On the front of the page:

Microglial cells stained with Iba-1 (green) in brain slice. Nuclei stained with DAPI (blue).

DIMORPHIC BRAIN REGION REGULATION OF MICROGLIA MORPHOLOGY AND BEHAVIORAL IMPLICATION

FOCUS ON THE CROSSTALK BETWEEN GLUCOCORTICOID AND ADENOSINE A_{2A} RECEPTORS

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“I have not failed.

I've just found 10000 ways that won't work.”

Thomas Edison

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Oral Communications

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ABSTRACT

Synthetic glucocorticoids, such as dexamethasone, are used in pregnancies at risk of premature delivery in order to promote fetal lung maturation. Animal and human studies show that this treatment leads to alterations in the normal growth and deleterious effects in the developing brain and other organs, but the high efficacy of these drugs and the significant decrease in mortality justify their wide use in clinical practice. Previous studies have also shown that exposure to stress, or administration of glucocorticoids during early phases of development, can contribute to the development of neuropsychiatric disorders in adulthood, such as anxiety, depression and addictive behavior. Moreover, studies with animal models using prenatal exposure to glucocorticoids have reported deleterious effects on neurons, such as neuronal apoptosis, synaptic loss and dendritic atrophy.

Microglia are cellular elements of the brain innate immune system, responsive to changes in the immune environment, as occurs by exposure to synthetic glucocorticoids, which are immunomodulators. These cells are affected in their morphologic phenotype in conditions of chronic stress and previous work from our laboratory shows that prenatal exposure to dexamethasone alters microglia morphology in the prefrontal cortex, an effect observed at post-natal days 1,7 and 90. Notably, the effect of dexamethasone on microglia morphology depends on the gender of the animal.

Adenosine is a neuromodulator, which activates different receptor subtypes, namely adenosine A_{2A} receptors, that are implicated in anxiety and depression and are also modulators of microglia morphology and function.

Previous *in vivo* and *in vitro* data from our laboratory suggest a possible crosstalk between glucocorticoid and adenosine A_{2A} receptors systems in microglial cells. This previous work highlights the gender-specific modulation of microglia and behavior (anxiety and recognition memory) by glucocorticoids and adenosine A_{2A} receptors.

In the present work we aimed at further dissecting the crosstalk between glucocorticoids and adenosine A_{2A} receptors, using three different approaches:

i) evaluation of the impact of prenatal exposure to dexamethasone in the dorsal hippocampus, in order to clarify if microglia morphology is regulated in a brain region specific manner (comparison with previous results obtained in the prefrontal cortex) and the ability of A_{2A}R blockade to interfere with dexamethasone effects (**Chapter 2.1)**;

ii) evaluation of the impact of the global genetic deletion of $A_{2A}R$ on anxiety, cognition (recognition memory) and glucocorticoid receptor levels in prefrontal cortex and hippocampus, regions implicated in behavioral performance in tests chosen for anxiety and cognition assessment **(Chapter 2.2)**;

iii) *in vitro* evaluation of $A_{2A}R$ ability to regulate subcellular translocation of glucocorticoid receptor to the nucleus and the subsequent transcription of inflammatory mediators in a microglia cell line **(Chapter 2.3)**.

In **Chapter 2.1**, using female Wistar rats prenatally exposed to dexamethasone in embryonic days 18 and 19 (1 mg/kg/day, subcutaneously) and chronically treated with an $A_{2A}R$ antagonist (0.1 mg/kg/day, intraperitoneally) for 21 consecutive days before PND 90, we report that prenatal exposure to dexamethasone is associated with long-lasting microglial hyper-ramification in the dorsal hippocampus and that the chronic blockade of $A_{2A}R$ is able to normalize microglia morphology. These results contrast with dexamethasone-induced changes in the prefrontal cortex of females, where we previously described a decrease in the length and in the number of processes, an effect not normalized by the chronic blockade of $A_{2A}R$ at adulthood. Altogether, these results suggest that dexamethasone and $A_{2A}R$ modulate microglia morphology in a brain region-specific manner.

In **Chapter 2.2**, we used female mice with a global genetic deletion of $A_{2A}R$ to further confirm: i) if $A_{2A}R$ modulate anxiety and recognition memory in a gender-specific manner, by comparison with published work in males; ii) if $A_{2A}R$ regulate glucocorticoid receptor levels in the prefrontal cortex and in the hippocampus, as occurs by pharmacological blockade of the receptor. We observed that these mice exhibit anxious-like behavior, without alterations in short-term memory. These results are in accordance with the literature: male knock-outs for this receptor have an anxiety-like phenotype and the pharmacological blockade of the receptor in males was able to recover memory deficits induced by chronic stress. We observed that the global genetic deletion of $A_{2A}R$ does not affect glucocorticoid receptor levels in prefrontal cortex and hippocampus, in opposition to what happens in females chronically treated with an $A_{2A}R$ antagonist in adulthood, that exhibited alterations in the density of glucocorticoid receptor levels in a brain region-specific manner, namely a general decrease in both regions.

In **Chapter 2.3**, we used a microglial cell line to explore a putative mechanism of interaction between glucocorticoid and A_{2A} receptors. In this case, we studied the translocation of the glucocorticoid receptor from the cytoplasm to the nucleus, induced by dexamethasone, in the

presence and in the absence of a selective $A_{2A}R$ antagonist. The blockade of $A_{2A}R$ impairs the translocation of glucocorticoid receptor to the nucleus induced by dexamethasone. Interestingly, the exposure to the antagonist of $A_{2A}R$ *per se* significantly decreased glucocorticoid receptor levels in the cytoplasm and the nucleus, indicating that adenosine, through $A_{2A}R$, modulate glucocorticoid receptor levels and translocation process in microglial cells.

Altogether, this study shows that exposure to glucocorticoids during development causes long-lasting morphological changes in microglial cells in the dorsal hippocampus of females. Moreover, microglia morphology is recovered with the chronic blockade of $A_{2A}R$ in adulthood, pointing towards a crosstalk between these two systems. The *in vitro* results support this hypothesis, showing that $A_{2A}R$ affects the translocation of the glucocorticoid receptors to the nucleus and the density of these receptors, which may affect glucocorticoid genomic effects.

Keywords: Microglia, Glucocorticoids, Neurodevelopment, Adenosine A_{2A} Receptor

RESUMO

Os glucocorticóides sintéticos, como a dexametasona, são frequentemente utilizados em situações de gravidez com risco de parto prematuro, com o objetivo de acelerar a maturação pulmonar fetal. Apesar de estudos em animais e humanos demonstrarem que este tratamento leva a um atraso no crescimento e outros efeitos nefastos no cérebro e em outros órgãos em desenvolvimento, a eficácia elevada destes fármacos e a diminuição significativa da mortalidade justifica o seu uso frequente na clínica. Estudos anteriores têm reportado que a exposição ao *stress*, ou a administração de glucocorticóides durante etapas cruciais do desenvolvimento, contribuem para o aparecimento de distúrbios neuropsiquiátricos, como a ansiedade, depressão e comportamento aditivo. De facto, estudos em modelos animais expostos durante o período pré-natal a glucocorticóides reportam efeitos prejudiciais nos neurónios, tais como apoptose neuronal, perda sináptica e atrofia dendrítica.

As células da microglia são células do sistema imunitário do cérebro, que respondem a alterações no ambiente celular, como a exposição a glucocorticóides sintéticos (imunomoduladores). Estas células são afetadas no seu fenótipo morfológico em condições de stress crónico, e estudos anteriores do nosso laboratório demonstraram que a exposição pré-natal a dexametasona altera a morfologia da microglia no córtex pré-frontal, um efeito observado nos dias 1, 7 e 90 após nascimento. Notavelmente, o efeito da dexametasona na morfologia da microglia é dependente do género do animal.

A adenosina é um neuromodulador que ativa diferentes subtipos de recetores, como os recetores A_{2A} de adenosina, que estão implicados na fisiopatologia da ansiedade e da depressão e também são moduladores da morfologia e função da microglia.

Resultados preliminares obtidos *in vivo* e *in vitro* no nosso laboratório sugeriram uma possível interação entre os recetores de glucocorticóides e de adenosina A_{2A}, demonstrando uma modulação dependente do género na microglia e no comportamento (ansiedade e memória).

Neste trabalho focámo-nos no estudo da interação entre os recetores de glucocorticóides e de adenosina A_{2A}, utilizando três abordagens diferentes:

i) avaliação do impacto da exposição pré-natal a dexametasona no hipocampo dorsal, com o objetivo de clarificar se a morfologia da microglia é regulada de uma maneira específica entre regiões do cérebro, comparando com resultados prévios obtidos no córtex pré-frontal e, se o bloqueio dos recetores $A_{2A}R$ é eficaz na reversão dos efeitos mediados pela dexametasona **(Capítulo 2.1)**;

ii) avaliação do impacto da deleção genética global dos $A_{2A}R$ na ansiedade, cognição (memória) e níveis de recetores de glucocorticóides no córtex pré-frontal e no hipocampo, regiões implicadas na ansiedade e na cognição, comportamentos avaliados nos testes comportamentais **(Capítulo 2.2)**;

iii) avaliação do *in vitro* a capacidade dos $A_{2A}R$ regularem a translocação subcelular dos recetores de glucocorticóides para o núcleo e subsequente transcrição de mediadores inflamatórios numa linha celular de microglia **(Capítulo 2.3)**.

No **capítulo 2.1**, utilizámos fêmeas de ratos Wistar expostas, no período pré-natal, a dexametasona nos dias embrionários 18 e 19 (1 mg/kg/dia, subcutaneamente) e cronicamente tratados com um antagonista de $A_{2A}R$ (0.1 mg/kg/dia, intraperitonealmente) durante 21 dias consecutivos antes do dia 90 após nascimento. Os animais expostos a dexametasona durante o desenvolvimento apresentaram alterações nas células da microglia (híper-ramificação) do hipocampo dorsal, as quais persistem até à idade adulta, no entanto, o bloqueio crónico dos $A_{2A}R$ foi capaz de normalizar a morfologia da microglia. Estes resultados contrastam com as alterações induzidas pela dexametasona no córtex pré-frontal de fêmeas, onde previamente descrevemos uma diminuição do comprimento e do número de processos, um efeito que não foi normalizado pelo bloqueio crónico dos $A_{2A}R$ na idade adulta. Estes resultados analisados em conjunto, sugerem que a dexametasona e os $A_{2A}R$ modulam a morfologia da microglia de uma maneira dependente da região do cérebro.

No **capítulo 2.2** foram utilizadas fêmeas com a deleção genética global de $A_{2A}R$ com o intuito de confirmar: i) se a modulação dos $A_{2A}R$ na ansiedade e na memória é dependente do género, por comparação com trabalhos publicados em machos; ii) se os $A_{2A}R$ regulam os níveis de recetores de glucocorticóides no córtex pré-frontal e no hipocampo, como ocorre no caso do bloqueio farmacológico deste recetor. Estes animais exibiram um comportamento ansioso, sem alterações na memória. Estes resultados estão de acordo com o que está descrito na literatura em que machos com a deleção genética global de $A_{2A}R$ possuem um fenótipo comportamental ansioso e o bloqueio farmacológico deste recetor em machos foi capaz de recuperar défices de memória induzidos pelo stress crónico. Por conseguinte, a deleção genética global não teve um impacto nos níveis de recetores de glucocorticóides no córtex pré-frontal e no hipocampo, contrastando com o que foi observado em fêmeas cronicamente tratadas com o antagonista dos $A_{2A}R$ na vida adulta, que exibem alterações na densidade dos recetores de glucocorticóides dependendo da região do cérebro analisada, nomeadamente, uma diminuição em ambas as regiões.

No **capítulo 2.3** foi utlizada uma linha celular de microglia com o objetivo de explorar os mecanismos de interação entre os recetores de glucocorticóides e de $A_{2A}R$. Neste caso, o mecanismo estudado foi a translocação do recetor de glucocorticóides do citoplasma para o núcleo, induzido pela dexametasona, na presença e na ausência de um antagonista seletivo dos $A_{2A}R$. O bloqueio dos $A_{2A}R$ afeta a translocação de recetores de glucocorticóides para o núcleo induzido pela dexametasona. Interessantemente, a exposição ao antagonista dos $A_{2A}R$ *per se* diminuiu significativamente os níveis de recetores de glucocorticóides no citoplasma e no núcleo, indicando que a adenosina, através da ativação dos $A_{2A}R$ modula os níveis de recetores de glucocorticóides e o seu processo de translocação nas células da microglia.

Concluindo, os resultados apresentados indicam que a exposição a glucocorticóides durante o desenvolvimento afeta a morfologia da microglia no hipocampo dorsal de fêmeas, alterações que persistem até à idade adulta. Adicionalmente, a morfologia da microglia foi recuperada com o bloqueio crónico dos $A_{2A}R$ na idade adulta, sugerindo assim, uma interação entre estes dois sistemas. Os resultados *in vitro* suportam esta hipótese, demonstrando que os $A_{2A}R$ afetam a translocação dos recetores de glucocorticóides para o núcleo e a sua densidade, podendo afetar os efeitos genómicos dos glucocorticóides.

Palavras-chave: Microglia, Glucocorticóides, Neurodesenvolvimento, Recetores de adenosina A_{2A}

ABBREVIATIONS LIST

A

A_1R	Adenosine A_1 receptor
$A_{2A}R$	Adenosine A_{2A} receptor
$A_{2B}R$	Adenosine A_{2B} receptor
A_3B	Adenosine A_3 receptor
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine triphosphate

B

BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin

C

CAPS	3-(Cyclohexylamino)-1-propane sulfonic acid
CNS	Central nervous system
CORT	Corticosterone
CRH	Corticotropin-releasing hormone

D

DAPI	4’,6-diamidino-2-phenylindole
DEX	Dexamethasone
DG	Dentate gyrus
dHip	Dorsal hippocampus
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid
DTT	Dithiothreitol

E

ECF	Enhanced chemifluorescence
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EPM	Elevated plus maze

F

FBS	Fetal bovine serum

G

GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response elemento

H

Hip	Hippocampus
HPA	Hypothalamic-pituitary-adrenal

I

Iba-1	Ionized calcium-binding adapter molecule -1
IL	Interleukin
Ip	Intraperitoneally

L

LPS	Lipopolysaccharide

M

MR	Mineralocorticoid receptor
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

N

NAcc	Nucleus accumbens
NO	Nitric oxide
NOR	Novel object recognition

O

OF	Open field

P

PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PND	Postnatal day
PVDF membrane	Polyvinylidene difluoride

R

RIPA	Radio-immunoprecipitation assay
RPMI	Roswell Park Memorial Institute
RT	Room temperature

S

SCH58261	7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4 triazolol[1,5c]pyrimidine
Sc	Subcutaneously
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean

T

TBS	Tris buffered saline solution supplemented with Tween
TEMED	Tetramethylethylenediamine
TNF	Tumour necrosis factor

Chapter 1

GENERAL INTRODUCTION

1. Microglia, cellular players in brain homeostasis and disease

1.1 Overview of microglial cells

Nissl was the first to identify microglial cells, describing them as reactive neuroglia similar to macrophages (Nissl 1899). Later, Ramón y Cajal, described these cells as the "third element of the nervous system" (non-astrocyte glial cells), due to the morphological differences of these cells when compared with the "first element" (neurons) and "second element" (astrocytes) (Cajal 1913 - 1914). However, it was Pío Del Río-Hortega (Hortega 1937), a student of Ramón y Cajal, that introduced the concept of microglia as a particular cell type, reviewed in (Tremblay et al. 2015).

Microglia are cellular elements of the brain immune system, responsible for the constant survey of potential threats and for responsive actions to changes of the surrounding environment. The surveilling function is supported by *in vivo* imaging studies, using mice with enhanced green fluorescent protein (EGFP)-expressing microglia, which demonstrated the ramified morphology of these cells with their fine processes in constant movement, extending and retracting within the brain parenchyma (Davalos et al. 2005; Nimmerjahn et al. 2005). Several studies indicate that microglial processes motility is guided by neuronal activity (Davalos et al. 2005; Li et al. 2012; Nimmerjahn et al. 2005; Tremblay et al. 2010; Wake et al. 2009). Microglial cells constantly monitor neuronal synapses, being capable to sense distress signals and to eliminate compromised neurons, thus having a vital role in central nervous system (CNS) homeostasis.

The responses of microglia to changes in the surrounding environment include immune functions, such as cytokine secretion, phagocytosis, antigen presentation and expression of several immune-related factors (Tremblay et al. 2011).Microglia morphology is important for surveillance and homeostasis of the brain, having a strong correlation with their functional state.

1.2 Role of microglia during neurodevelopment

Microglia originate from progenitor cells with mesodermal origin found in the yolk sac that invade the brain in the early development (Ginhoux et al. 2010; Ginhoux et al. 2013). During development, these cells are much less ramified than those found in the adult brain, having an amoeboid morphology. Only after the invasion of the brain parenchyma, these cells start to proliferate and become more ramified, originating mature microglia, as present in the adult brain (Dalmau et al. 1997; Dalmau et al. 1998a)and reviewed in (Prinz and Priller 2014).

In addition to their immune functions, microglial cells have a critical role during CNS development (Tremblay et al. 2011). During brain development, a period of remarkable plasticity, with the formation of supranumerary synapses, microglia exerts a central role in the elimination of immature synapses and in the strengthening of mature synapses (Hoshiko et al. 2012; Paolicelli et al. 2011; Ji et al. 2013; Schafer et al. 2012; Zhan et al. 2014). These functions may require direct contact of microglial cells with synapses (Tremblay et al. 2010) (Wake et al. 2009) or by the release of molecules, such as complement factors, cytokines or trophic factors (Paolicelli et al. 2011; Schafer et al. 2012).

In 2009, Wake and colleagues, in conditions of ischemia, characterized the physical interaction between microglia and neurons *in vivo* during adulthood, showing that microglial processes became enlarged after contacting synaptic elements and, in some cases, the synaptic structure was eliminated (Wake et al. 2009). Moreover, Tremblay and colleagues showed, by *in vivo* imaging, that microglia play an active role as a regulator of synapses, by establishing brief contacts with synaptic structures, in adolescent mouse visual cortex, in physiological conditions (Tremblay et al. 2010).

Paolicelli and colleagues reported the key role of microglia, namely in synapse elimination, during development (Paolicelli et al. 2011), showing that microglia can engulf and eliminate synapses during this period, and alterations of this function result in the increase in the number of dendritic spines and immatures synapses.

Other studies described that C1q and C3, elements of the complement system, when localized at synaptic compartments, tag these synapses for engulfment by microglia, which express C1q and C3 receptors (Brown and Neher 2014; Schafer et al. 2012; Stevens et al. 2007). In fact, mice lacking the receptor for C3 chemokine, CR3, in microglial cells, exhibit increased number of spines, suggesting that synaptic pruning by microglia is affected (Schafer et al. 2012). Additionally, these alterations in microglia functionality lead to weak synaptic transmission,

decreased functional brain connectivity and behavior abnormalities, and to alterations associated with neurodevelopmental and neuropsychiatric disorders, including autism spectrum disorders, schizophrenia and depression (Zhan et al. 2014).

Microglia also contribute to pre-synaptic differentiation (Cristovao et al. 2014) and synaptogenesis through the release of cytokines, namely IL-10 (Lim et al. 2013) and neurotrophic factors, as the brain-derived neurotrophic factor (BDNF) (Parkhurst et al. 2013). In fact, these observations highlight the undisputable role of microglia during neurodevelopment in the formation/maintenance of brain circuits.

It is well established that the morphology of microglial cells is associated with their functional state (Hinwood et al. 2012; Kreisel et al. 2014). In fact, alterations in microglia morphology have been associated with functional impairment of these cells, which can lead to alterations in brain circuits and to neuropsychiatric disorders, such as depression and anxiety disorders (Rial et al. 2015; Yirmiya et al. 2015). Therefore, the close relation of microglial cells to synaptic function and maintenance of neuronal circuits, suggest that any disturbance, namely the exposure to immunomodulators, as high levels of glucocorticoids (GCs) during development, could contribute to alterations in the functionality of these cells and consequently to neuropsychiatric conditions.

2. Glucocorticoids, modulators of stress-related conditions and microglial cells

2.1. Endogenous production and regulation of glucocorticoids

Stress is defined as any perturbation of homeostasis of the organism, both psychological or physiological (Goldstein and Kopin 2007). Stressful events are present through life, causing physiological responses that involve changes at peripheral and central levels coordinated by the CNS, throughout the hypothalamus-pituitary-adrenal (HPA) axis (Herman and Cullinan 1997). This system is constituted by three endocrine glands: the hypothalamus and the pituitary gland, located in the CNS and the adrenal glands which is a peripheral tissue (Habib et al. 2001).

The activation of the HPA axis leads to a cascade of signaling pathways (Figure 1) that begins with the release of the corticotropin-releasing hormone (CRH) by neurosecretory neurons of the paraventricular nuclei of the hypothalamus (Herman et al. 1995). CRH binds to its receptor in the pituitary gland and induces the secretion of adrenocorticotropic hormone (ACTH) (Plotsky and Meaney 1993), which promotes the release, by adrenal glands, of anti-inflammatory agents, GCs (cortisol in humans or corticosterone -CORT- in rodents), to the systemic circulation. After a stressful condition, elevated levels of circulating GCs act on the hypothalamus and pituitary (to suppress CRH and ACTH production) in a negative feedback cycle (Weiser et al. 2011).

In addition to stress response, glucocorticoids are also regulated by circadian rhythm, peaking prior to the active period (day for humans and night for rodents (Chung et al. 2011; Ota et al. 2012).

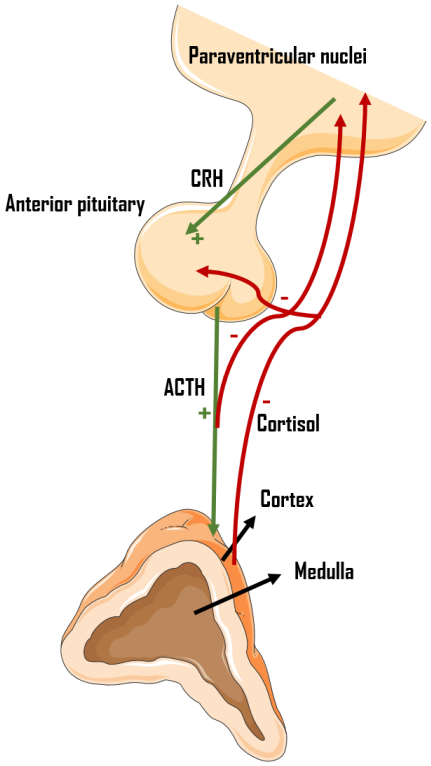

Figure 1. Illustration of the HPA axis circuitry and feedback mechanisms of regulation. When circadian or stress signals activate the HPA axis, a positive feedback is induced, represented in green. HPA axis activation stimulate the cells of the PVN in the hypothalamus to release CRH. CRH activates the pituitary, which in turn, releases ACTH, that acts on the adrenal cortex, resulting in increased levels of glucocorticoids into the systemic circulation. These GCs act in a negative feedback loop, represented in red, to suppress the production of ACTH and CRH in the pituitary gland and hypothalamus, respectively.

2.2. Glucocorticoids in physiology and stress conditions

After being released into the systemic circulation, GCs exert a varied spectrum of effects that are critical in stress adaptive response. These include cardiovascular activation, suppression of immunity or inflammation, activation of the metabolic system (lipolysis, proteolysis and gluconeogenesis) and effects on the reproductive (Munck et al. 1984; Sapolsky et al. 2000). However, prolonged exposure to elevated levels of GCs lead to immune dysfunction (Sorrells and Sapolsky 2007), endocrine dysregulation (Sapolsky et al. 2000) and to neuropathology (Sapolsky 1999).

In chronic stress conditions, there is a disturbance of the HPA axis, characterized by increased levels of GCs levels, that leads to an increase of glucocorticoid receptor (GR) activation, with several consequences for brain structure and function. HPA axis abnormalities contribute to the development of neuropsychiatric disorders, such as depression and anxiety (Oliveira et al. 2006; Sousa et al. 2008).

Previous studies using rodent models of prenatal stress showed that the exposure to stress conditions during the course of neurodevelopment, increases the vulnerability to diseases, namely cardio-metabolic disorders and neuroendocrine dysfunction, as well as increased risk to develop neuropsychiatric disorders in adulthood, reviewed in (Moisiadis and Matthews 2014).

Moreover, early life stress leads to impaired growth and differentiation of several developing systems, namely the CNS, reviewed in (Cottrell and Seckl 2009). Neuroarchitectural changes and reduced number of neurons, in several regions of the CNS have been described, including the hippocampus (Hip) (Sousa et al. 2000), the prefrontal cortex (PFC) (Cerqueira et al. 2005) and the amygdala (Oliveira et al. 2012). In fact, these structures are crucially involved in emotional and cognitive behavioral functions, that when altered, may lead to hyperanxious phenotype (Oliveira et al. 2006), an increase in the propensity to develop depression (Roque et al. 2011) and addictive-like behavior (Rodrigues et al. 2012). Additionally, the exposure to GCs affects the coherence of the communication between cortico-limbic structures (medial PFC and ventral Hip) (Oliveira et al. 2013).

Repeated stress during adulthood in rodents also impacts on the morphology of microglial cells, particularly, causing an atrophy and a decrease in the complexity of these cells in the hippocampus (Kreisel et al. 2014). Additionally, in mice subjected to early life stress, it was found an increase in the number and motility of microglial cell processes in the somatosensory cortex (Takatsuru et al. 2015), suggesting that high levels of GCs/stress have an impact in the morphology and consequently functionality of microglial cells.

2.3 Glucocorticoids as prenatal therapy: molecular, structural and behavioral outcomes

GCs are involved in the development and maturation of several fetal organs, reviewed in (Moisiadis and Matthews 2014). During late gestation, occurs an increase in the levels of GCs in the fetal circulation, a process that is essential for the normal maturation of several systems, namely fetal lung maturation and production of pulmonary surfactant, among others. For this reason and because synthetic GCs, such as DEX (dexamethasone), are able to cross the placenta, these hormones are used in neonatal care, to promote maturation of the fetal lungs in case of premature delivery. This therapeutic approach drastically reduces the rates of neonatal death associated with disorders, such as respiratory distress syndrome (Brownfoot et al. 2013; Holland and Taylor 1991).

Despite the beneficial effects of the use of GCs in prenatal therapy, some studies describe that the prenatal exposure to GCs leads to a reduction in offspring birth weight and causes hypertension, insulin resistance, type 2 diabetes and cardiovascular diseases, reviewed in (Drozdowicz and Bostwick 2014). In fact, it was already demonstrated that prenatal exposure of GCs can act permissively to accelerate some developmental processes, as eye opening in the offspring rats, while delaying others, such as body growth (Oliveira et al. 2006), suggesting alterations in normal development.

Synthetic GCs are different from endogenous GCs in their chemical structure and pharmacokinetics, having higher affinity for GR than mineralocorticoid receptors (MRs), the two types of corticosteroid receptors (Sorrells and Sapolsky 2007). The deleterious effects of these GCs occur by the fact that the fetus has limited capacity to degrade GCs and because 11β-hydroxysteroid dehydrogenase, present in the local of maternal-fetus exchange and responsible for the inactivation of endogenous GCs from the mother, is unable to metabolize synthetic GCs, facilitating the passage through the placenta and accessing the brain (White 2001). Furthermore, animals prenatally exposed to DEX display an anxious-like phenotype, susceptibility to depression and signs of compromised GCs negative feedback in adulthood which was not observed in animals exposed to CORT (Oliveira et al. 2006). These apparently contradictory effects can be explained: DEX is a specific ligand for GR and differential effects of DEX and CORT have been attributed to the selective activation of GR by DEX, causing an impact in the programming of behavioral functions (Oliveira et al. 2006). Additionally, animals prenatally treated with DEX have a reduction in the number and volume of neuronal cells in the nucleus accumbens (Nacc) and in ventral tegmental area (VTA), where dopaminergic cell bodies

are present; the decrease of these cells resulted in a reduction of the dopaminergic enervation (Leao et al. 2007). It was also observed that prenatal exposure to DEX delays neuronal differentiation and migration in cerebral cortex (Fukumoto et al. 2009). In the same animal model, it was also described alterations in dendritic arborization in the amygdala (increase in the number of spines), a modulatory region of anxiety and fear behavior (Oliveira et al. 2012) and in the Nacc region (Rodrigues et al. 2012).

In summary, several studies describe the deleterious effects of synthetic GCs (Figure 2), which disturb the highly plastic and sensitive brain, during critical phases of neurodevelopment. Although GCs are important as prophylactic treatment in preterm birth, there are still several important issues about the use of antenatal GCs that should be further studied, reviewed in (Moisiadis and Matthews 2014).

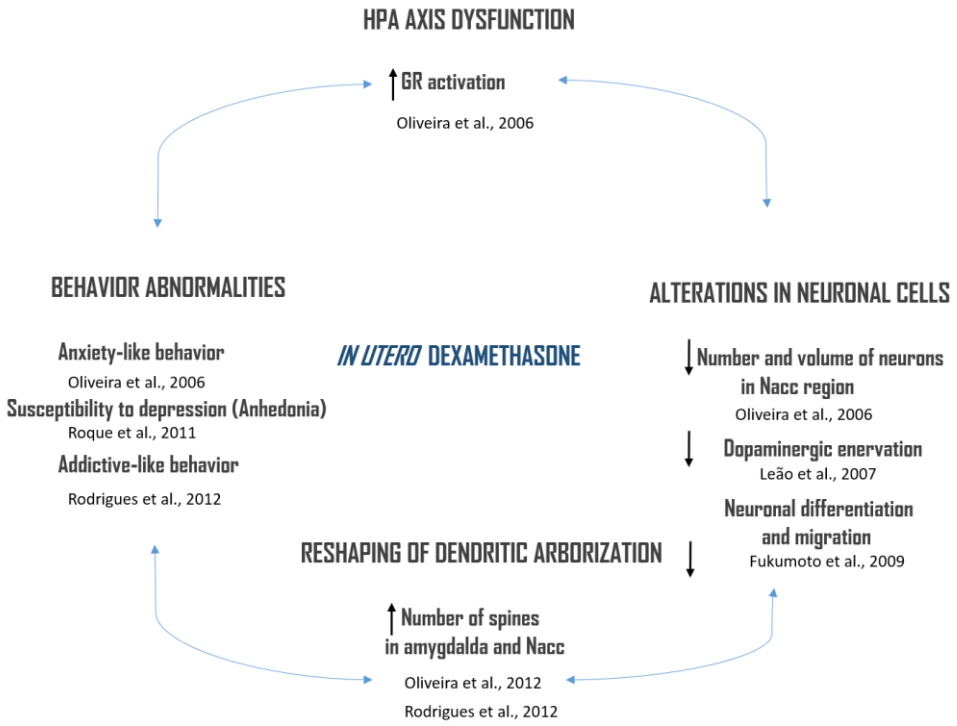

Figure 2. Schematic representation of the impact of prenatal exposure to dexamethasone. Prenatal exposure to dexamethasone leads to an increase in glucocorticoid receptor activation. Moreover, exposure to DEX *in utero* causes alterations in neurons, namely a decrease in the number and volume in Nacc region and a reduction in the dopaminergic enervation, leading to consequent effects in neuronal differentiation and migration and reshaping of dendritic arborization (increase in the number of spines). These alterations result in behavioral impairments, such as anxiety-like behavior, susceptibility to depression and addictive-like behavior.

2.4. Glucocorticoids: cellular and molecular actions

There are two types of corticosteroid receptors differentially distributed in the brain, reviewed in (Reul and de Kloet 1985): the high-affinity MRs, that GCs bind during periods of basal secretion, and the low-affinity GR, that are occupied and activated during periods of elevated secretion of GCs, as occurs following stress (Arriza et al. 1988); and reviewed in (Sapolsky et al. 1984). Regarding their brain distribution, MRs are restricted to the lateral septum and hippocampus, while GR have a more ubiquitous distribution, being present in the Nacc and cortical structures, such as the PFC, along with hypothalamus and Hip (McGimsey et al. 1991) (Figure 3).

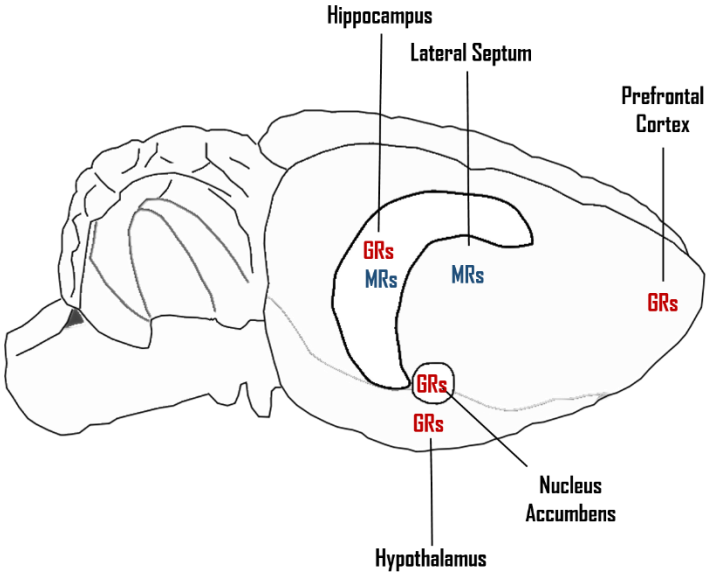

Figure 3. Schematic representation of the distribution of glucocorticoid and mineralocorticoid receptors in the adult rat brain. Corticosteroid receptors have a brain region-specific distribution. This figure shows the regions that have high levels of GR, represented in red, and MRs, represented in blue. GR are expressed in higher density in the PFC, Nacc, hypothalamus and Hip and MRs are expressed in higher density in Hip and in the lateral septum.

GR are mainly composed by three specific domains: N-terminal transactivation domain, a DNA-binding domain, and a C-terminal ligand-binding domain (Oakley and Cidlowski 2011; Vandevyver et al. 2012).

Conventionally, under basal conditions, GR are present in the cytoplasm, interacting with chaperones, such as heat shock protein 90 (hsp 90), that block the nuclear localization sequence (NLS) present in GR and therefore inhibiting GR nuclear translocation. However, when ligands bind to GR, it activates their translocation to the nucleus, leading to the regulation of the expression of different genes (Kadmiel and Cidlowski 2013).

GR control the expression of target genes [transcriptional activation (transactivation) or transcriptional repression (transrepression)], by direct binding to glucocorticoid response elements (GRE) in the promotor region of specific genes (Figure 4). To mediate transactivation, GR bind to the GRE and activates the production of anti-inflammatory cytokines. In the case of transrepression, GR interact with negative GRE (nGRE) and inhibit pro-inflammatory response, reviewed in (Cruz-Topete and Cidlowski 2015) (direct genomic effect).

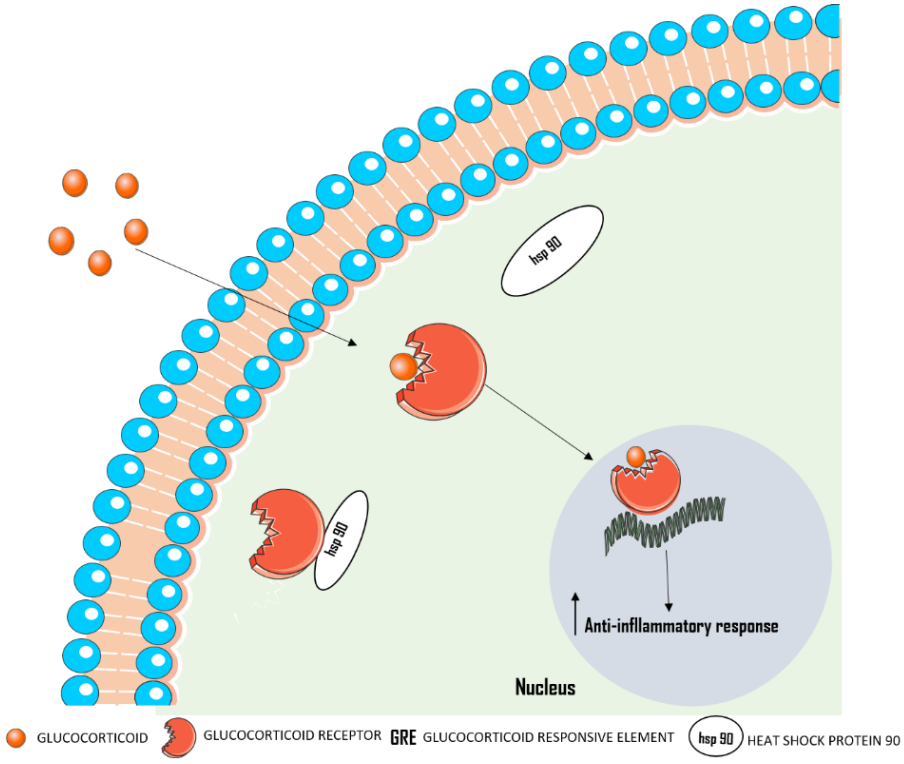

Figure 4. Schematic representation of glucocorticoid receptor activation. GR in their inactive state are present in the cytoplasm, in a multimeric complex with chaperone proteins, including heat shock proteins, such as hsp 90. Endogenous or synthetic GCs bind to GR and GR-ligand complex dissociates from the complex of chaperone proteins and translocate to the nucleus, where it regulates gene expression by binding to GRE and activates an anti-inflammatory response.

GCs can also regulate transcription without binding directly to DNA, but by associating with other transcription factors that regulate transcriptional activity. By inhibiting nuclear factor kappa beta (NF-κβ) or activator protein 1 (AP-1), transcriptional factors associated with pro-inflammatory response, and consequently, inhibiting the pro-inflammatory response (indirect genomic effect). The repressor effect of these transcriptional factors is the main basis for the anti-inflammatory and immunosuppressive effects of GCs, reviewed in (Busillo and Cidlowski 2013).

GCs can also exert non-genomic actions, by the activation of GR localized in the plasma membrane (mGR) and directly modulating signal transduction pathways, namely interacting with kinases, such as the extracellular signal-regulated kinases, p38 isoforms (p38s) and mitogen-activated protein kinases (MAPKs). Although these non-genomic actions are not fully understood, they have been proposed to save as a mediator of the "rapid" actions of GR (Ayroldi et al. 2012). In addition, GR can also translocate to mitochondria and enhance their calcium buffering capacity, regulate mitochondrial oxidation, free radical formation and membrane potential, reviewed in (Du et al. 2009).

GR are phosphoproteins that, upon ligand binding, become hyperphosphorylated, a key signal for ubiquitination and proteossomal activation, the main degradation process for these receptors (Wallace and Cidlowski 2001). However, He and colleagues reported that when GR are present in the cytoplasm, their degradation can also be regulated by the lysosomal pathway (He et al. 2011). So, GR are degraded by lysosomes when present in the cytoplasm, but when translocated into the nucleus, the main degradation process is the proteasome-ubiquitin pathway. These pathways also contribute to regulate GR activity: when one of these systems is inhibited, GR signaling is potentiated (He et al. 2011; Wallace and Cidlowski 2001).

GR are present in neural and glial cells, including microglial cells. In fact, GR is the most abundant steroid hormone receptor present in microglial cells (Sierra et al. 2008) and GCs can suppress inflammation mediated by microglia (Goujon et al. 1996). It was also demonstrated that the exposure of a microglial cell line to GCs before an inflammatory stimulus, lipopolysaccharide (LPS), classically used as an activator of microglial responses, inhibits the production of pro-inflammatory mediators (Drew and Chavis 2000). Interestingly, disrupting the glucocorticoid signaling using GR antagonists *in vivo* results in an exacerbated and prolonged inflammatory response to the effects of LPS (Nadeau and Rivest 2003).

Similar findings were described in mice lacking GR in macrophage/microglia: an exacerbated inflammatory response after a LPS stimulus (Carrillo-de Sauvage et al. 2013). These GR KO mice present alterations in microglia morphology, namely a more amoeboid shape, and decrease motility after LPS infection, demonstrating a role of GR in the regulation of motility and morphology of these cells. However, when microglia become sensitized by GCs over-secretion, GCs no longer activate an anti-inflammatory response, but instead promote a pro-inflammatory response, namely in cases of neurodegenerative diseases (Frank et al. 2012).

3. Adenosine, a modulator of microglia

3.1 Overview of adenosine and adenosine receptors

Adenosine is a homeostatic substance that belongs to the family of purines, being present in all cell types, namely neurons and glial cells (Ribeiro et al. 2002). This substance is a well-known neuromodulator and homeostatic regulator in the brain (Cunha 2001). It has several roles in the brain, from the modulation of synaptic transmission to the control of synaptic plasticity (Dias et al. 2013).

There are four different adenosine receptors, designated as A_1, A_{2A}, A_{2B} and A_3 receptors, and all of them are metabotropic (G-protein coupled) receptors, with seven transmembrane domains (Fredholm et al. 2001). In accordance with the plethora of functions regulated by the adenosinergic system, adenosine receptors expression is variable between brain regions (Ribeiro et al. 2002) (Figure 5). A study of the distribution of adenosine-binding sites in the cat visual cortex showed changes in the distribution of adenosine receptors during development (analyzed from 3 days until 95 days of age), that stabilizes in adult cat, showing that adenosine receptors density varies during development (Shaw et al. 1986).

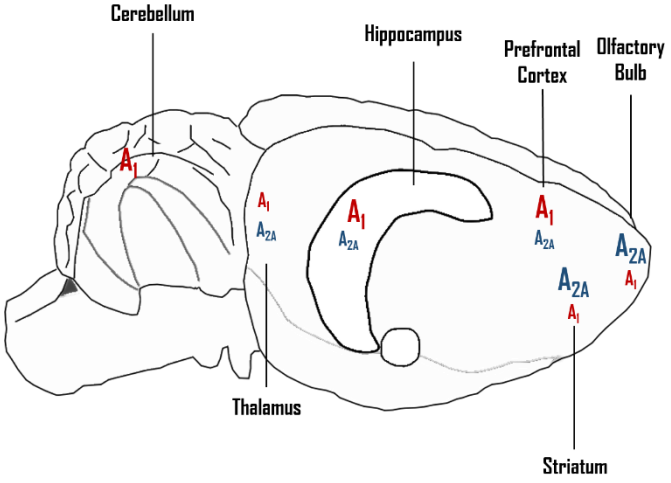

Figure 5. Schematic representation of the distribution of adenosine A_1 and A_{2A} receptors in the brain. Adenosine A_1 receptors are highly expressed in the prefrontal cortex, hippocampus and cerebellum, while adenosine A_{2A} receptors are predominant in the striatal areas and olfactory bulb. This figure shows the regions that expressed A_1 receptors, represented in red, and A_{2A} receptors, represented in blue. High levels of expression are indicated by bigger alphabets, while low levels are indicated with smaller alphabets.

In the case of $A_{2A}R$, previous studies have shown that they are is widely expressed throughout the body, with the highest level of expression in the striatum and olfactory bulb. The

levels of $A_{2A}R$ are not constant throughout life: the striatum is the only brain region that expresses high levels since the embryonic development that persist until adult age (Weaver 1993). In fact, it was described in this study that in other regions, namely in the intermediate lobe pituitary, the levels of $A_{2A}R$ expression decrease after PND 7 and become stable in the adult brain. Notably, in aged rat, the levels of $A_{2A}R$ in striatum, hippocampus and cortex increase again (Cunha et al. 1995).

3.2 Modulatory role of $A_{2A}R$ in microglial cells

Adenosine is produced and released by most cells, including neurons and glial cells. In fact, microglial cells are equipped with all adenosine receptor subtypes (Dare et al. 2007).

Dalmau and his colleagues show that microglia express enzymes related with purine metabolism in the developing brain and that this expression is associated with microglia morphology (Dalmau et al. 1998b).

Several studies have shown that microglia morphology can be regulated by adenosine (Gyoneva et al. 2009; Gyoneva et al. 2014; Orr et al. 2009; Wollmer et al. 2001). For instance, Orr and co-workers shows that adenosine, through the activation of $A_{2A}R$, causes a retraction of microglial processes by the exposure to LPS and the treatment with an $A_{2A}R$ selective antagonist induces the re-extension of microglial cell processes (Orr et al. 2009). In addition, local application of ATP to LPS-activated microglia caused a rapid retraction of microglial cells processes, an effect that is dependent on adenosine (a product of ATP metabolic degradation) and mediated by $A_{2A}R$ (Gyoneva et al. 2009; Gyoneva et al. 2014). Altogether, these data suggest that microglia morphology is controlled by purines, namely adenosine, through $A_{2A}R$ activation.

A_{2A} receptors not only have the ability to control microglia morphology, but also microglia proliferation. It was described, *in vitro* study, that exposure to LPS increases microglia proliferation, an effect blocked in the presence of an $A_{2A}R$ antagonist, suggesting that $A_{2A}R$ also regulates microglia proliferation in non-physiological conditions (Gomes et al. 2013; George et al. 2015).

$A_{2A}Rs$ are also important regulators of other microglial functions, such as the release of inflammatory mediators as nitric oxide (NO) (Saura et al. 2005) and neurotrophic factors as brain–derived neurotrophic factor (BDNF) (Gomes et al. 2013). Additionally, Dai and colleagues show that the activation of $A_{2A}R$ controls microglia inflammatory response and that this response is dependent on glutamate levels (Dai et al. 2010).

4. Aims of the thesis

Microglial cells are the resident innate immune cells of the CNS, that play crucial roles in physiological and pathological conditions. Among these functions, microglial cells are indispensable for the definition of the number of functional synapses and circuit organization during neurodevelopment.

Several findings support that the exposure to GCs during development induce an overall spectrum of abnormalities and neuropsychiatric conditions. In fact, DEX has deleterious effects in neurons and behavior, but the impact of this synthetic glucocorticoid in microglial cells remain unclear.

Considering the well known ability of adenosine $A_{2A}R$ to regulate microglia morphology and function and the crosstalk between $A_{2A}R$ and GR, the main objectives of the present thesis were:

- To evaluate eventual changes in microglia morphology in the dorsal hippocampus (dHip) of adult females prenatally exposed to DEX and the ability of $A_{2A}R$ pharmacologic manipulation to normalize these alterations. The impact of DEX in microglia morphology is an important issue, that we tried to clarify, performing tridimensional reconstructions of microglial cells from animals prenatally exposed to DEX and treated chronically with an $A_{2A}R$ antagonist in adulthood, a technique that allows the evaluation of microglia morphometry (**Chapter 2.1**).

- To characterize $A_{2A}R$ KO female mice in terms of behavior and GR density in PFC and Hip. For that, we performed behavior analysis, to evaluate anxiety and recognition memory and GR density by western blot in Hip and PFC, regions implicated in behavioral tests chosen for anxiety and cognition assessment (**Chapter 2.2**).

- To study cellular mechanisms of GR-$A_{2A}R$ crosstalk in microglial cells, with focus on GR cellular trafficking and transcriptional regulation of inflammatory mediators. For that, we used a microglia cell line (BV-2) and performed manual fractionation to evaluate the density of GR in subcellular fractions in the presence and in the absence of a selective $A_{2A}R$ antagonist and levels of expression of inflammatory mediators by real-time PCR analysis (**Chapter 2.3**).

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Chapter 2

EXPERIMENTAL WORK

Chapter 2.1

Brain region regulation of microglia morphology by adenosine A_{2A} receptors in female adult rats prenatally exposed to glucocorticoids

1. Rationale

Synthetic GCs, such as DEX, are anti-inflammatory drugs commonly used in obstetrics to enhance fetal lung maturation in pregnancies at risk of preterm delivery (Brownfoot et al. 2013). Although these drugs have clinical benefits, they are also mediators of stress responses and have a negative impact on the developing brain, namely a reduction in the volume and number of neuronal cells (Oliveira et al. 2012) and an impact upon neuronal differentiation and migration during critical phases of neurodevelopment in the cortex (Fukumoto et al. 2009). Abnormalities in behavior, such as anxiety-like behavior (Oliveira et al. 2006), susceptibility to depression (Roque et al. 2011) and addition-like behavior (Rodrigues et al. 2012) have been described as deleterious consequences of the use of GCs. Moreover, DEX induces brain region-specific morphological changes in dendritic arborization, triggering dendritic atrophy and synaptic loss in regions associated with stress, namely in the amygdala (Oliveira et al. 2012).

The Hip is a brain region particularly susceptible to stress effects. Several studies showed that prenatal exposure to DEX induces neuronal apoptosis and reduces neurogenesis in the subgranular subregion of the Hip – dentate gyrus (DG), reviewed in (Gould et al. 2000). Furthermore, the chronic treatment with GCs in adulthood causes dendritic atrophy and synaptic loss in the dHip, while in the ventral hippocampus GCs cause dendritic hyper-ramification (Pinto et al. 2015), which are correlated with functional deficits in hippocampal-dependent functions, namely cognitive deficits.

Microglia morphology, which is highly dynamic (cellular processes constantly retract and extend), is critical for brain functioning and undergoes profound remodelling in neuropsychiatric diseases.

In a rat model of prenatal exposure to dexamethasone, the offspring exhibits anxious-like phenotype and cognitive deficits at adulthood. Using this model, we previously reported gender-specific changes in microglia morphology, namely hyper-ramification of microglial cell processes in males and a de-ramification in females in the prefrontal cortex, a brain region involved in the pathophysiology of anxiety. We also observed that the chronic treatment with a selective $A_{2A}R$ antagonist normalizes microglia morphology in the prefrontal cortex and anxiety in males, but not in females, whereas it was able to ameliorate cognitive deficits in both genders.

Females prenatally exposed to DEX have an impact in cognition at the level of short-term recognition memory, namely a decrease in the recognition index in the novel object recognition test (NOR) was detected in females exposed prenatally to DEX (unpublished data). Additionally, *in vivo* electrophysiological analysis revealed a disruption of the coherence between the PFC and the dHip (unpublished data). Remarkably, A_{2A}R antagonist chronic treatment reverted the effects of DEX in both parameters.

Considering the impact of microglia remodeling in the PFC on anxiety and the ability of A_{2A}R to modulate both microglia morphology and behavior, we now study the impact of DEX on microglia morphology in the dHip. As A_{2A}R are regulators of microglia morphology (Gyoneva et al. 2009; Orr et al. 2009) and their blockade is well known as cognitive enhancer (Li et al. 2015) (Machado et al. 2016), we also studied the ability of chronic A_{2A}R blockade to alter DEX-induced changes in microglia morphology of female rats.

2. Working hypothesis

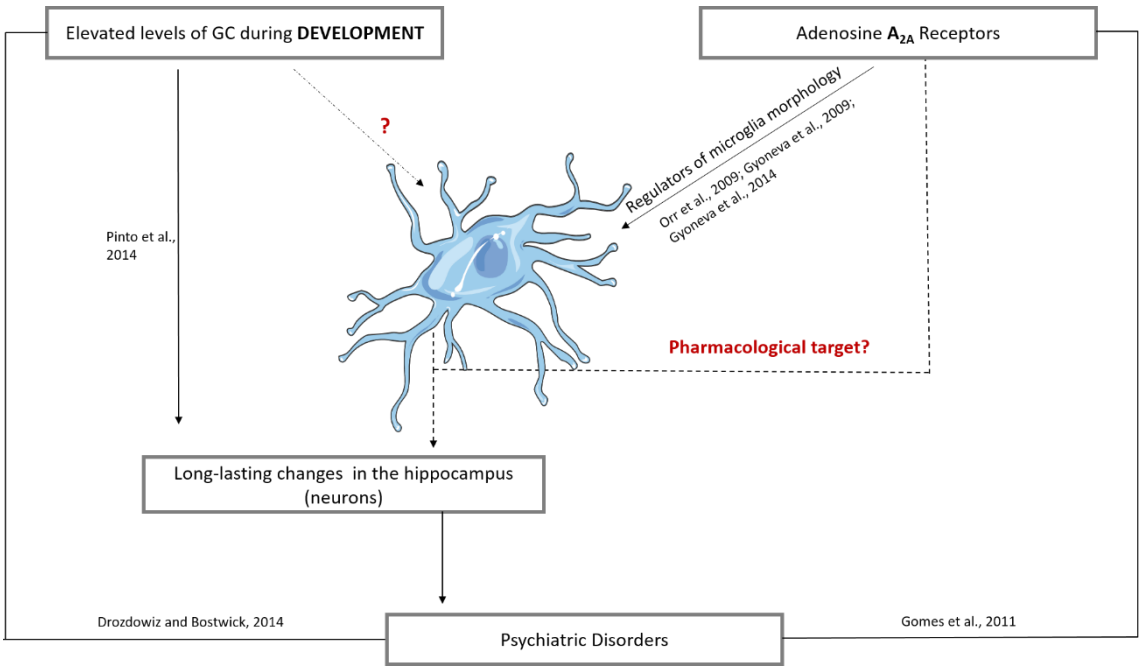

Figure 6. Schematic representation of adenosine A_{2A} receptor modulation of microglia morphology in the hippocampus of animals prenatally exposed to glucocorticoids. During development, high levels of GCs, that can result from a stressful event or GCs therapy, lead to neuronal changes in Hip and consequently to psychiatric conditions. We hypothesize that these changes involve microglial cells. Our work focus on microglia and their regulation by $A_{2A}R$, once these cells influence the formation of neuronal circuits and behavior. $A_{2A}R$, as microglia modulators, are good candidates as pharmacologic targets. Thereby, our main goal is to dissect the brain-specific response to *in utero* GCs treatment, focusing on microglia and $A_{2A}R$. Broken arrows indicate connections that are not fully characterized and were addressed in the present work. The full arrows represent the connection described in the literature with the respective reference.

3. Methods

3.1 Animals and pharmacological treatment

Mice were handled according to European Union and National legislation. Pregnant Wistar females (Charles River, Barcelona, Spain) were injected subcutaneously (sc) with DEX (1 mg/kg/day) (Acros Organics, Geel, Belgium – 230300010) or saline at days 18 and 19 of pregnancy, as described in (Oliveira et al. 2006). Female animals from the offspring were treated during the three last weeks prior to post-natal day (PND) 90 with the selective A_{2A}R antagonist, SCH 58261 (0.1 mg/kg/day) (Tocris, United Kingdom - 2270) or saline, by intraperitoneal injection (ip) (Figure 7). This protocol of chronic administration has been described as anxiolytic in adult rodents subjected to stress protocols (Kaster et al. 2015). Animals were placed under standard laboratory conditions (room temperature (RT); food and water *ad libitum*; light/dark cycle 12/12 hours). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Figure 7. Schematic representation of the animal model treated with DEX and A_{2A}R antagonist. Pregnant female Wistar rats were injected with DEX (1 mg/kg/day, sc) or saline on days 18 and 19 of gestation. Animals from the offspring were chronically treated with SCH58261 (0.1 mg/kg/day, ip) or saline for 3 weeks before PND 90.

3.2 Immunohistochemistry

Animals were deeply anesthetized with an ip injection of sodium pentobarbital (60 mg/kg) and transcardially perfused with heparinized saline, followed by 4% paraformaldehyde (PFA). After perfusion, brains were removed, kept in 4% PFA overnight and transferred to 30% sucrose solution in PBS overnight at 4°. After fixation, brains were cryo-preserved and sectioned (50 µm) in the cryostat.

Slices from the dHip, located at the stereotaxic coordinates of interaural 5.20 mm and bregma - 3.80 mm (Paxinos and Watson, 1998) were selected and subjected to a free floating

immunohistochemical procedure. Brain sections were incubated for 2 h at RT with blocking solution [5% bovine serum albumin - (BSA) and 0.1% Triton X-100] in phosphate buffered saline (PBS: NaCl 137 mM, KCl 2.1 mM, KH_2PO_4 1.8 mM and $Na_2HPO_4.2H_2O$ 10 mM, at pH 7.4). Incubation with primary antibody (Table 1) was performed for 48 h in the blocking solution at 4°C, with mild agitation. After being washed in PBS (3 x 10 min), the sections were incubated with the secondary antibody (Table 1) for 2 h. Then, sections were washed with PBS (3 x 10 min) and incubated with the nuclear dye ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI, 1:5000) and washed again with PBS (3 x 10 min). Sections were mounted using glycergel (Dako mounting medium).

Table 1. Primary and secondary antibodies

Antibody	Source	Company	Dilution
Iba-1	Rabbit	Wako	1:1000
Anti-rabbit	Donkey	Invitrogen	1:1000

3.3 Morphometric data analysis

Images of microglial cells (Z-stacks) were acquired using a confocal microscope (Zeiss Confocal Microscope LSM 710 Meta) with the 63x objective (Plan-Apochromat 63x/1.40 Oil DIC M27) (Figure 8).

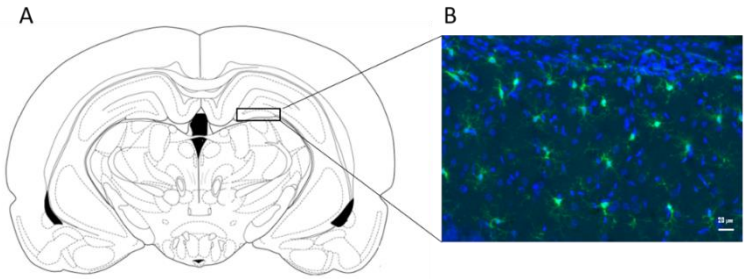

Figure 8. Schematic diagram with the localization of microglial cells acquired for tridimensional reconstruction. A) Schematic diagram of a section from the rat brain, adapted from the Rat Brain Atlas, with coordinates of interaural 5.20 mm and bregma - 3.80 mm (Paxinos, G. and Watson, C., 1998) (B) Representative image, obtained by the author in confocal microscope with 20x magnification, of the region where the images for tridimensional reconstruction of microglia were acquired. Nuclei were stained with DAPI, blue, and microglia with Iba-1, green.

To perform the tridimensional reconstruction of microglia cells from the dHip, the cells were manually drawn in several planes of the same image, using the Neurolucida software. For each animal, ten microglial cells were analyzed, in a total of 40 cells *per* condition. The tridimensional analysis of the reconstructed microglial cells was performed using NeuroExplored software. To assess changes in microglia morphology, we quantitatively analyzed the number and length of ramifications (Figure 9).

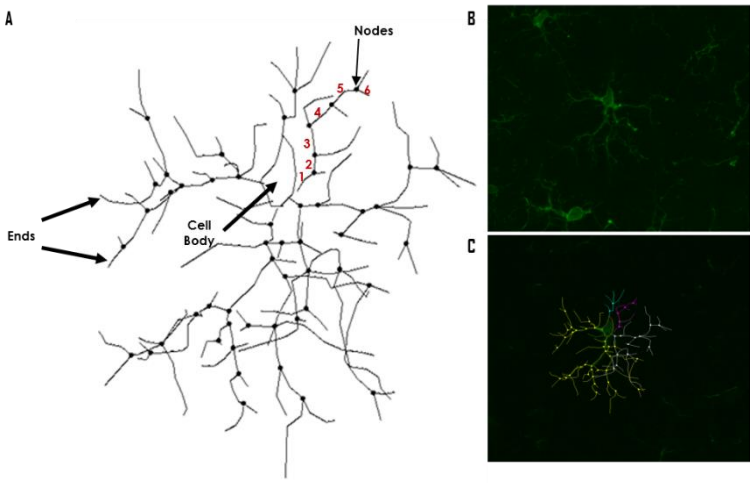

Figure 9. Illustrative scheme showing the tridimensional reconstruction of an adult microglial cell and the respective image acquired and reconstructed. (A) The nodes and ends of microglia are specified in the figure. Numbers correspond to the branch order, starting in the cell body (1-6). The principle established for the reconstruction of the processes was that all ramifications, regardless their length, must be taken into consideration, in order to get a final result analogous to the original cell. (B) Microglia labelled with Iba-1. (C) Tridimensional reconstructed cell using Neurolucida software.

3.4 Data analysis

Statistical analysis was conducted using GraphPad Prism 5. Differences between groups were analyzed by Student's t-test, when comparing two conditions or one-way analysis of variance (ANOVA), when comparing more than two conditions. The level of significance was set at $p<0.05$. Results are presented as mean ± SEM (standard error of the mean).

4. Results

4.1. Dexamethasone led to long-lasting alterations in microglia morphology in dorsal hippocampus

Animals exposed to DEX during development have an impact in microglia morphology in the PFC correlated with anxiety-like behavior (Caetano 2014; Henriques 2015) and cognitive deficits (unpublished data). Therefore, it is plausible that microglia morphology is also altered in the dHip of animals exposed to DEX when compared with animals treated with saline (Saline). To confirm this hypothesis, we analyzed the morphometry of microglia in DG of females at PND 90 (Figure 10 A). The DG was chosen because the effects of chronic or prenatal treatment with DEX in neurons is well described, namely apoptosis and a reduction in neurogenesis (Noorlander et al. 2014; Sousa et al. 1999). As depicted in Fig. 10 A and quantified in Fig 10 B (left panel), the number of ramifications significantly increased in microglia from animals exposed to DEX (order 1: 7.9 ± 0.4; order 2: 15.3 ± 0.9; order 3: 20.6 ± 1.7; order 4: 21.9 ± 1.5), when compared with Saline animals (order 1: 6.1 ± 0.8; order 2: 12.1 ± 1.1; order 3: 16.1 ± 1.5; order 4: 17.2 ± 1.7). However, the treatment with DEX did not affect the length of the ramifications (Figure 10 B, right panel).

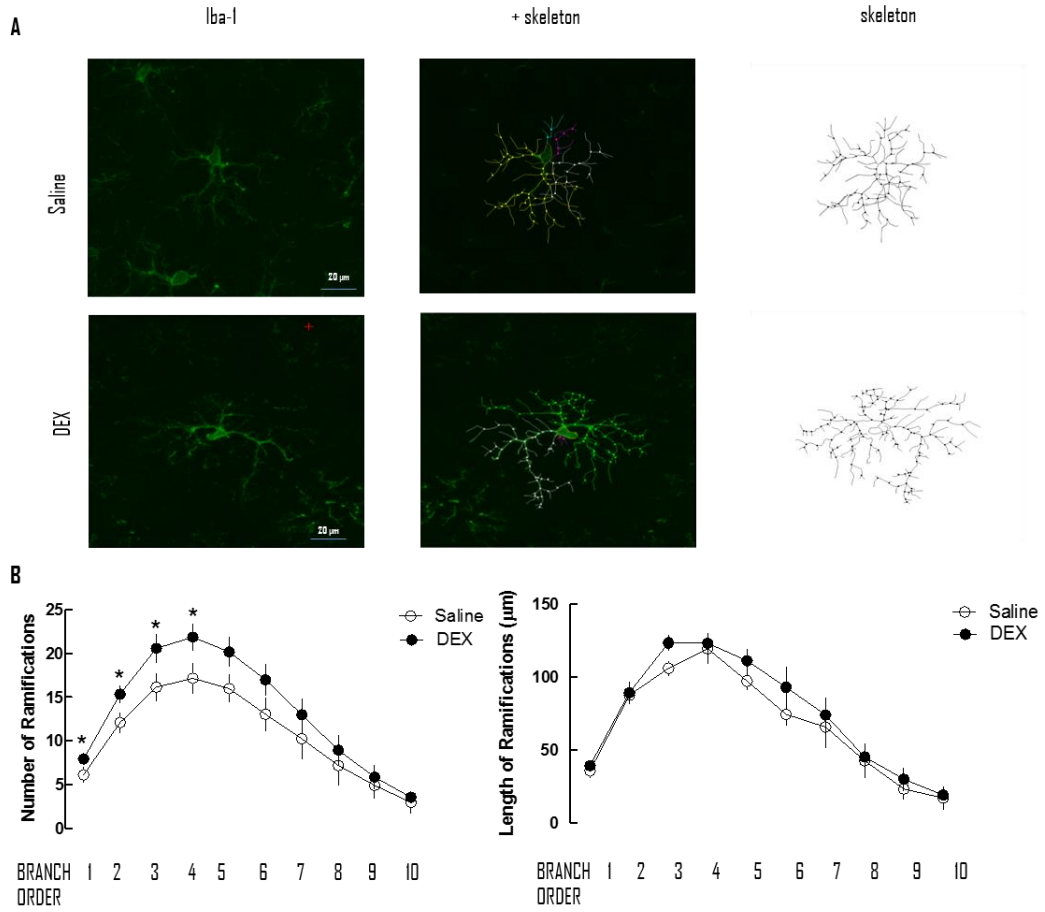

Figure 10. Effect of prenatal DEX treatment in the number and length of processes of microglia in the dHip at PND90. Pregnant female Wistar rats were injected with DEX (1 mg/kg/day, sc) or saline on days 18 and 19 of gestation. Microglial cells of females at PND90 were stained with Iba-1 and tridimensional reconstructions were performed using Neurolucida software. The animals that were exposed to DEX showed an increase in the number of ramifications compared to animals treated with saline. (A) Representative images obtained from the Iba-1 staining of microglia: Iba-1 immunoreactivity (Iba-1, green); stained microglia merged with manual reconstruction (+ skeleton) and isolated manual reconstruction (skeleton). (B) Quantification of the number and length of ramifications of microglia from DEX animals (n=3) and Saline (n=4); Comparison between two independent means was done by a Student's t test with $p < 0.05$ (*). Error bars are SEM.

4.2 Adenosine A_{2A}R selective blockade normalized GC-induced changes in microglia morphometry

Since A_{2A}R are important regulators of microglia morphology (Caetano 2014; Gyoneva et al. 2009; Gyoneva et al. 2014; Henriques 2015; Orr et al. 2009), we addressed whether the modulation of A_{2A}R could revert the effects triggered by DEX in microglia morphology.

Conversely to what happens in the PFC (Henriques 2015), where the A_{2A}R blockade was unable to normalize DEX-induced changes, in the dHip, we observed that the animals chronically treated with SCH 58261 after DEX-exposure (DEX + SCH), show a significant reduction in the number of ramifications (order 3: 15.9 ± 1.6; order 4: 17.0 ± 1.6; order 7: 8.1 ± 0.9) compared with the animals exposed only to DEX (order 3: 20.6 ± 1.7; order 4: 21.9 ± 1.5; order 7: 13.0 ± 1.8) (Figure 11 B, left panel). Furthermore, the length of processes of order 3, was also diminished in animals chronically treated with SCH 58261 after DEX exposure (order 3: 88.1 ± 6.6) compared with animals exposed only to DEX (order 3: 117.4 ± 7.1). These data indicate that the blockade of A_{2A}R was effective in recovering microglia morphology in dHip.

Importantly, the blockade of A_{2A}R *per se,* exerted a marked effect (reduction) in the length of some microglial processes (order 3: 77.0 ± 4.9; order 4: 79.4 ± 4.0) compared with Saline (order 3: 104.8 ± 4.9; order 4: 114.8 ±7.8) (Figure 11 B, panel right), although not affecting the number of processes. The numeric results associated with the morphometric analysis are summarize in table 6 in Chapter 4. Interestingly, these results contrast with the morphometric analysis of microglia from PFC, where the chronic treatment with A_{2A}R antagonist *per se* decreases the number and the length of microglial processes (Henriques 2015).

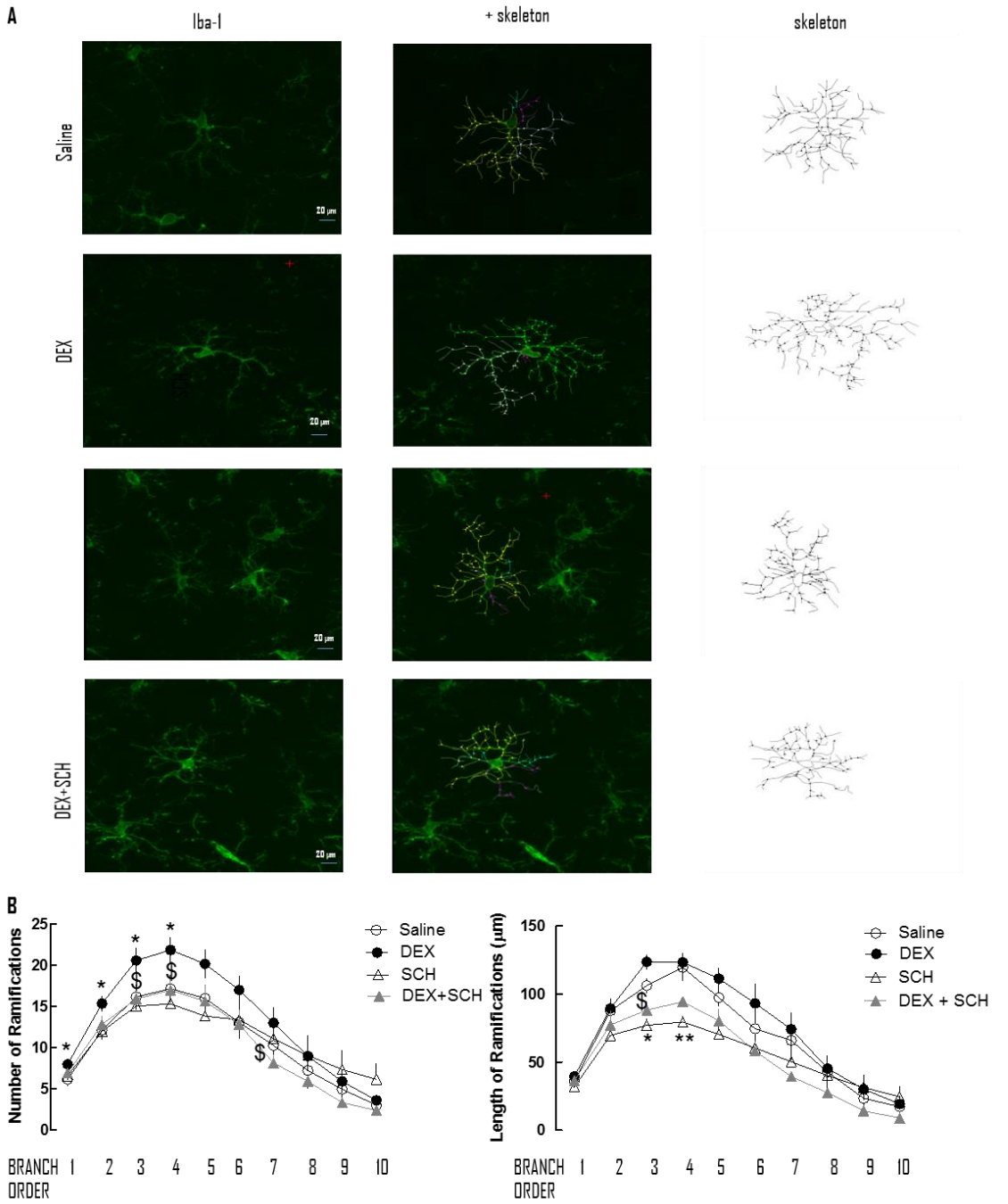

Figure 11. Effect of the chronic blockade of A_{2A}R in the number and length of processes of microglia in the dHip at PND90. Pregnant female Wistar rats were injected with DEX (1 mg/kg/day, sc) or saline on days 18 and 19 of gestation. Animals from the offspring were chronically treated with SCH 58261 (0.1 mg/kg/day, ip) or saline for 3 weeks before PND90. Microglial cells of females at PND90 were stained with Iba1 and tridimensional reconstructions were performed using Neurolucida Software. A_{2A}R blockade was able to revert DEX-induced changes. (A) Representative images obtained from the Iba-1 staining of microglia: Iba-1 immunoreactivity (Iba-1, green); stained microglia merged with manual reconstruction (+ skeleton) and isolated manual reconstruction (skeleton). (B) Quantification of number and length of

ramifications of microglia from DEX animals (n=3), Saline (n=4), SCH (n=4) and DEX + SCH (n=4); One-way ANOVA followed by a Turkey's multiple comparisons test with $p < 0.05$ (*), different from control and $p<0.05$ ($), different from DEX. Error bars are SEM.

4.3 Brain region-specific morphology: microglia from prefrontal cortex exhibited a more complex morphology than microglia from the dorsal hippocampus

The differential effect of DEX in the dHip (present work) and in the PFC (Henriques 2015), led us to analyze in detail the morphology of these cells in these brain regions in physiological conditions.

In this work we compared the morphology of female microglial cells in dHip and PFC (Figure 12 A). Microglial cells from PFC exhibited a more complex and ramified morphology, with higher number of ramifications (order 3: 22.1 ± 1.7; order 4: 26.6 ± 2.5; order 5: 27.4 ±3.1; order 6: 23.8 ± 3.3; order 10: 9.0 ± 1.8) and in the length (order 5: 132.3 ± 8.6; order 6: 108.0 ± 8.7; order 9: 47.5 ± 5.5) compared to microglial cells from the dHip (number of ramifications: order 3: 16.1 ± 1.5; order 4: 17.2 ± 1.8; order 5: 16.00 ± 1.6; order 6: 13.0 ± 1.9; order 10: 2.9 ± 1.9; length of the ramifications: order 5: 97.5 ± 6.4; order 6: 74.6 ± 7.9; order 9: 23.4 ± 7.3) (Figure 12 B panel left and right).

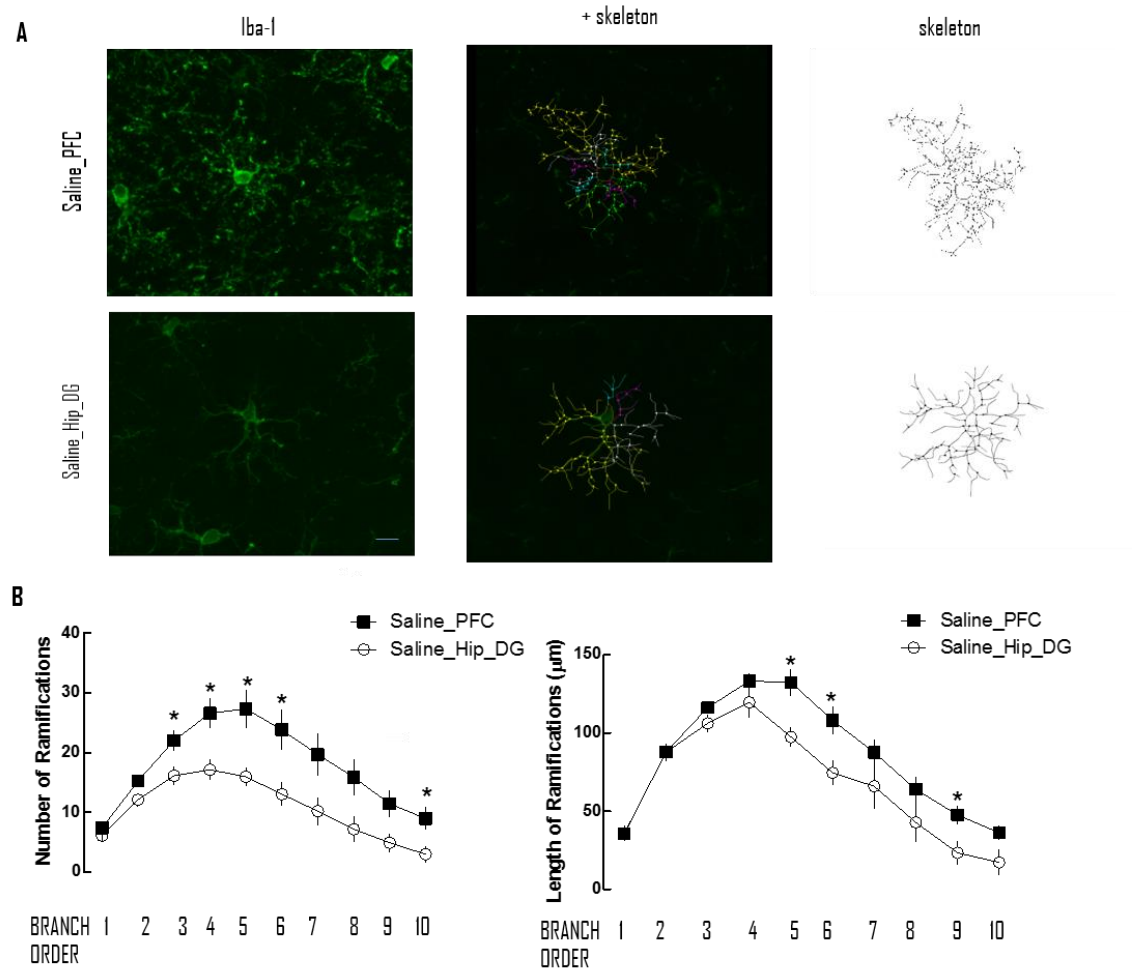

Figure 12. Microglia morphology in the dHip and in the PFC. Microglial cells from PFC or dHip of females at PND 90 were stained with Iba-1 and tridimensional reconstructions were performed using Neurolucida Software. (A) Representative images obtained from Iba-1 stained microglia: Iba-1 immunoreactivity (Iba-1, green); Iba-1 immunoreactivity merged with manual reconstruction (+ skeleton) and isolated manual reconstruction (skeleton). (B) Quantification of the number and the length of ramifications of microglia from PFC Saline animals (n=6) and Hip_DG Saline animals (n=4); Comparison between two independent means was done by a Student's t test with $p < 0.05$ (*). Error bars are SEM.

5. Discussion

It was generally accepted for decades that microglia are highly plastic cells, acquiring diverse morphological and functional phenotypes in response to different environmental stimuli. However, distinct microglia transcriptional identities between brain regions, namely the cortex and the hippocampus, it was only recently described (Grabert et al. 2016).

The dHip is a region particularly susceptible to stress and several studies described the impact of stress or GCs therapy (DEX) in neurons of this region (Pinto et al. 2015; Sousa et al. 2000; Woolley et al. 1990). Females prenatally exposed to DEX have an anxiety-like phenotype, cognitive deficits in recognition memory and a decrease in synchronization between PFC – dHip. Remarkably, the pharmacological blockade of A_{2A}R did not normalize DEX-induced changes in microglia morphology in the PFC, as well as anxiety, but was able to recover cognition deficits and the desynchronization between PFC-dHip, suggesting that in regions associated with cognition the blockade of A_{2A}R may be effective. So, we analyzed microglia morphology in the dHip, in order to clarify if these functional uncoupling between anxiety and cognition in females prenatally exposed to DEX and treated with A_{2A}R antagonist at adulthood could be explained by a dual brain region effect of A_{2A}R in the control of microglia morphology.

In this study, we observed that DEX leads to a long-lasting hyper-ramification of microglial cell processes from the dHip of young adult females. Interestingly, the opposite morphological profile was previously described by our group in PFC of females (de-ramification of microglial cell processes) (Henriques 2015). Besides the duality of effects according to the brain region, and eventually explaining this duality, we also reported differences in the morphology in physiological conditions, with the PFC exhibiting cells with a higher degree of morphological complexity. These data are in line with previous studies reporting regional differences in microglia (Lawson et al. 1990; Schwarz et al. 2012). Lawson and co-workers reported that microglia density is dependent on the brain region, having maximum density in Hip, olfactory bulb, basal ganglia and substantia nigra (Lawson et al. 1990) and that microglia morphology is dependent on their location, suggesting distinct profiles of vulnerability when subjected to different stimulus; however, they used a rudimentary approach to study cell morphology. Schwarz and colleagues, when describing gender-specific morphology of microglia, also analyzed different brain regions, namely hippocampus, amygdala, cortex and hypothalamus, and microglia morphology was also slightly different, although the differences were not explored by the authors (Schwarz et al. 2012). Our data strongly point towards the

concept that microglial cells exhibit different morphologic phenotypes in different regions. We demonstrate these differences using a quantitative study of specific morphologic features, as assessed by the tridimensional reconstructions of microglia morphology (morphometry).

Regional differences were also observed in response to the chronic treatment with an A_{2A}R antagonist, that was unable to correct microglia atrophy in the PFC, but reverted microglia hyper-ramification in the dHip. The mechanism behind these differences is not yet clarified, but regional gradients of A_{2A}R density may explain these differences. Indeed, we previously observed a tendency to a decrease of A_{2A}R density in the PFC of females prenatally treated with DEX whereas in the dHip we did not observe alterations (data not shown).

The observation that microglia morphology from dHip is affected by DEX raises the possibility that prenatal exposure to GCs may have an impact on learning and memory of children treated with GCs. In fact, observations by Kamphuis and colleagues revealed adverse effects of prenatally DEX in spatial memory, that can be associated with deficits in dHip (Kamphuis et al. 2003).

In conclusion, the data obtained show that DEX, in conditions mimicking the clinical use of GCs, in early periods of brain development, induces alterations in microglial morphology, in a brain region-specific manner, particularly in the dHip and in the PFC, with impact in behavior. Here we observed that microglia morphology remodeling in the dHip is a morphological correlate of cognitive deficits observed in our animal model. Females prenatally treated with DEX have poor performance in recognition memory tasks, namely in the NOR test; this cognitive impairment is preceded by the lack of coherence between the dHip and the PFC. Both behavior changes and electrophysiological abnormalities correlate with microglia morphology remodeling; specifically, the functional recovery by A_{2A}R blockade correlated with the morphological recovery of microglia in the dHip, a brain region strictly involved in memory consolidation (Fanselow and Dong 2010). These data are also in line with the well described role of A_{2A}R antagonist, including the non-selective compound caffeine as cognitive enhancer (Borota et al. 2014; Machado et al. 2016)

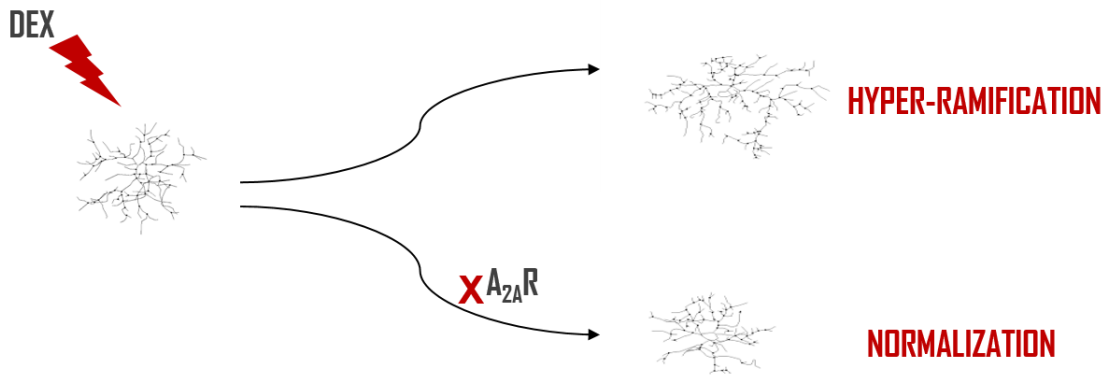

Figure 13. Schematic representation of the model of long-term effects of prenatal exposure to dexamethasone in microglia morphology from dorsal hippocampus of females. The exposure to DEX during early phases of neurodevelopment induce morphological alterations of microglial cells (Hyper-ramification). In addition, the chronic blockade of $A_{2A}R$ restore the morphological alterations induced by the exposure to DEX (Normalization).

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Chapter 2.2

Unravelling the role of $A_{2A}R$ on anxiety and cognition in females

1. Rationale

The role of adenosine A_{2A} receptors in the pathophysiology of stress-related disorders, namely anxiety, it is involved in controversy. Indeed, $A_{2A}R$ KO male mice have been shown to display anxiety-like behavior (Kaster et al. 2015), in line with a pharmacological study showing that the adenosine A_{2A} receptor agonist, CGS21680, reduces the anxiogenic effect of theophylline (Imaizumi et al. 1994). Surprisingly, A_{2A} receptor overexpression does not alter anxiety-like responses (Coelho et al. 2014).

Several studies support that $A_{2A}R$ blockade can be used as a neuroprotective strategy to manage the negative impact of chronic stress in mood and memory (Batalha et al. 2013; Kaster et al. 2015; Yamada et al. 2014). In fact, using pharmacological or knockout mice, several studies described the impact of $A_{2A}R$ in cognitive functions. Animal studies confirmed that caffeine and selective $A_{2A}R$ antagonists have a prophylactic benefit on memory dysfunction, elicited in experimental models of aging (Prediger et al. 2005), Alzheimer's disease (Cunha et al. 2008; Dall'Igna et al. 2007) and Parkinson's disease (Gevaerd et al. 2001). Furthermore, the activation of $A_{2A}R$ signaling in the Hip, using an optogenetic approach, triggers memory dysfunction, suggesting a role in cognition (Li et al. 2015). Moreover, the selective inactivation of $A_{2A}R$ enhances learning and memory functions (Shen and Chen 2009) and mice lacking $A_{2A}R$ display improved spatial recognition memory (Wang et al. 2006).

Importantly, in these studies the gender was not specified and recent work from our laboratory shows that the chronic blockade of $A_{2A}R$ is anxiogenic for young adult females (but not for males), without affecting the short-term recognition memory behavior (neither in males nor in males). Furthermore, we recently observed that chronic treatment with a selective antagonist of $A_{2A}R$ exacerbates the anxious-phenotype associated to the prenatal treatment with the synthetic glucocorticoid, dexamethasone, while ameliorating cognitive deficits caused by this neurodevelopment manipulation in females. In males, the treatment reverted anxiety and cognitive impairment. These data suggest that $A_{2A}R$ may have different effects in physiological and non-physiological conditions, in males and females. Thus, it was considered of importance to characterize the behavior (anxiety and short-term recognition memory) of female mice with a global genetic deletion of $A_{2A}R$ and to evaluate glucocorticoid receptor density in the brain, in particular, in the hippocampus and prefrontal cortex, regions implicated in behavioral tests chosen for anxiety and cognition assessment.

2. Working Hypothesis

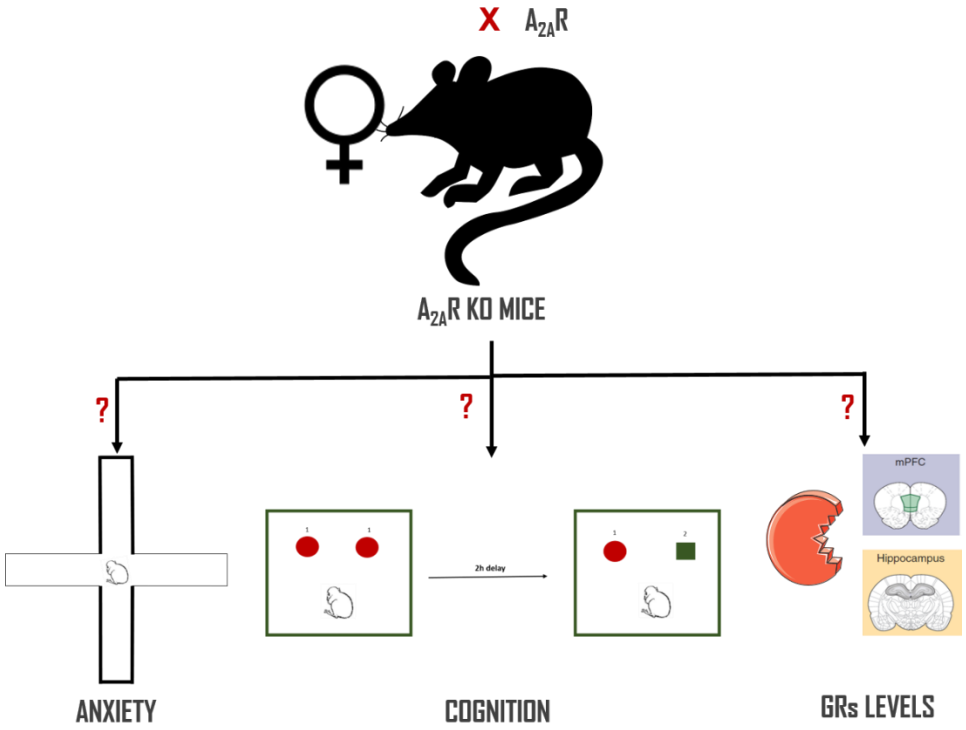

Fig. 14. Schematic representation of the possible impact of the genetic deletion of $A_{2A}R$ in behavior and glucocorticoid receptor levels. The impact of the global genetic deletion of $A_{2A}R$ in anxiety, cognition (short-term recognition memory) and GR levels in PFC and Hip, regions associated with anxiety and cognition, respectively, was evaluated using female $A_{2A}R$ KO mice with three months age, the same age of the animal model used in Chapter 2.1.

3. Methods

3.1 Animals

Mice were handled according to European Union and National legislation. Female C57BL/6 mice with three months age were used. The animals were under standard laboratory conditions (RT; food and water ad libitum; light/dark cycle 12/12 hours).

Mouse genotyping was performed using genomic DNA extracted from ear clipping in the Center of Neuroscience and Cell Biology (CNC) animal facility. All efforts were made to minimize animal suffering and reduce the number of animals used.

3.2 Behavioral tests

All animals were submitted to behavior analysis at three months of age, the same age of the animals subjected to the chronic blockade of $A_{2A}R$ in Chapter 2.1. Animals were habituated to the environment where the tests were performed for 1 h in a dark and quiet room, with controlled temperature and ventilation. The animals were submitted to different tests in the following order: first, the animals were tested for locomotor activity in the open field (OF); next, the animals were tested in the elevated plus maze (EPM) for anxiety and, last, the novel object recognition (NOR) was used to assess short-term recognition memory. An overhead video-camera was used to monitor mice behavior during the test. All devices were wiped clean with 76% ethanol solution and dried before evaluating the other animal.

3.2.1 Open field

The open field test is used to evaluate the locomotor activity. The open field area consists of an empty and bright square arena, surrounded by walls to prevent animal from escaping. The animal was placed at the center of the arena and the behavior was recorded for 10 min. The locomotor activity was measured by the distance traveled and velocity, as described in (Machado et al. 2016).

3.2.2 Elevated plus maze

The elevated plus maze is an elevated maze with four arms (two open and two closed) that are settled to form a plus shape (Figure 15). This test was used to assess the degree of anxiousness: animals were placed in the intersection of the arms and tested for 5 min. Time

spent in the open arms and the number of total entries were measured and used to assess the anxious behavior, as described in (Machado et al. 2016).

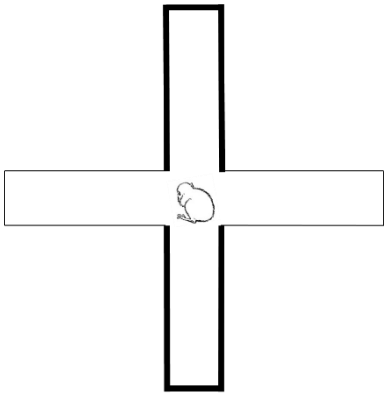

Figure 15. Schematic representation of the elevated plus maze test. The EPM test was performed to calculate the time spent in open arms *per* total time of the assay, to evaluate anxiety-related behavior of female $A_{2A}R$ KO mice. Open arms are represented by a thin line, while closed arms by a bolder line. The mice were placed in the center, where all arms are connected.

3.2.3 Novel object recognition

The novel object recognition test (Figure 16) was used to evaluate the ability to distinguish between a familiar and a novel object, being an indicator of short-term recognition memory. Briefly, plastic objects with the same shape, size and color were used in the first test during 10 min. After 2 h, the second test started with two objects placed: a familiar object, which has been used in the first test and a novel object, which the animal has not seen before, with different shape and color, but similar size. The recognition index was measured by [time spent in novel object / (time spent in familiar object + time spent in novel object), as described in (Machado et al. 2016).

Figure 16. Schematic representation of the novel object recognition test. Short-memory performance was evaluated using the NOR test. Mice first underwent a training session, being exposed during 10 min to two identical objects (familiar objects). The test session was performed 120 min after, with a familiar and a novel object. 1: Familiar object; 2: Novel object.

3.3 Western blotting

Animals were sacrificed and the PFC and Hip were dissected in ice-cold Hank's balanced salt solution (HBSS: 137 nM NaCl, 5.4 nM KCl, 0.45 nM KH_2PO_4, 0.34 nM NaHPO, 4 nM $NaHCO_3$, 5 nM glucose; pH 7.4) and kept in dry ice until freezing at -80ºC. The samples were lysed and homogenized in radio-immunoprecipitation assay (RIPA) buffer [(150 mM NaCl, 50 mM Tris, 5 mM Ethylene glycol tetraacetic acid, 1% Triton X-100, 0,5% Deoxycholate (DOC), 0,1% sodium dodecyl sulfate (SDS)] supplemented with 1 mM dithiothreitol (DTT) and complete miniprotease inhibitor cocktail tablets. The resulting homogenate was sonicated for 1 min and centrifuged at 16 100 x g for 10 min at 4ºC. The supernatant was collected and stored at -80ºC until use.

Protein concentration was measured by the colorimetric bicinchoninic acid (BCA) (Pierce, Rockford, USA) assay. A standard concentration curve of BSA (2 mg/mL) was prepared by serial dilutions in milliQ water. Samples were diluted in milliQ water and incubated with BCA reagent for 30 min at 37ºC, protected from light. After the determination of protein concentration, the samples were denatured with 6x concentrated sample buffer (0.5 M Tris-HCL, 30% glycerol, 10% SDS, 0.6 M DTT, 0,02% bromophenol blue, pH 6.8) at 95ºC, for 5 min.

Equal amounts (25 µg) of protein were resolved on 8% SDS-polyacrylamide gels [8% bisacrylamide, trizma- HCL (1.5 M, pH 8.8), 10% SDS, 10% ammonium persulfate (APS), 1% tetramethylethylenediamine (TEMED) with a 4% stacking gel [4% bisacrylamide, trizma HCL (0.5 M, pH 6.8), 10% SDS, 10% APS, 2% TEMED). The separation of proteins occurred in a bicine-buffered solution [25 mM trizma, 25 mM bicine, 0.1% SDS, ph8.3). Samples were stacked by running at 60 V for 15 min and resolved at 140 V for 1 h. Proteins were transferred from the gel into the polyvinylidene difluoride (PVDF, Millipore) membrane at 750 mA current for about 1h30 min, using a CAPS solution [3-(cyclohexylamino)-1-propane-sulfonic acid] buffered solution with methanol (10 mM CAPS, 10% methanol, pH 11.0). Membranes were blocked with a 5% milk solution in Tris-buffered saline (10 mM Tris, 150 mM NaCl) with Tween-20 (TBS-T) for 1 h at RT, and incubated overnight at 4ºC with a primary antibody (Table 2) diluted in 1% milk in TBS-T. After washing (3 x 15 min in TBS-T), membranes were incubated with secondary antibodies (Table 2) conjugated with horseradish peroxidase or with alkaline phosphatase for 2h at RT and washed with TBS-T (3 x 15 min). Then, the bands were visualized with a commercial enhanced chemiluminescence detection method (ECL, Advantsa) kit (glyceraldehyde 3-phosphate dehydrogenase, GAPDH) or enhanced chemofluorescent detection method (ECF, GE Healthcare) (GR). The fluorescence was detected on an imaging system (Typhoon FLA 9000, GE Healthcare or ImageQuantTM LAS 500, GE Healthcare) and band quantification was performed using Image

Quant 5.0 software (Molecular Dynamics, Amersham Biosciences). Data are presented as a ratio between the immunoreactivity of GR and GAPDH (loading control) and expressed as percentage of control.

Table 2. Primary and secondary antibodies used in western blotting.

Antibody	Source	Company	Dilution
GR (M-20)	Rabbit	Santa Cruz Biotechnology	1:1000
GAPDH	Goat	Sicgen	1:5000
Anti-rabbit	Goat	GE Healthcare	1:10 000
Anti-Goat	Rabbit	Life Technologies	1: 10 000

3.7 Data analysis

Statistical analysis was conducted using GraphPad Prism 5. Differences between groups were analyzed by Student's t-test. The level of significance was set at $p<0.05$. Results are presented as mean ± SEM.

4. Results

4.1 $A_{2A}R$ genetic deletion did not affect locomotor behavior

Spontaneous locomotor behavior was evaluated with the OF, namely the velocity and the distance travelled by the animals. WT and $A_{2A}R$ KO mice did not perform differently in terms of total distance travelled (WT: 1891.7 ± 291.4; $A_{2A}R$ KO: 1705.2 ± 473.9, cm) (Figure 17 A) and velocity (WT: 3.3 ± 0.5; $A_{2A}R$ KO: 2.9 ± 0.8, cm/sec) (Figure 17 B).

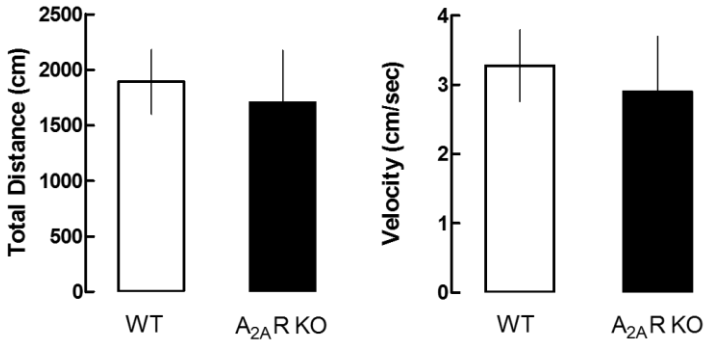

Figure 17. Effect of the genetic deletion of $A_{2A}R$ in locomotor behavior. The OF test was performed to evaluate the locomotor behavior in $A_{2A}R$ KO female mice compared to WT mice at PND90. (A) Represents the total distance travelled by the animals. (B) Represents the velocity of the animals. WT, control mice; KO, $A_{2A}R$ KO mice. All data represent mean ± SEM of n= 4-5 per condition. Comparison between two independent means was done by a Student's t test.

4.2 $A_{2A}R$ genetic deletion was associated to anxious-like behavior in female mice

Since $A_{2A}R$ pharmacological blockade exerts gender-specific effects on behavior (unpublished data), we considered important to assess whether A_2R KO females also exhibit anxious-like behavior, as described for male mice (Kaster et al. 2015). To address this question, the animals were submitted to the EPM test.

$A_{2A}R$ KO female mice manifested anxious-related behavior (spent less time in open arms/total time) (0.1 ± 0.04, Figure 18 A) and made fewer entries (3.5 ± 1.2, Figure 18 B), as compared with WT animals (time spent in open arms/total time: 0.2 ± 0.02; number of entries in open arms: 7.00 ± 0.8). The number of entries in closed arms did not differ between groups (Figure 18 B).

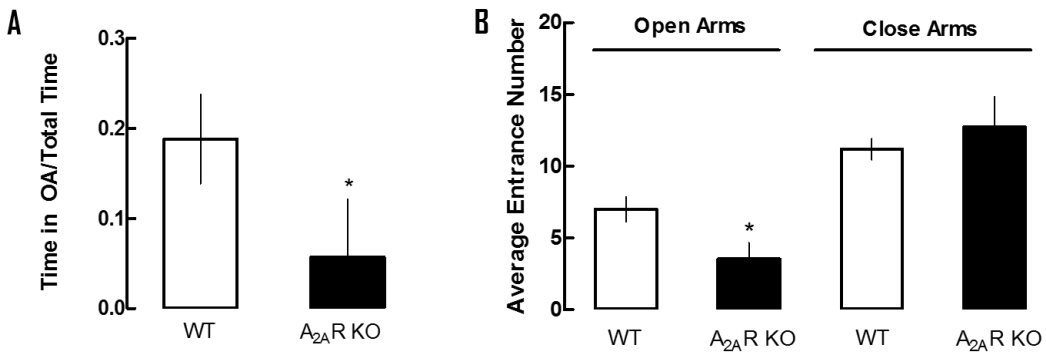

Figure 18. Effect of the genetic deletion of $A_{2A}R$ in anxiety behavior. The EPM test was performed to evaluate anxiety-related behavior of $A_{2A}R$ KO female mice compared to WT mice at PND90. (A) Represents the time spent in open arms *per* total time of the assay. (B) Represents the number of entries in the open and closed arms. WT, control mice; KO, $A_{2A}R$ KO mice. All data represent mean ± SEM of n= 4-5 per condition. Comparison between two independent means was done by a Student's t test with $p < 0.05$ (*).

4.3 $A_{2A}R$ genetic deletion did not affect short-term recognition memory

Previous data from our group indicate that the pharmacologic treatment with an $A_{2A}R$ antagonist restores cognition defects induced by prenatal exposure to DEX, further strengthening a positive modulatory effect of $A_{2A}R$ in cognition (unpublished data). So, the impact of the genetic deletion on hippocampal-dependent memory was evaluated using the NOR test in $A_{2A}R$ KO female mice.

During the first trial, none of the animals tested had a preferential object (data not shown). In the second trial, $A_{2A}R$ KO mice (0.4 ± 0.02) did not show an impairment on hippocampal dependent memory when compared with WT animals (0.4 ± 0.03), assessed by the time spent in the novel object (recognition index) (Figure 19).

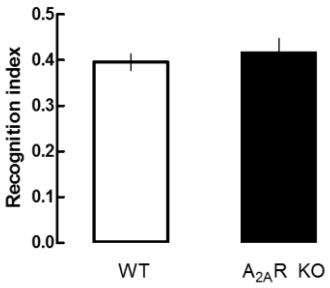

Figure 19. Influence of the genetic deletion of $A_{2A}R$ on short-term recognition memory. The NOR test was performed to evaluate cognitive deficits in $A_{2A}R$ KO female mice compared to WT mice at PND 90. The graphic represents the time spent in the novel object *per* total time spent in the novel and familiar objects. All data represent mean ± SEM of n= 4-5 per condition.

4.4 The genetic deletion of $A_{2A}R$ did not affect glucocorticoid receptor levels in the hippocampus and in the prefrontal cortex

With the hypothesis that $A_{2A}R$ and GR interact with each other, we aimed to understand if the deletion of $A_{2A}R$ alters GR density. Based on the behavioral analysis performed, related with cognition and anxiety, we decided to study the expression levels of GR in Hip (cognition) and PFC (anxiety) by western blot. These results showed that the levels of GR were similar between $A_{2A}R$ KO mice (Hip: 121.4 ± 13.0; PFC: 100.0 ± 10.9; % of control) and WT mice in both regions (Hip: 100.0 ± 14.6; PFC: 100.0 ± 29.4, % of control) (Figure 20 A, B).

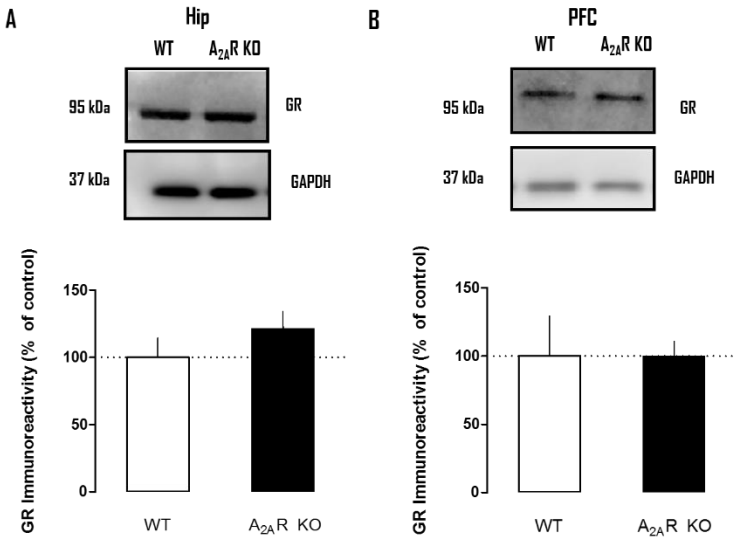

Figure 20. Effect of genetic deletion on glucocorticoid receptor protein levels. The protein levels of GR were assessed by immunoblotting in total protein extracts of the Hip (A) and PFC (B) in $A_{2A}R$ KO female mice compared to WT mice at PND 90. Representative bands are presented above the graphs, with the respective loading controls (GAPDH), to confirm that identical amounts of protein were loaded into the gel. Data are presented as ratio a (GR/GAPDH) and represented Control (n=4-5) and $A_{2A}R$ KO (n=4-7). Each bar is the mean ± SEM.

5. Discussion

Adenosine A_{2A} receptors have been implicated in anxiety and cognition. Although the blockade of $A_{2A}R$ is globally accepted as a cognitive enhancer and anxiolytic, in animal models of disease, the majority of the studies were performed in males. This topic is of particular relevance, considering our previous work, where we describe gender differences in anxious behavior associated with the pharmacological blockade of $A_{2A}R$. Furthermore, the pharmacological modulation of the receptor in an animal model of chronic anxiety (caused by prenatal exposure to DEX, as described in Chapter 2.1), differentially impact on the behavioral performance of males and males.

Altogether, these data raise new questions about the physiological role of $A_{2A}R$ in behavioral differences between males and females and about the role of this receptor in the modulation of GR density in brain areas critically implicated in anxiety and cognition. Thus, we decided to characterize anxiety and cognition in $A_{2A}R$ KO female mice. The present study shows that $A_{2A}R$ KO female mice have an anxious-like phenotype, in accordance with previous results from our group showing that the chronic blockade of $A_{2A}R$ *per se* leads to an anxious-like behavior in females, but not in males (unpublished data). Our results are also in line with a previous study that shows that the genetic deletion of $A_{2A}R$ triggers an anxiety-like behavior, which was exclusively performed in males (Kaster et al. 2015).

Concerning memory, the genetic deletion of $A_{2A}R$ in females was not effective as a cognitive enhancer. However, these results may also be justified by the fact that WT females did not learn the task in our study. In fact, other studies using WT female animals, with the same background, report variable recognition indexes, between 0.4 (Frye et al. 2013) to 0.8 (Oliveira et al. 2015), suggesting that some control animals have a low recognition index.

In $A_{2A}R$ KO animals (females) we did not observe statistically significant alterations in the levels of GR in Hip and PFC compared to WT animals. These results contrast with what happens in females chronically exposed to a selective $A_{2A}R$ antagonist during adulthood, that showed variations in the density of GR in a brain region-specific manner.

The results described highlight the urgent need to carefully analyze the output of screening tests for the efficacy and safety of molecules with therapeutic potential, considering that the vast majority of these tests are only performed in males. Our study also emphasizes that conclusions taken by comparison between genetic and pharmacological blockade

modulation of receptors also deserve particular attention, since the outcomes are not necessarily the same.

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Chapter 2.3

Adenosine A_{2A} receptors modulate glucocorticoid receptor levels in subcellular fractions of microglial cells

1.Rationale

Dexamethasone has high affinity to GR, which are well known modulators of immune responses, including those mediated by microglia, immunocompetent cells of the CNS. Adenosine is a neuromodulator, which activates different receptor subtypes, including A_{2A}R. These receptors are expressed by microglial cells and control microglia morphology and function, namely proliferation and secretion of inflammatory mediators.

A functional interaction between GCs and A_{2A}R systems was already described, using a stress model of maternal separation (Batalha et al. 2013). This study reported that GR levels decrease in hippocampal extracts of rats submitted to maternal separation and that the chronic blockade of A_{2A}Rs is able to restore these alterations (Batalha et al. 2013). We also showed, using brain extracts from rodents exposed to DEX in the intrauterine period (a model of chronic anxiety), alterations in the density of A_{2A}R and that animals exposed chronically to an A_{2A}R antagonist have alterations in the density of GR (unpublished data). These alterations are dependent on the brain region and animal age. Moreover, the density of A_{2A}R is affected by GCs, an effect dependent on the concentration and exposure time to DEX in a microglial cell line (Caetano 2014). The cellular specificity of this crosstalk was not studied and, particularly in microglia cells, the mechanisms underlying A_{2A}R-GR interactions were not explored so far.

We have previously found that prenatal exposure to immunosupressants, such as GCs, induces changes in microglia morphology in a brain region and gender-specific manner and that the pharmacological blockade of A_{2A}R normalizes GC-induced changes in microglia morphology, also in a brain region and gender-specific manner, further suggesting a crosstalk between these two systems in microglial cells.

Considering the relevance of the action of GR upon inflammatory response and the modulatory action of A_{2A}R in microglial cells, this study was focused in: i) the ability of A_{2A}R pharmacological manipulation to interfere with the cytoplasm-nucleus translocation of GR with DEX treatment; ii) the effect of DEX in the expression of inflammatory mediators by microglia cells; iii) the effect of A_{2A}R antagonist in the expression of anti-inflammatory mediators in the presence and in the absence of DEX.

2. Working Hypothesis

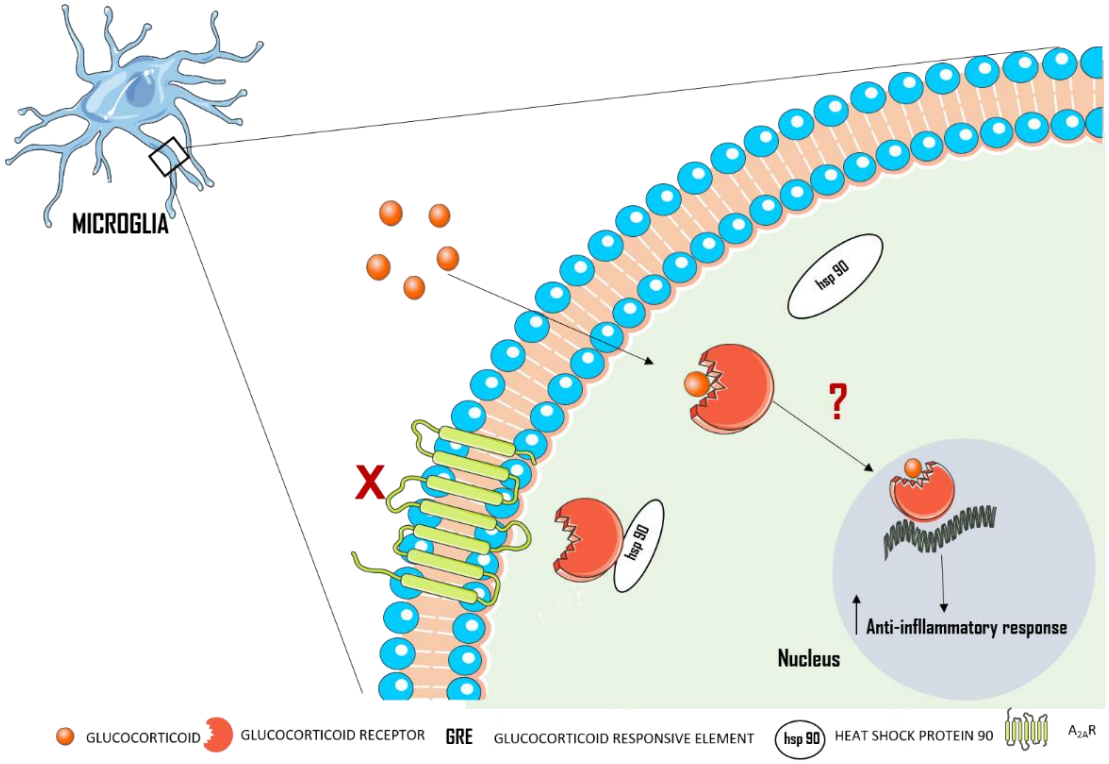

Fig. 21. Schematic representation of the possible mechanism behind the crosstalk between A_{2A}R and GR in microglial cells. GR, in their inactive state, are present in the cytoplasm. Endogenous or synthetic GCs bind to GR and GR-ligand complex suffers a conformational change, becoming active. Then, GC-GR translocate to the nucleus, where they regulate gene expression by binding to GRE and activate an anti-inflammatory response.

3. Methods

3.1 Microglia cell culture

Murine microglial cell line (BV-2) were grown in Roswell Park Memorial Institute (RPMI) supplemented with 10% heat inactivated bovine serum (FBS) and 1% penincillin-streptomycin at 37°C in a humidified incubator. Cells were allowed to grow up to 80-90% confluency and plated on 24 well plates at a density of 2 x 10^4 cells/well for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and on 6 well plates at a density of 6 x 10^4 cells/well for western blot analysis.

3.2 Microglial cells pharmacologic treatment

BV-2 cells were allowed to stabilize for 24 h after plating and before any pharmacological treatment. The cell line was first treated with 1 µM of DEX (Acros Organics, Belgium)[(concentration used in previous results from our laboratory (Caetano 2014)] for 15 min, 30 min, 45 min and 60 min of exposure to evaluate the exposure time of DEX that triggers the translocation of GR to the nucleus.

The effect of DEX was studied in the presence of the selective $A_{2A}R$ antagonist, SCH 58261 (50 nM) (Tocris, United Kingdom), added 15 min before DEX (it was previously reported that in this concentration and time, SCH 58261 selectively blocks $A_{2A}R$) (George et al. 2015; Gomes et al. 2013) (Figure 22).

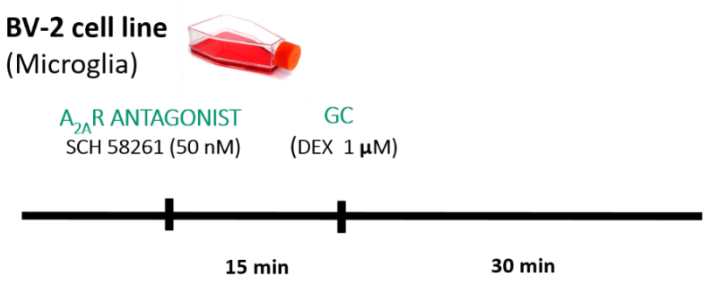

Figure 22. Schematic representation of the pharmacological treatment of BV-2 cell line with DEX and $A_{2A}R$ antagonist. BV-2 cells were pretreated with $A_{2A}R$ antagonist (SCH 58261, 50 nM for 15 min) and/or with a GR ligand (DEX, 1 µM for 30 min). Then, the cells were lysed to perform manual fractionation or RNA extraction.

3.3 Assessment of cell viability

The MTT assay was used to assess cell viability. The tetrazolium ring of MTT is cleaved by dehydrogenases present in the cytosol and in active mitochondria and precipitates as a purple formazan product. The extension of the reaction was quantified colorimetrically (absorbance at 570 nm, with a reference filter at 620 nm). Thus, higher absorbance values are related to a greater number of viable cells or the same number, but enzymatically more active.

The metabolic activity of BV-2 cells exposed to DEX (1 µM for 30 min) and SCH (50 nM for 45 min) was evaluated. Cells were incubated for 1h30 min with MTT (0.5 mg/mL) directly dissolved in the culture media. After incubation, medium was discarded and MTT formazan crystals were dissolved by addition of 1 mL isopropanol/HCl 0.04 M. Results were expressed as percentage of control.

3.4 Manual fractionation protocol

Cells were washed with RPMI medium and scraped into ice-cold buffer A [composed by: 10 nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH7.9; 10 mM KCl, 0.1 mM EDTA supplemented with protease inhibitors and 1 mM DTT; 0,4% nonyl phenoxypolyethoxylethanol (NP-40)]. The mixture was incubated for 30 min on ice and then centrifuged at 16 100 x g for 5min at 4^{o}C. The supernatant was collected (cytoplasmic fraction) and the pellet was vigorously resuspended with a solution of 20 nM of HEPES, pH 7.9, 420 mM NaCl, 1 mM of EDTA, 1mM of DTT and 10% glycerol. This mixture was incubated for 60 min on ice and centrifuged at 16 100 x g for 5 min at 4^{o}C; the supernatant was collected (nuclear fraction). Both extracts were stored at -80^{o}C.

3.5 Western blotting

Protein concentration was measured by the BCA assay and denaturized, as described in Chapter 2.2. Equal amounts (25 µg) of protein were resolved on 10% SDS-polyacrylamide gels [10% bisacrylamide, trizma- HCL (1.5 M, pH 8.8), 10% SDS, 10% APS, 1% TEMED] with a 4% stacking gel. The separation of proteins occurred in a bicine-buffered solution. Samples were stacked by running at 60 V for 15 min and resolved at 140 V for 1 h. Proteins were transferred from the gel into the polyvinylidene difluoride membrane at 750 mA current for about 1h30 min, using a CAPS solution buffered solution with methanol. Membranes were blocked with a 5% milk solution in TBS-T for 1 h at RT and incubated overnight at 4^{o}C with a primary antibody (Table 3) diluted in 1% milk in TBS-T. After washing (3 x 15 min in TBS-T), membranes were incubated

with secondary antibodies (Table 3) conjugated with horseradish peroxidase or to alkaline phosphate for 2 h at RT and washed with TBS-T (3 x 15 min) and bands were visualized with ECF. The fluorescence was detected on Typhoon FLA 9000 and quantification was performed using Image Quant 5.0 software. Data was expressed as percentage of control.

Table 3. Primary and secondary antibodies used in western blotting.

Antibody	Source	Company	Dilution
GR (M-20)	Rabbit	Santa Cruz Biotechnology	1:1000
GAPDH	Rabbit	Abcam	1:1000
Actin	Mouse	Sigma	1:1000
Lamin B1	Rabbit	Abcam	1:1000
Anti-rabbit	Goat	GE Healthcare	1:10 000
Anti-mouse	Goat	Biorad	1:10 000

3.5 RNA extraction

RNA was isolated from the BV-2 cell line treated with DEX, SCH 58261 and both (Figure 22) using the Trizol reagent, frozen in dry ice and kept at -80°C until processing. Briefly, the samples were defrosted and chloroform (200 µL) was added. The tubes were homogenized and kept 5 min on ice. Then, the samples were centrifuged at 12 000 x g for 15 min at 7°C to assure phase separation. Aqueous phase was collected to a new tube and RNA was precipitated with isopropanol (400 µL). Glycogen (20 µg) was added in order to facilitate the precipitation. Samples were kept at -20°C overnight and centrifuged at 12 000 x g for 30 min at 4°C. Pellets were washed twice with ethanol and RNA was ressuspended in RNA-free water. The concentration and purity of total RNA was assessed by NanoDrop spectrophotometer. A 260/280 nm absorbance ratio of 1.8 or higher was accepted for purity of total RNA. Then, the RNA samples were treated with deoxyribonuclease I to remove possible DNA contamination. Total RNA was used for cDNA synthesis.

3.6 cDNA synthesis

First-strand cDNA was synthesized using NZY First-Strand cDNA Synthesis Kit (Nzytech, Portugal), following supplier's instructions. NZYRT 2x Master Mix and NZYRT Enzyme Mix were added to each RNA sample followed by incubation for 10 min at 25°C. This was followed by a 30 min step at 50°C and the reaction was inactivated at 85°C for 5 min. The next step was the treatment with NZY RNase H (E.coli) for 20 min at 37°C to degrade the RNA template. Samples were kept to 4°C and then stored at -20°C.

To evaluate the purity of cDNA, a qualitative polymerase chain reaction (PCR) was performed for β-actin, using intron-spanning primers. Briefly, cDNA was subjected to a 35-cycle PCR amplification using a MyTaq Red MIX (Bioline) and forward and reverse primers (Table 4). The electrophoresis was performed using 1.5% agarose gel containing 0.005% ethidium bromide and the samples were visualized in a transilluminator (Versadoc, Biorad). After, the cDNA was diluted 1:2 in TrisEDTA (TE) buffer and stored at -20°C.

3.7 Real Time -qPCR

Real time -qPCR was performed to evaluate protein expression of pro- and anti-inflammatory cytokines, using the iTaq Supermix (Bio-Rad) and the following primers (Table 4).

Table 4. Primers used for detect mRNA expression of different pro- and anti- inflammatory cytokines.

Target	Forward	Reverse
TNF (MM_013693)	5′ - CCCAATCTGTGTCCTTCT - 3′	3′ - TTCTGAGCATCGTAGTTGT - 5′
IL-1β (MM_008361)	5′ - ATAGAAGTCAAGACCAAAGTG - 3′	3′ - GACCATTGCTGTTTCCTAG - 5′
IL-10 (MM_010548)	5′ – CTTTGCATGGTGTCCTTTCA -3′	3′- TCTCCCTGGTTTCTCTTCC – 5′
GAPDH (MM_008084)	5′ – CGACTTCAACAGCAACTC – 3′	3′ – TGTAGCCGTATTCATTGTCATA – 5′
β-actin (MM_031144)	5′ – GCTCCTCCTGAGCGCAAG – 3′	3′- CATCTGCTGGAAGGTGGACA – 5′

A mix solution was prepared (7 µL H_2O autoclaved, 10 µL Syber Green, 0.4 µL of mix primers (200 nM) (presented on Table 4) to a final volume of 18 µL/sample, taking into account the number of cDNA samples and control. After pipetting 18 µL of the mix reaction into PCR plate, 2 µL of 1:2 diluted cDNA sample was added, and the real-time qPCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, USA).

3.8 Data analysis

Statistical analysis was conducted using GraphPad Prism 5. The differences between groups were analyzed by Student's t-test or ANOVA. The level of significance was set at $p<0.05$. Results are presented as mean ± SEM.

4.Results

4.1 Exposure to dexamethasone triggered the translocation of glucocorticoid receptor to the nucleus

The aim of this set of experiments was to assess the impact of blocking $A_{2A}R$ in GR translocation from the cytoplasm to the nucleus. In order to do that, we first need to determine the time of exposure to DEX necessary for the translocation of the receptor to the nucleus. We exposed the cells to 1 µM of DEX for different exposure times: 15, 30, 45 and 60 min, and evaluated the density of GR in cytoplasmic and nuclear fractions (Figure 23). As expected, DEX induced the translocation of GR, an effect already observed at 15 and 30 min of exposure. At exposure times superior to 30 min, GR in the nucleus were clearly reduced, an effect likely related with degradation processes, as previously described in other cell types (Dvorak et al. 2005).

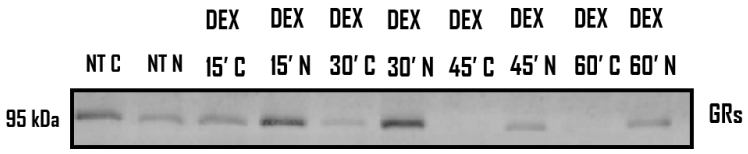

Figure 23. Effect of different exposure time to dexamethasone in the translocation of glucocorticoid receptor from the cytoplasm to the nucleus in a microglial cell line. BV-2 cells were treated with the agonist of GR (DEX, 1 µM) for several time-points: 15, 30, 45 and 60 min. Cells were lysed and subcellular fractions (cytoplasm and nucleus) were separated by manual fractionation, immunoblotted and probed for GR antibody. The time point of 30 min of exposure to 1 µM of DEX triggered the translocation of GR to the nucleus. The figure shows the representative Western blotting. (NT- cells without pharmacological treatment).

4.2 Dexamethasone and $A_{2A}R$ antagonist did not affect microglial viability

MTT assay was performed in order to clarify if DEX or the selective $A_{2A}R$ antagonist affect cell viability. BV-2 cells were treated with DEX (1 µM) for 30 min and with $A_{2A}R$ antagonist (50 nM) for 45 min. The viability was estimated by modifications in the ability of the cells to reduce MTT. As presented in Figure 24, incubation with DEX or SCH 58261 did not affect the ability of cells to reduce MTT and, consequently, cell viability (DEX: 111.0 ± 10.5; SCH: 101.3 ± 9.4, % of control).

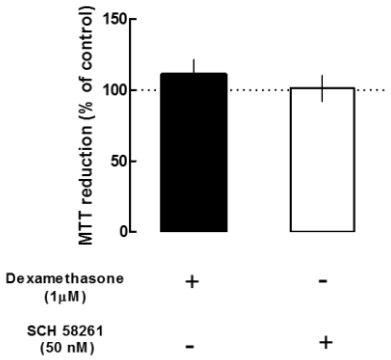

Figure 24. Effect of A_{2A}R antagonist or dexamethasone in the viability of the cells. BV-2 cells were exposed for 30 min to DEX (10 μM) or 45 min to SCH 58261 (50 nM). After incubation, the MTT assay was performed and the capacity of the cells to reduce MTT was evaluated. Dexamethasone and A_{2A}R antagonist at the concentration and exposure time used did not affect the viability of the cells. Results are presented as percentage of control (cells without pharmacological treatment). Each bar is the mean ± SEM n=3-5 per condition.

4.3 A_{2A}R blockade impaired DEX-induced glucocorticoid receptor translocation from the cytoplasm to the nucleus

To study the subcellular localization of GR, taken as an indicator of translocation, cytoplasmic and nuclear fractions were separated with a fractionation protocol, based in sequential centrifugations.

To assess the effect of A_{2A}R blockade in GR translocation process, western blot was performed and the density of GR was evaluated in the cytoplasmic and nuclear fractions. First, 30 min of exposure to DEX at 1 μM induced the translocation of GR from the cytoplasm to the nucleus, since a significant reduction of GR levels in the cytoplasm (DEX C, 59.6 ± 7.8, % of control) and an increase in the nuclear fraction (DEX N, 150.9 ± 10.5, % of control) were observed (Figure 25 A, B). This is in line with the literature (Dvorak et al. 2005; Sackey et al. 1996), although not described so far for microglial cells.

Considering the effect of A_{2A}R blockade in GR translocation after DEX treatment (DEX + SCH), it was observed that the levels of GR in the cytoplasmic fraction did not reach the expected decrease (DEX + SCH C, 76.0 ± 8.6, % of control). Moreover, blocking A_{2A}R impaired the translocation process, preventing the increase of GR levels in the nucleus (DEX + SCH N, 88.1 ± 12.3) (Figure 25 A, B).

The antagonist of $A_{2A}R$ *per se* decreased GR levels in both fractions (SCH C, cytoplasm: 55.0 ± 6.9; SCH N, nucleus: 73.7 ± 4.8), suggesting that adenosine, through $A_{2A}R$ activation, is important for the maintenance of GR levels in the cytoplasm and in the nucleus (Figure 25 A, B). These effects also suggest that $A_{2A}R$ may activate degradation processes of GR.

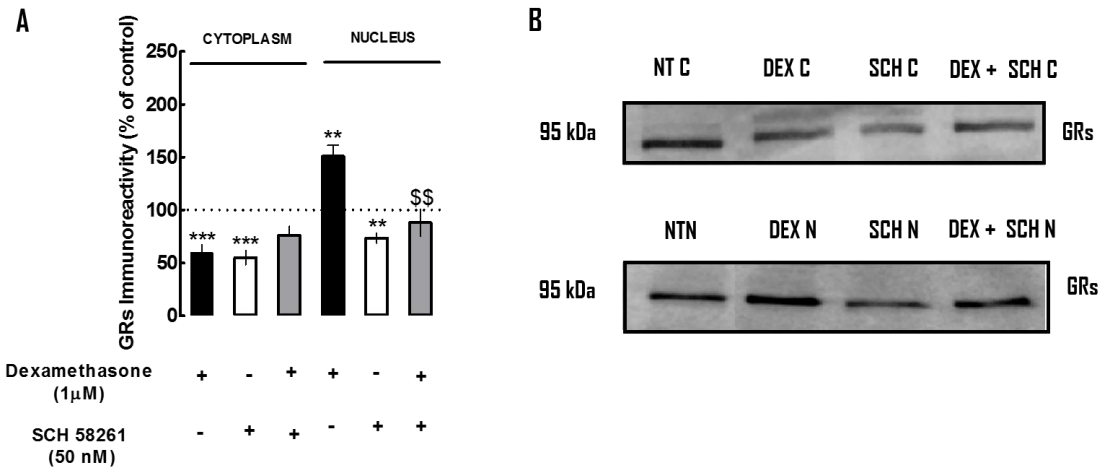

Figure 25. Influence of A_{2A} receptor blockade on GR density in microglia cells. BV-2 cells were pretreated with $A_{2A}R$ antagonist (SCH 58261, 50 nM for 15 min), and/or with agonist of GR (DEX, 1 μM for 30 min). The cells were lysed, the subcellular fractions (cytoplasm and nucleus) were separated by manual fractionation, immunoblotted and probed for GR antibody. The blockade of $A_{2A}R$ impaired the translocation of GR from the cytoplasm to the nucleus and the $A_{2A}R$ *per se* leads to decrease levels of GR in cytoplasmic and nuclear fractions. Results are presented as percentage of control (cells without pharmacological treatment). Each bar is the mean ± SEM n=8-11 per condition. (A). One-way ANOVA followed by a Turkey's multiple comparisons test with $p < 0.001$ (***), $p< 0.01$ (**), different from control and $p< 0.01$; ($$), different from DEX. Error bars are SEM. (B) Representative Western blotting (NT- cells without pharmacological treatment).

It is important to emphasize that, we were not able to find an adequate protein to use as loading control for none of the fractions (cytoplasm and nucleus). GAPDH, Lamin B1 and β-actin were tested as loading controls for cytoplasmic fractions, nuclear fractions and both, respectively, but all demonstrated to be affected by our experimental conditions (Figure 26 A, B, C). We did not find a good loading control for these experiments, so it was decided not to normalize the samples.

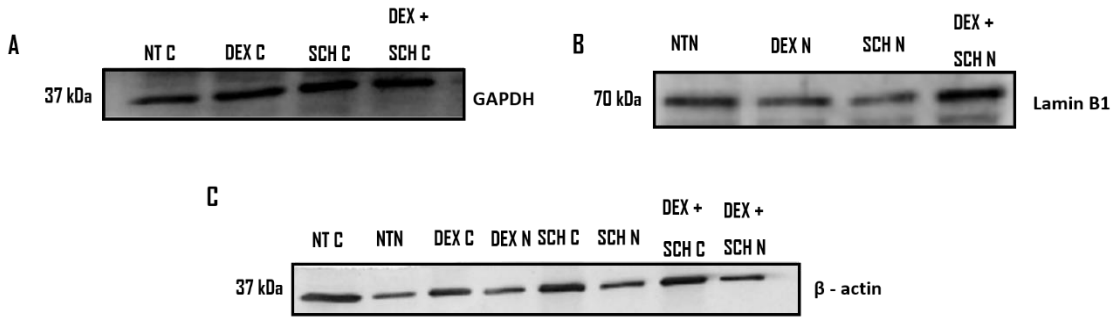

Figure 26. Evaluation of GAPDH, Lamin B1 and β-actin as loading controls for the experiments with dexamethasone and A_{2A}R antagonist in microglial cells. Representative Western Blotting of BV-2 cells pretreated with A_{2A}R antagonist (SCH 58261, 50 nM for 15 min) and/or with the agonist of GR (DEX, 1 μM for 30 min). The cells were lysed, the subcellular fractions (cytoplasm and nucleus) were separated by manual fractionation, immunoblotted and probed for GAPDH (A), Lamin B1 (B) and β-actin (C).

4.4 A_{2A}R blockade tended to control the expression of inflammatory mediators

The blockade of A_{2A}R in the brain has been described as anti-inflammatory (Rebola et al. 2011) and able to impair microglia secretion of inflammatory mediators (Dai et al. 2010). The main goal of this set of experiments was to evaluate the impact of A_{2A}R blockade upon the expression of inflammatory mediators and to test if this impact is altered in the presence of DEX. Note that DEX prevents the production of inflammatory cytokines, such as TNF and IL-1β, usually in the presence of an inflammatory stimulus, such as LPS (Carrillo-de Sauvage et al. 2013). Thus, we analyzed the immunological profile of BV-2 cells treated with DEX, SCH 58261 or both, assessing the mRNA expression of several cytokines by real time-qPCR. The cytokines analyzed were: IL-10 (anti-inflammatory cytokine) (Couper et al. 2008), TNF (Zelova and Hosek 2013) and IL-1β (Palomo et al. 2015) (pro-inflammatory cytokines), all produced and released by microglial cells (Carrillo-de Sauvage et al. 2013; Drew and Chavis 2000; Ledeboer et al. 2002).

A_{2A}R antagonist *per se* induced a slight increase of the IL-10 mRNA expression, as compared with control conditions, although it was not statistically significant (1.2 ± 0.2, fold-change of the control) (Figure 27 A). Regarding pro-inflammatory cytokines, there was a slight decrease of Il-1β and TNF mRNA expression compared to control conditions (IL-1β: 0.7 ± 0.1; TNF: 0.8 ± 0.1, fold-change of the control) (Figure 27 B, C). Although not statistically significant, these results point to a global trend as anti-inflammatory, a hypothesis that deserves further investigation.

As expected, the exposure of BV-2 microglial cells to DEX did not alter the pro- and anti-inflammatory cytokines transcript levels, both in the presence (IL-10: 0.8 ± 0.2; IL-1β: 1.0 ± 0.2; TNF: 0.8 ± 0.1, fold-change of the control) or absence (IL-10: 1.0 ± 0.1; IL-1β: 0.9 ± 0.2; TNF: 1.2 ± 0.1, fold-change of the control) of the A_{2A}R antagonist. Even though, in the presence of A_{2A}R, IL-10 and TNF expression tends to decrease, without alterations in the expression of IL-1β.

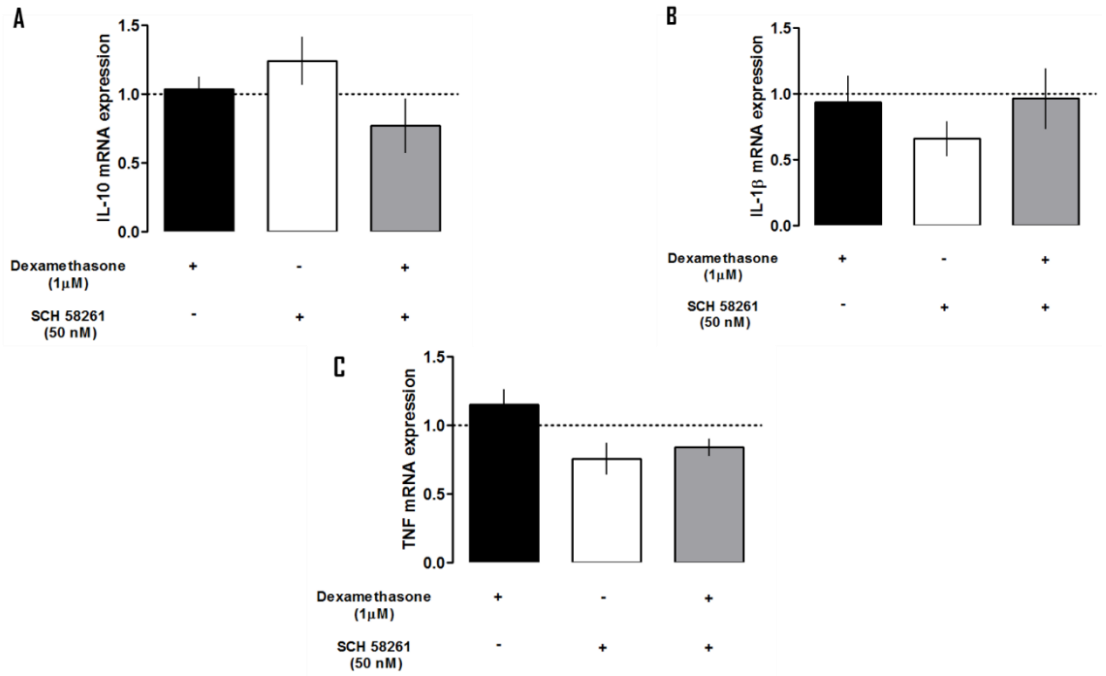

Figure 27. The effect of A_{2A}R blockade in the expression of inflammatory mediators. BV-2 cells were pretreated with A_{2A}R antagonist (SCH 58261, 50 nM for 15 min) and/or with the agonist of GR (DEX, 1 μM for 30 min). The cells were lysed with Trizol and the RNA were extracted and mRNA expression from IL-10 (A), IL-1 β (B) and TNF (C) was quantified by real time -qPCR. Results are presented as percentage of control (cells without pharmacological treatment). Each bar is the mean ± SEM n=3-5 per condition.

5. Discussion

The experiments described in this chapter were designed to evaluate the interaction between $A_{2A}R$ and GR systems in microglial cells. The main findings achieved were: i) adenosine, through $A_{2A}R$ activation, have a role in the stability of GR levels in the cytoplasm and in the nucleus; ii) the translocation of GR to the nucleus is dependent on the tonic activation of $A_{2A}R$ by endogenous adenosine; iii) $A_{2A}R$ blockade tends to increase the expression of IL-10 and to decrease the expression of TNF and IL-1β; iv) $A_{2A}R$ blockade, in the presence of DEX, tends to decrease the expression of IL-10 and TNF, without affecting IL-1β expression.

These results and previous results from our laboratory showed DEX-induced alterations in $A_{2A}R$ density in microglial cells in culture (Caetano 2014); prenatal DEX and chronic administration of $A_{2A}R$ antagonist *per se* in adulthood altered the density of $A_{2A}R$ and GR in a brain region-specific manner (unpublished data), further strengthening the crosstalk between these systems. Additionally, other authors also demonstrated that the chronic blockade of $A_{2A}R$ affects GR levels in hippocampus extracts of rats subjected to early life stress (maternal separation) (Batalha et al. 2013).

The molecular mechanisms that underlie GR intracellular pathway were well described (reviewed in (Kadmiel and Cidlowski 2013)). Indeed, GR signaling includes the activation of GRE and, consequently, the recruitment of coactivators to induce an anti-inflammatory response and the activation of nGRE that inhibits transcription factors, repressing pro-inflammatory responses. Taking this into consideration, we evaluated the impact of $A_{2A}R$ blockade on the expression of inflammatory mediators by determining the expression of pro- and anti-inflammatory cytokines. DEX treatment (1 μM for 30 min) *per se* did not have an effect when compared with control conditions. In the literature, the anti-inflammatory response of DEX (100 nM, 40 min) in microglial cells was only described in the presence of an inflammatory stimulus, LPS (Carrillo-de Sauvage et al. 2013). Therefore, the absence of an anti-inflammatory effect in DEX treatment (1 μM for 30 min) was somehow expected. We choose 30 min of incubation with 1 μM of DEX without any inflammatory stimulus to correlate with the previous conditions of western blotting experiments, where an effect of $A_{2A}R$ blockade in the translocation of GR to the nucleus was observed in these conditions. Further studies are needed to confirm if prolonged exposure to DEX, or the same time of exposure, but in the presence of an inflammatory stimulus, such as LPS, alters the expression of inflammatory mediators.

A_{2A}R antagonist *per se* tended to enhance the expression of the anti-inflammatory cytokine, IL-10, and to decrease the expression of pro-inflammatory cytokines, TNF and IL-1β, although it was not statistically significant. A_{2A}R blockade, conversely to what happens with DEX (that seems to exert anti-inflammatory effects only in the presence of inflammatory stimuli), tends to repress the expression of inflammatory and to trigger the expression of anti-inflammatory mediators, even in the absence of additional stimulus. In fact, in the literature it is described that the blockade of A_{2A}R, using a selective antagonist, also inhibit pro-inflammatory response of microglial cells, in the presence of LPS (Dai et al. 2010). Additionally, A_{2A}R activation by a selective agonist, potentiates the pro-inflammatory response in microglial cells, namely nitric oxide release, but not TNF, in the presence of LPS (Saura et al. 2005). These observations point to a neuroprotective role for A_{2A}R blockade through the control of microglia-mediated inflammation. In line with the already described physiological role of A_{2A}R in the control of microglia function and morphology, the present results suggest that tonic activation of A_{2A}R by endogenous adenosine contribute to the basal expression of inflammatory mediators, as confirmed by the alterations observed when blocking A_{2A}R.

Finally, we show that the neuromodulator adenosine, through the activation of A_{2A}R, controls the levels of GR in the cytoplasm and in the nucleus and it is also necessary for DEX-induced translocation of GR from the cytoplasm to the nucleus. Further experiments are needed in order to verify the functional impact of these effects of A_{2A}R blockade. In fact, the blockade of A_{2A}R could be:

i) inducing the degradation of GR, and consequently the decrease of GR levels in both fractions;
ii) affecting the synthesis of GR, that may justify the observed decreased levels of GR in the cytoplasm. Reduced synthesis of GR would lead to less translocation of the receptor to the nucleus and to the decrease in GR density in this subcellular fraction;
iii) affecting the levels of endogenous GC, eventually leading to less translocation of the receptor to the nucleus.

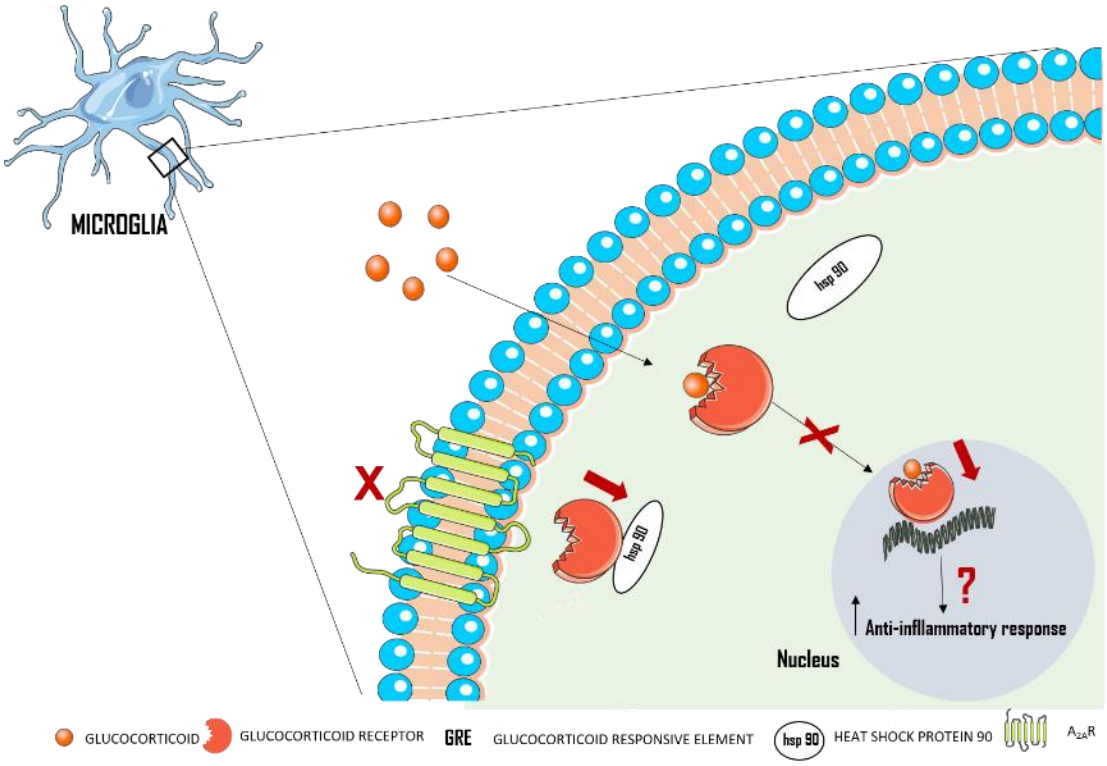

Figure 28. Schematic representation of the impact of A_{2A}R blockade in glucocorticoid receptor in microglial cells. A_{2A}R blockade affects the levels of GR in cytoplasm and nucleus, as well as the translocation process of the receptor from the cytoplasm to the nucleus. These results suggest a role of adenosine, through A_{2A}R, in the stabilization and regulation of the translocation process of the GR to the nucleus in microglial cells.

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Chapter 3

GENERAL CONCLUSIONS

1.General conclusion

The main goal of this study was to investigate the crosstalk between $A_{2A}R$ and GR systems using *in vivo* and *in vitro* approaches.

The impact of *in utero* DEX in neurons is well described (**Chapter 2.1**). It has been observed that prenatal exposure to DEX leads to alterations in neuronal migration (Fukumoto et al. 2009), dendritic arborization (Cerqueira et al. 2007; Oliveira et al. 2013; Pinto et al. 2015; Rodrigues et al. 2012; Oliveira et al. 2012), inducing also neuronal death (Gould et al. 2000). Microglia have an important role during brain development, contributing in a large extent to the maintenance of appropriate number and functionality of synapses (Cristovao et al. 2014; Lim et al. 2013; Paolicelli et al. 2011; Parkhurst et al. 2013). This fundamental role was one of the reasons why microglia was considered in the present work as a candidate for mediating the deleterious effects of prenatal exposure to DEX, once these cells are equipped with GR (Sierra et al. 2008) and responsive to immunomodulators.

The effect of DEX in microglia morphology from dHip was addressed by the tridimensional reconstruction of microglial cells performed in rats exposed to DEX during neurodevelopment (**Chapter 2.1**). Prenatal treatment with DEX significantly increases the number of microglial processes (hyper-ramification). In fact, knowing the correlation between morphology and functionality of microglial cells, these results suggest that microglia functionality can also be affected. An alteration in the function of these cells during neurodevelopment could result in brain circuits changes and consequently in neuropsychiatric conditions, as anxiety and susceptibility to depression, behavior abnormalities observed in animals exposed to DEX during brain development (Cerqueira et al. 2005; Oliveira et al. 2006).

$A_{2A}R$ are well-recognized modulators of microglia morphology and function (Gyoneva et al. 2009; Gyoneva et al. 2014; Orr et al. 2009). So far, previous results from our group show an ability of $A_{2A}R$ to regulate microglial morphology in the PFC in a gender-specific manner (unpublished data). Moreover, the results described in **Chapter 2.1** showed that the blockade of $A_{2A}R$ was able to reverse DEX-induced morphological alterations, suggesting that microglia morphology is modulated by $A_{2A}R$ in a gender and brain region-specific manner. Thus, this can be correlated with the fact that the expression of $A_{2A}R$ is variable between genders (unpublished data) and brain regions, reviewed in (Cunha 2005).

The results described in **Chapter 2.1** suggests that the functional uncoupling between anxiety and cognition in females prenatally exposed to dexamethasone and treated with a selective $A_{2A}R$ antagonist may be explained, by a differential regulation of microglia morphology by $A_{2A}R$ in different brain regions.

Moreover, if $A_{2A}R$ were important modulators of GR, we expected to observe alterations in GR levels in $A_{2A}R$ KO mice, although similar levels of GR were found in PFC of $A_{2A}R$ KO mice, compared to WT animals (**Chapter 2.2**). We also observed that $A_{2A}R$ KO mice exhibit anxious-like behavior, without alterations in short-term memory.

The results described in **Chapter 2.3** show that adenosine, through the activation of $A_{2A}R$, have an important role in the translocation of GR from the cytoplasm to the nucleus and in the stabilization of GR levels, observed by the fact that the blockade of $A_{2A}R$ induces a decrease in the levels of GR in the cytoplasm and in the nucleus.

To conclude, future work is essential for the understanding of these specific mechanisms, as microglial cells are main players during CNS neurodevelopment. Identification of different approaches capable of modulating their performance is a promising target for future therapeutic development. Our work has provided new insights into specific players ($A_{2A}R$) that could be used in the regulation of these innate immune cells.

2. Future perspectives

Microglia undergoes several changes upon prenatal exposure to GCs. This work was designed to unravel the molecular mechanisms behind microglia alterations and modulation by $A_{2A}R$. Therefore, in the future, several important questions should be addressed.

First, using the animal model described in **Chapter 2.1**, it would be interesting to describe the impact of microglia changes in neurons, namely dissecting if not only the morphology, but also the functionality of microglial cells is altered during brain development, namely synaptic pruning, formation and maturation of synapses. For that, the number of spines and their morphology must be analyzed in this animal model in early time-points of development. In order to do that, markers of synaptic pruning, such as C1qa and C3 could be an approach to observe if in early stages of development this animal model present high levels of this complement factors, suggesting an alteration in synaptic pruning by microglia, as described by Lui and co-workers (Lui et al. 2016). Another approach could be inducing the elimination of microglial cells in an organotypic cell culture, by incorporation of clodronate liposomes, which induce microglial apoptosis, without interfering with the viability of neurons or other glial cells (Markovic et al. 2005) and expose the slices to DEX, so we could eliminate the contribution of microglia in the alterations induced by DEX in neurons, namely in synaptogenesis.

Knowing that $A_{2A}R$ are modulators of microglial morphology, it will be of interest to evaluate the impact of the genetic deletion of $A_{2A}R$, using $A_{2A}R$ KO mice in microglia morphology and in synapse (number and maturation) (**Chapter 2.2)**.

The crosstalk between GR and $A_{2A}R$ must be explored using a microglial cell line. Therefore, several experiments need to be performed:

- Experiments with longer time points with DEX in the presence of SCH 58261 to clarify the impact of blocking $A_{2A}R$ in the expression of inflammatory mediators;
- The use of the agonist of $A_{2A}R$, in order to access the role and the relationship between GR function and $A_{2A}R$, not only in the inflammatory response, but also in the translocation mechanism;
- To understand if blocking $A_{2A}R$ is inducing the degradation of GR, by performing experiments with MG 132, an inhibitor of proteosome degradation (the main degradation process described for GR);

- The levels of CORT will be measured in the presence of SCH 58261, to observe if the blockade of $A_{2A}R$ induces alterations in endogenous GCs
- Measurement of mRNA expression of GR in the presence of an $A_{2A}R$ antagonist, to understand if the blockade of $A_{2A}R$ is affecting the expression of this receptor;
- Experiments with adenosine deaminase, the enzyme responsible for adenosine degradation, to eliminate the endogenous adenosine and validate if endogenous adenosine has an important role in the stabilization of GR in cytoplasm and nucleus, in order to complement the results showed in **Chapter 2.3;**
- It would also be interesting to explore if GR levels change in microglial cells (*in vitro*), in an animal model that $A_{2A}R$ are overexpressed in forebrain neurons (Coelho et al. 2014).

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Chapter 4

ANNEX

1.Reagents

Table 5. Reagents

Reagents	Supplier
Ammonium persulfate ($(NH_4)_2S_2O_8$, APS)	Sigma-Aldrich (Portugal)
Bicine ($C_6H_{13}NO_4$)	Sigma-Aldrich (Portugal)
Bisacrylamide ($C_7H_{10}N_2O_2$)	Bio-Rad Laboratories (USA)
Bovine serum albumin (BSA)	Sigma-Aldrich (Portugal)
Bromophenol blue ($C_{19}H_{10}Br_4O_5S$)	M&B (Mumbai)
[3-(Cyclohexylamino) - 1 - propane sulfonic acid] ($C_6H_{11}NH(CH_2)_3SO_3H$, CAPS)	Sigma-Aldrich (Portugal)
Complete miniprotease inhibitor cocktail tablets	Roche (Germany)
Deoxycholic acid ($C_{24}H_{40}O_4$, DOC)	Sigma-Aldrich (Portugal)
Dithiothreitol ($C_4H_{10}O_2S_2$, DTT)	Sigma-Aldrich (Portugal)
di-Sodium Hydrogen Phosphate 7-hydrate PA-ACS ($Na_2HPO_4.7H_2O$)	Sigma-Aldrich (Portugal)
Deoxyribonuclease I (Dnase I)	Life Techonologies (USA)
Ethylenediamine tetraacetic acid (EDTA)	Sigma-Aldrich (Portugal)
Ethylene glycol tetraacetic acid (EGTA)	Sigma-Aldrich (Portugal)
Fetal Bovine Serum (FBS)	Invitrogen (Spain)
Glucose ($C_6H_{12}O_6$)	Sigma-Aldrich (Portugal)
Glycerol ($C_3H_8O_3$)	Sigma-Aldrich (Portugal)
Glycogen ($C_{24}H_{42}O_{21}$)	Invitrogen (Spain)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ($C_8H_{18}N_2O_4S$, HEPES)	Sigma-Aldrich (Portugal)
Methanol (CH_3OH)	Sigma-Aldrich (Portugal)
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ($C_{18}H_{18}BrN_5S$, MTT)	Sigma-Aldrich (Portugal)
Nonyl phenoxypolyethoxylethanol (NP-40)	Sigma-Aldrich (Portugal)
Paraformaldehyde (PFA)	Sigma-Aldrich (Portugal)
penicillin-streptomycin	Sigma-Aldrich (Portugal)
Potassium chloride (KCl)	Merck (Germany)
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich (Portugal)
ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI)	Invitrogen (Spain)
Roswell Park Memorial Institute (RPMI)-1640 medium (R1383)	Sigma-Aldrich (Portugal)
Sodium Bicarbonate ($NaHCO_3$)	Sigma-Aldrich (Portugal)
Sodium chloride (NaCl)	Sigma-Aldrich (Portugal)
Sodium dodecyl sulfate ($NaC_{12}H_{25}SO_4$,SDS)	Bio-Rad Laboratories (USA)
Sodium Phosphate ($NaHPO_4$)	Sigma-Aldrich (Portugal)
Tetramethylethylenediamine ($C_6H_{16}N_2$, TEMED)	Bio-Rad Laboratories (USA)
Trizma base [$NH_2C(CH_2OH)_3$]	Sigma-Aldrich (Portugal)
Trizol	Life Techonologies (USA)
Triton X-100	Sigma-Aldrich (Portugal)
Tween 20 ($C_{58}H_{114}O_{26}$)	Merck (Germany)

2.Supplementary data

Table 6. Summary of the morphometric analysis (number of ramifications) of microglia from the results described in Chapter 2.1, 4.2. *Differences between saline animals; § Differences between DEX animals

Order	Saline (n=4)	DEX (n=3)	SCH (n=4)	DEX + SCH (n=4)
1	6.1 ± 0.8	**7,9 ± 0.4 ***	6.6 ± 0.7	6.9 ± 0.4
2	12.1 ± 1.1	**15.3 ± 0.9 ***	12.0 ± 1.0	12.8 ± 1.3
3	16.1 ± 1.5	**20.6 ± 1.7 ***	15.0 ± 1.8	**15.9 ± 1.6 $**
4	17.2 ± 1.7	**21.9 ± 1.5 ***	15.3 ± 1.6	**17.0 ± 1.6 $**
5	16.0 ± 1.6	20.2 ± 1.7	13.8 ± 2.7	15.6 ± 2.0
6	13.0 ± 1.9	17.0 ± 1.7	13.4 ± 2.6	**12.8 ± 1.7 $**
7	10.2 ± 2.3	13.0 ± 1.8	11.1 ± 2.4	8.1 ± 0.9
8	7.2 ± 2.1	9.0 ± 1.7	9.0 ± 2.5	5.9 ± 0.6
9	5.0 ± 1.5	5.9 ± 1.4	7.3 ± 1.5	3.3 ± 0.4
10	3.0 ± 1.2	3.6 ± 0.4	6.1 ± 2.0	2.3 ± 0.5

Table 7. Summary of the morphometric analysis (length of ramifications) of microglia from the results described in Chapter 2.1, 4.2. *Differences between saline animals; § Differences between DEX animals

Order	Saline (n=4)	DEX (n=3)	SCH (n=4)	DEX + SCH (n=4)
1	33.4 ± 4.4	39.7 ± 1.2	31.9 ± 2.7	36.5 ± 2.8
2	84.4 ± 5.9	84.1 ± 7.3	69.6 ± 4.5	77.4 ± 10.1
3	**104.8 ± 4.9**	**117.4 ± 7.1**	**77.0 ± 4.9 ***	**88.1 ± 6.6 $**
4	**114.8 ± 7.8**	116.5 ± 8.3	**79.4 ± 4.0 ****	94.3 ± 2.3
5	97.0 ± 6.0	100.6 ± 12.1	70.4 ± 11.0	80.4 ± 8.8
6	75.0 ± 8.3	85.1 ± 12.9	60.5 ± 10.6	58.1 ± 6.9
7	57.4 ± 6.7	65.8 ± 11.9	50.3 ± 9.7	40.0 ± 2.5
8	41.1 ± 10.8	43.9 ± 5.4	40.1 ± 10.1	27.5 ± 2.9
9	24.0 ± 7.8	27.4 ± 6.2	31.4 ± 9.4	14.1 ± 1.9
10	16.2 ± 7.6	16.5 ± 3.8	24.5 ± 7.9	8.9 ± 2.1