugd, A., Ahmed, T., Burnouf, S., Belarbi, K., Hamdame, M., Grosjean, M.E., Humez, S., Balschun, D., Blum, D., Buée, L. and D'Hooge, R. (2011) Hippocampal tauopathy in tau transgenic mice coincides with impaired hippocampus-dependent learning and memory, and attenuated late-phase long-term depression of synaptic transmission. Neurobiol. Learn. Mem. **95**, 296–304
19 Van der Jeugd, A., Vermaercke, B., Derisbourg, M., Lo, A.C., Hamdane, M., Blum, D., Buée, L. and D'Hooge, R. (2013) Progressive age-related cognitive decline in tau mice. J. Alzheimer's Dis. **37**, 777–778
20 Lo, AC, Iscru, E., Blum, D., Tesseur, I., Callaerts-Vegh, Z., Buée, L., De Strooper, B., Balschun, D. and D'Hooge, R. (2013) Amyloid and tau neuropathology differentially affect prefrontal synaptic plasticity and cognitive performance in mouse models of Alzheimer's disease. J. Alzheimer's Dis. **37**, 109–125
21 Burnouf, S., Martire, A., Derisbourg, M., Laurent, C., Belarbi, K., Leboucher, A., Fernandez-Gomez, F.J., Troquier, L., Eddarkaoui, S., Grosjean, M.E. et al. (2013) NMDA receptor dysfunction contributes to impaired brain-derived neurotrophic factor-induced facilitation of hippocampal synaptic transmission in a tau transgenic model. Aging Cell **12**, 11–23
22 Guerreiro, R., Wojtas, A., Bras, J., Carrasquillo, M., Rogaeva, E., Majounie, E., Cruchaga, C., Sassi, C., Kauwe, J.S., Younkin, S. et al. (2013) TREM2 variants in Alzheimer's disease. N. Engl. J. Med. **368**, 117–127

23 Heneka, M.T. and O'Banion, M.K. (2007) Inflammatory processes in Alzheimer's disease. J. Neuroimmunol. **184**, 69–91
24 Wyss-Coray, T. and Rogers, J. (2012) Inflammation in Alzheimer disease: a brief review of the basic science and clinical literature. Cold Spring Harb. Perspect. Med. **2**, a006346
25 Fredholm, B.B., Battig, K., Holmen, J., Nehlig, A. and Zvartau, E.E. (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev. **51**, 83–133
26 Haskell, C.F., Kennedy, D.O., Wesnes, K.A. and Scholey, A.B. (2005) Cognitive and mood improvements of caffeine in habitual consumers and habitual non-consumers of caffeine. Psychopharmacology **179**, 813–825
27 Riedel, W., Hogervorst, E., Leboux, R., Verhey, F., van Praag, H. and Jolles, J. (1995) Caffeine attenuates scopolamine-induced memory impairment in humans. Psychopharmacology **122**, 158–168
28 Lorist, M.M. and Tops, M. (2003) Caffeine, fatigue, and cognition. Brain Cogn. **53**, 82–94
29 Takahashi, R.N., Pamplona, F.A. and Prediger, R.D. (2008) Adenosine receptor antagonists for cognitive dysfunction: a review of animal studies. Front. Biosci. **13**, 2614–2632
30 van Gelder, B.M., Buijsse, B., Tijhuis, M., Kalmijn, S., Giampaoli, S., Nissinen, A. and Kromhout, D. (2007) Coffee consumption is inversely associated with cognitive decline in elderly European men: the FINE Study. Eur. J. Clin. Nutr. **61**, 226–232
31 Ritchie, K., Carriere, I., de Mendonca, A., Portet, F., Dartigues, J.F., Rouaud, O., Barberger-Gateau, P. and Ancelin, M.L. (2007) The neuroprotective effects of caffeine: a prospective population study (the Three City Study). Neurology **69**, 536–545
32 Maia, L. and de Mendonca, A. (2002) Does caffeine intake protect from Alzheimer's disease? Eur. J. Neurol. **9**, 377–382
33 Lindsay, J., Laurin, D., Verreault, R., Hebert, R., Helliwell, B., Hill, G.B. and McDowell, I. (2002) Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging. Am. J. Epidemiol. **156**, 445–453
34 Eskelinen, M.H., Ngandu, T., Tuomilehto, J., Soininen, H. and Kivipelto, M. (2009) Midlife coffee and tea drinking and the risk of late-life dementia: a population-based CAIDE study. J. Alzheimer's Dis. **16**, 85–91
35 Cao, C., Loewenstein, D.A., Lin, X., Zhang, C., Wang, L., Duara, R., Wu, Y., Giannini, A., Bai, G., Cai, J. et al. (2012) High blood caffeine levels in MCI linked to lack of progression to dementia. J. Alzheimer's Dis. **30**, 559–572
36 Gelber, R.P., Petrovitch, H., Masaki, K.H., Ross, G.W. and White, L.R. (2011) Coffee intake in midlife and risk of dementia and its neuropathologic correlates. J. Alzheimer's Dis. **23**, 607–615
37 Ross, G.W. and Petrovitch, H. (2001) Current evidence for neuroprotective effects of nicotine and caffeine against Parkinson's disease. Drugs Aging **18**, 797–806
38 Simonin, C., Duru, C., Salleron, J., Hincker, P., Charles, P., Delval, A., Youssov, K., Burnouf, S., Azulay, J.P., Verny, C. et al. (2013) Association between caffeine intake and age at onset in Huntington's disease. Neurobiol. Dis. **58**, 179–182
39 Arendash, G.W., Schleif, W., Rezai-Zadeh, K., Jackson, E.K., Zacharia, L.C., Cracchiolo, J.R., Shippy, D. and Tan, J. (2006) Caffeine protects Alzheimer's mice against cognitive impairment and reduces brain β-amyloid production. Neuroscience **142**, 941–952
40 Arendash, G.W., Mori, T., Cao, C., Mamcarz, M., Runfeldt, M., Dickson, A., Rezai-Zadeh, K., Tane, J., Citron, B.A., Lin, X. et al. (2009) Caffeine reverses cognitive impairment and decreases brain amyloid-β levels in aged Alzheimer's disease mice. J. Alzheimer's Dis. **17**, 661–680
41 Prasanthi, J.R., Dasari, B., Marwarha, G., Larson, T., Chen, X., Geiger, J.D. and Ghribi, O. (2010) Caffeine protects against oxidative stress and Alzheimer's disease-like pathology in rabbit hippocampus induced by cholesterol-enriched diet. Free Radic. Biol. Med. **49**, 1212–1220
42 Dostal, V., Roberts, C.M. and Link, C.D. (2010) Genetic mechanisms of coffee extract protection in a *Caenorhabditis elegans* model of β-amyloid peptide toxicity. Genetics **186**, 857–866
43 Trinh, K., Andrews, L., Krause, J., Hanak, T., Lee, D., Gelb, M. and Pallanck, L. (2010) Decaffeinated coffee and nicotine-free tobacco provide neuroprotection in *Drosophila* models of Parkinson's disease through an NRF2-dependent mechanism. J. Neurosci. **30**, 5525–5532
44 Currais, A., Kato, K., Canuet, L., Ishii, R., Tanaka, T., Takeda, M. and Soriano, S. (2011) Caffeine modulates tau phosphorylation and affects Akt signaling in postmitotic neurons. J. Mol. Neurosci. **43**, 326–332
45 Blum, D., Laurent, C., Burnouf, S., Ferry, B., Marciniak, E., Derisbourg, M., Eddarkaoui, S., Parrot, S., Demeyer, D., Ledent, C. et al. (2013) Effects of caffeine intake and adenosine A_{2A} receptor deletion in a transgenic model of Alzheimer's disease-like tau pathology. Neuroscience 2013 Meeting, San Diego, CA, U.S.A. 9–13, November 2013, Abstract 523.14/H3
46 Sebastiao, A.M., Ribeiro, F.F. and Ribeiro, J.A. (2012) From A_1 to A_3 en passant through A_{2A} receptors in the hippocampus: pharmacological implications. CNS Neurol. Disord. Drug Targets **11**, 652–663
47 Cunha, R.A., Constantino, M.C., Sebastiao, A.M. and Ribeiro, J.A. (1995) Modification of A_1 and A_{2a} adenosine receptor binding in aged striatum, hippocampus and cortex of the rat. NeuroReport **6**, 1583–1588
48 Lopes, L.V., Cunha, R.A. and Ribeiro, J.A. (1999) Cross talk between A_1 and A_{2A} adenosine receptors in the hippocampus and cortex of young adult and old rats. J. Neurophysiol. **82**, 3196–3203
49 Rebola, N., Rodrigues, R.J., Lopes, L.V., Richardson, P.J., Oliveira, C.R. and Cunha, R.A. (2005) Adenosine A_1 and A_{2A} receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. Neuroscience **133**, 79–83
50 Diogenes, M.J., Costenla, A.R., Lopes, L.V., Jeronimo-Santos, A., Sousa, V.C., Fontinha, B.M., Ribeiro, J.A. and Sebastiao, A.M. (2011) Enhancement of LTP in aged rats is dependent on endogenous BDNF. Neuropsychopharmacology **36**, 1823–1836
51 Gimenez-Llort, L., Schiffmann, S.N., Shmidt, T., Canela, L., Camon, L., Wassholm, M., Canals, M., Terasmaa, A., Fernandez-Teruel, A., Tobena, A. et al. (2007) Working memory deficits in transgenic rats overexpressing human adenosine A_{2A} receptors in the brain. Neurobiol. Learn. Mem. **87**, 42–56
52 Batalha, V.L., Pego, J.M., Fontinha, B.M., Costenla, A.R., Valadas, J.S., Baqi, Y., Radjainia, H., Muller, C.E., Sebastiao, A.M. and Lopes, L.V. (2013) Adenosine A_{2A} receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation. Mol. Psychiatry **18**, 320–331
53 Lopes, L.V., Sebastiao, A.M. and Ribeiro, J.A. (2011) Adenosine and related drugs in brain diseases: present and future in clinical trials. Curr. Top. Med. Chem. **11**, 1087–1101
54 Dall'Igna, O.P., Porciuncula, L.O., Souza, D.O., Cunha, R.A. and Lara, D.R. (2003) Neuroprotection by caffeine and adenosine A_{2A} receptor blockade of β-amyloid neurotoxicity. Br. J. Pharmacol. **138**, 1207–1209
55 Dall'Igna, O.P., Fett, P., Gomes, M.W., Souza, D.O., Cunha, R.A. and Lara, D.R. (2007) Caffeine and adenosine A_{2a} receptor antagonists prevent β-amyloid (25–35)-induced cognitive deficits in mice. Exp. Neurol. **203**, 241–245
56 Cunha, G.M., Canas, P.M., Melo, C.S., Hockemeyer, J., Muller, C.E., Oliveira, C.R. and Cunha, R.A. (2008) Adenosine A2A receptor blockade prevents memory dysfunction caused by β-amyloid peptides but not by scopolamine or MK-801. Exp. Neurol. **210**, 776–781
57 Canas, P.M., Porciuncula, L.O., Cunha, G.M., Silva, C.G., Machado, N.J., Oliveira, J.M., Oliveira, C.R. and Cunha, R.A. (2009) Adenosine A_{2A} receptor blockade prevents synaptotoxicity and memory dysfunction caused by β-amyloid peptides via p38 mitogen-activated protein kinase pathway. J. Neurosci. **29**, 14741–14751
58 Hasko, G., Pacher, P., Vizi, E.S. and Illes, P. (2005) Adenosine receptor signaling in the brain immune system. Trends Pharmacol. Sci. **26**, 511–516
59 Orr, A.G., Orr, A.L., Li, X.J., Gross, R.E. and Traynelis, S.F. (2009) Adenosine A_{2A} receptor mediates microglial process retraction. Nat. Neurosci. **12**, 872–878
60 Matos, M., Augusto, E., Machado, N.J., dos Santos-Rodrigues, A., Cunha, R.A. and Agostinho, P. (2012) Astrocytic adenosine A_{2A} receptors control the amyloid-β peptide-induced decrease of glutamate uptake. J. Alzheimer's Dis. **31**, 555–567
61 Hindley, S., Herman, M.A. and Rathbone, M.P. (1994) Stimulation of reactive astrogliosis *in vivo* by extracellular adenosine diphosphate or an adenosine A_2 receptor agonist. J. Neurosci. Res. **38**, 399–406
62 Ke, R.H., Xiong, J., Liu, Y. and Ye, Z.R. (2009) Adenosine A_{2a} receptor induced gliosis via Akt/NF-κB pathway *in vitro*. Neurosci. Res. **65**, 280–285
63 Nishizaki, T. (2004) ATP- and adenosine-mediated signaling in the central nervous system: adenosine stimulates glutamate release from astrocytes via A_{2a} adenosine receptors. J. Pharmacol. Sci. **94**, 100–102
64 Pignataro, G., Maysami, S., Studer, F.E., Wilz, A., Simon, R.P. and Boison, D. (2008) Downregulation of hippocampal adenosine kinase after focal ischemia as potential endogenous neuroprotective mechanism. J. Cereb. Blood Flow Metab. **28**, 17–23

65 Saura, J., Angulo, E., Ejarque, A., Casado, V., Tusell, J.M., Moratalla, R., Chen, J.F., Schwarzschild, M.A., Lluis, C., Franco, R. and Serratosa, J. (2005) Adenosine A_{2A} receptor stimulation potentiates nitric oxide release by activated microglia. J. Neurochem. **95**, 919–929
66 Fiebich, B.L., Biber, K., Lieb, K., van Calker, D., Berger, M., Bauer, J. and Gebicke-Haerter, P.J. (1996) Cyclooxygenase-2 expression in rat microglia is induced by adenosine A_{2a}-receptors. Glia **18**, 152–160
67 Yu, L., Shen, H.Y., Coelho, J.E., Araujo, I.M., Huang, Q.Y., Day, Y.J., Rebola, N., Canas, P.M., Rapp, E.K., Ferrara, J. et al. (2008) Adenosine A_{2A} receptor antagonists exert motor and neuroprotective effects by distinct cellular mechanisms. Ann. Neurol. **63**, 338–346
68 Dai, S.S., Zhou, Y.G., Li, W., An, J.H., Li, P., Yang, N., Chen, X.Y., Xiong, R.P., Liu, P., Zhao, Y. et al. (2010) Local glutamate level dictates adenosine A_{2A} receptor regulation of neuroinflammation and traumatic brain injury. J. Neurosci. **30**, 5802–5810
69 Rebola, N., Simoes, A.P., Canas, P.M., Tome, A.R., Andrade, G.M., Barry, C.E., Agostinho, P.M., Lynch, M.A. and Cunha, R.A. (2011) Adenosine A_{2A} receptors control neuroinflammation and consequent hippocampal neuronal dysfunction. J. Neurochem. **117**, 100–111
70 Silva, C.G., Métin, C., Fazeli, W., Machado, N.J., Darmopil, S., Launay, P.S., Ghestem, A., Nesa, M.P., Bassot, E., Szabó, E. et al. (2013) Adenosine receptor antagonists including caffeine alter fetal brain development in mice. Sci. Transl. Med. **5**, 197ra04
71 Postuma, R.B., Lang, A.E., Munhoz, R.P., Charland, K., Pelletier, A., Moscovich, M., Filla, L., Zanatta, D., Rios Romenets, S., Altman, R. et al. (2012) Caffeine for treatment of Parkinson disease: a randomized controlled trial. Neurology **79**, 651–658
72 Simola, N., Morelli, M. and Pinna, A. (2008) Adenosine A_{2A} receptor antagonists and Parkinson's disease: state of the art and future directions. Curr. Pharm. Des. **14**, 1475–1489
73 Morelli, M., Carta, A.R. and Jenner, P. (2009) Adenosine A_{2A} receptors and Parkinson's disease. Handb. Exp. Pharmacol. 589–615
74 Pinna, A. (2009) Novel investigational adenosine A_{2A} receptor antagonists for Parkinson's disease. Expert Opin. Invest. Drugs **18**, 1619–1631

Received 17 September 2013
doi:10.1042/BST20130229