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Purinergic involvement in microglial responses to immunomodulation during neurodevelopment

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Catarina Alexandra dos Reis Vale Gomes (Centro de Neurociências e Biologia Celular) e a orientação institucional do Professor Doutor Ângelo José Ribeiro Tomé (Universidade de Coimbra).

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The experimental work described in the present thesis was performed at the Center for Neuroscience and Cell Biology (CNC), University of Coimbra, in collaboration with the Institute for Biomedical Imaging and Life Sciences (IBILI), University of Coimbra and with the Life and Health Sciences Research Institute (ICVS), University of Minho.

O trabalho experimental descrito na presente tese foi realizado no Centro de Neurociências e Biologia Celular (CNC), Universidade de Coimbra, em colaboração com o Instituto Biomédico de Investigação da Luz e Imagem (IBILI), Universidade de Coimbra e com o Instituto de Investigação em Ciências da Vida e Saúde (ICVS), Universidade do Minho.

A ideia

De onde ela vem? De que matéria bruta
Vem essa luz que sobre as nebulosas
Cai de incógnitas criptas misteriosas
Como as estalactites duma gruta?!

Vem da psicogenética e alta luta
Do feixe de moléculas nervosas,
Que, em desintegrações maravilhosas,
Delibera, e depois, quer e executa!

Augusto dos Anjos

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"Aqueles que passam por nós, não vão sós, não nos deixam sós.
Deixam um pouco de si, levam um pouco de nós."
Antoine de Saint-Exupéry

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TABLE OF CONTENTS

FIGURES AND TABLES INDEX

FIGURES

1. Introduction

3. Materials and Methods

4. Results

TABLES

1. Introduction

3. Materials and Methods

4. Results

ABBREVIATIONS LIST

A_1R	Adenosine A_1 receptor
$A_{2A}R$	Adenosine A_{2A} receptor
$A_{2B}R$	Adenosine A_{2B} receptor
A_3R	Adenosine A_3 receptor
ACTH	Adrenocorticotropic hormone
ADA	Adenosine deaminase
AK	Adenosine kinase
AMP	Adenosine monophosphate
AMY	Amygdala
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
Ca^{2+}	Calcium
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CBG	Corticosterone-binding globulin
CD11b	Cluster of differentiation molecule 11b
CNS	Central nervous system
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CRH	Corticotropin-releasing hormone
CT	Chamber temperature
Cu	Copper
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
dSTR	Dorsal striatum
EDTA	Ethylenediamine tetraacetic acid
EPM	Elevated plus maze test
FBS	Fetal bovine serum

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
GD	Gestational day
G_i	Guanosine nucleotide binding protein with inhibitory function
GR	Glucocorticoid receptor
G_s	Guanosine nucleotide binding protein with stimulatory function
HIP	Hippocampus
HPA axis	Hypothalamic-pituitary-adrenal axis
Iba1	Calcium binding adaptor molecule 1
IL-1β	Interleukin 1β
iNOS	Inducible nitric oxide synthase
IR	Immunoreactivity
K^+	Potassium ion
MC	Mineralocorticoid
MR	Mineralocorticoid receptor
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAcc	Nucleus accumbens
NaCl	Sodium chloride
NaF	sodium fluoride
NGF	Nerve growth factor
NO	Nitric oxide
O_2	Oxygen
OCT	Optimum cutting temperature compound
OT	Object temperature
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PGE_2	Prostaglandin E2
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PND	Postnatal day

PRM	Primitive ramified microglia
PVDF	polyvinylidene difluoride membrane
RIPA	Radio-immunoprecipitation assay
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute culture medium
RT	Room temperature
SAH	S-adenosyl homocysteine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBS-T	Tris buffered saline solution supplemented with Tween-20
TEMED	Tetramethylethylenediamine
TNF-α	Tumor necrosis factor α

ABSTRACT

Dexamethasone (DEX) is an immunomodulator used in neonatal care to enhance fetal lung maturation in pregnancies at risk of preterm delivery. Despite of this clinical benefit for the newborn, DEX also causes unwanted effects in the central nervous system (CNS), namely behavioral changes (e.g. depression and hyper-anxious phenotype). DEX is a synthetic glucocorticoid with high affinity to glucocorticoid receptors, which are well known mediators of stress responses, becoming detrimental for the immature brain. In rodents, these behavioral changes were associated with several morphological and functional changes in neurons, such as an increase in the number of morphologically immature synapses. However, to date it was not clarified if these changes result from a direct neuronal effect or if they could be mediated by microglia. Since microglia are key player cells in neuroinflammatory processes, and DEX is an anti-inflammatory compound, it was intended to know if there was also an effect upon microglia, in particular in the morphology of these cells. Nevertheless, more than understanding, it would be desirable to prevent and/or rectify these unwanted effects in synaptic dysfunction and microglia reactivity. In this context, adenosine receptors emerged as potential pharmacological targets, namely adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$), taking into consideration their involvement in the pathophysiology of anxiety and depression, as well as in the control of microglia morphology and function.

In the present study, a microglial cell line (N9 cells) was incubated with different concentrations of DEX (0.1 µM, 1 µM and 10 µM) during 3, 6, 24 and 48 hours, and the density of A_1R, $A_{2A}R$ and glucocorticoid receptors (GR) was determined by western blot analysis of cell lysates. The same analysis was performed in brain extracts (prefrontal cortex, striatum, nucleus accumbens, hippocampus and amygdala) at postnatal day 1 and 7 in Wistar rats treated *in utero* with DEX 1 mg kg^{-1}. The short- and long-term impact of DEX upon microglia morphology was evaluated through tridimensional reconstructions of microglia Iba-1 immunoreactivity in the prefrontal cortex of *in utero* DEX-treated rats at postnatal day 1, 7 and at 3 months age (young adult females). Anxiety-like profile was evaluated in young adult Wistar females by the elevated plus maze test.

In the *in vitro* study performed in the cell line, DEX induced changes in the density of $A_{2A}R$, an effect that was dependent on the concentration and exposure time to DEX. The ex vivo analysis of brain sections from *in utero* DEX-treated animals, the density of A_1R, $A_{2A}R$ and GR was also affected in a manner dependent on the brain region and the age of the animal. The morphology of microglia was also affected by the prenatal DEX treatment, a long-term effect

that persisted until adult age. Adult animals exhibited a decrease in the number and length of microglial cell processes, although morphometric features of the cell body were not affected by DEX. Behavioral analysis confirmed that adult females treated *in utero* with DEX exhibit a hyper-anxious phenotype.

In conclusion, the present results indicate that besides neurons, antenatal DEX also affects microglia, namely in morphological features (cell processes) strictly implicated in the main function of these cells as sensors of the brain parenchyma homeostasis. These changes in microglial processes were observed immediately after birth and were not reversible, persisting up to adulthood. Importantly, both *in vitro* and *ex vivo* studies showed that DEX interferes with the adenosinergic system, an important regulator of microglia morphology and function, also involved in the pathophysiology of neuropsychiatric conditions, such as anxiety and depression.

Key words: microglia, neurodevelopment, corticosteroids, adenosine

RESUMO

A dexametasona (DEX) é um imunomodulador utilizado durante o período neonatal para promover a maturação pulmonar do feto em gravidezes de risco de parto prematuro. Apesar deste claro benefício para o recém-nascido, a DEX apresenta efeitos secundários a nível do sistema nervoso central (SNC), nomeadamente alterações comportamentais (ex. depressão e ansiedade). A DEX é um corticosteróide sintético com elevada afinidade para os recetores de glucocorticóides (GR), conhecidos mediadores de respostas relacionadas com o stress*, sendo prejudiciais para o desenvolvimento do SNC. Em roedores, estas alterações comportamentais foram associadas a alterações morfológicas e funcionais dos neurónios, como por exemplo o aumento do número de sinapses imaturas. No entanto, até à data desconhece-se se os referidos efeitos resultam de uma ação neuronial direta da DEX ou se são mediados pelas células da microglia. Como a microglia é um importante mediador de respostas inflamatórias no SNC e a DEX é um composto anti-inflamatório, pretende-se clarificar se a microglia é afetada pela DEX, nomeadamente a nível morfológico. Mais do que tentar perceber estes efeitos, seria desejável prevenir e/ou reverter quer os efeitos a nível sináptico, quer a nível da reatividade da microglia. Neste contexto, os recetores da adenosina (em particular os recetores A_1 e A_{2A}, A_1R e $A_{2A}R$) têm potencial como alvos farmacológicos, uma vez que são importantes reguladores da morfologia e da função da microglia e estão envolvidos na fisiopatologia da depressão e ansiedade.*

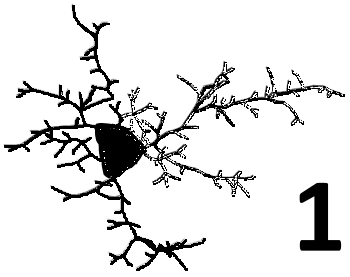
No presente estudo, uma linha celular de microglia (células N9) foi exposta a diferentes concentrações de DEX (0.1 µM, 1 µM and 10 µM) durante 3, 6, 24 e 48 horas, e a densidade dos recetores A_1R, $A_{2A}R$ e GR, foi determinada através de análise western blot *dos lisados celulares. O mesmo tipo de análise foi realizado em extratos totais de regiões isoladas do cérebro (córtex pré-frontal, estriado, núcleo accumbens, hipocampo e amígdala) em diferentes períodos de desenvolvimento (dias pós-natal 1 e 7), de ratos* Wistar *tratados* in utero *com uma dose de 1 mg kg^{-1} de DEX. O impacto da DEX na morfologia da microglia foi avaliado a curto e longo prazo através de reconstruções tridimensionais da microglia marcada com Iba-1. Esta análise foi realizada no córtex pré-frontal e em diferentes períodos de desenvolvimento (dias pós-natal 1 e 7) e na idade adulta (fêmeas Wistar com 3 meses de idade). O perfil ansiogénico nas fêmeas adultas foi testado através do teste do labirinto em cruz elevado.*

Os estudos in vitro *indicaram alterações na densidade dos recetores $A_{2A}R$ da microglia, alteração dependente da concentração de DEX utilizada e do tempo de exposição ao fármaco. No modelo animal foram detetadas alterações na densidade dos recetores em estudo após o tratamento pré-natal com DEX, sendo variáveis em função da região do cérebro e da idade do*

animal. A nível morfológico também se observaram alterações na microglia, alterações que se mantiveram na idade adulta. Aos 3 meses de idade, a microglia apresentou um menor número de processos de menor comprimento, embora a análise morfométrica do corpo celular não tenha revelado diferenças entre o grupo tratado com DEX e o grupo controlo. Os resultados da análise comportamental confirmaram um perfil ansioso nos animais tratados in utero *com DEX.*

Concluindo, os resultados apresentados indicam que, para além dos neurónios, a administração de DEX durante o neurodesenvolvimento também afeta a microglia, nomeadamente em parâmetros morfológicos (processos celulares) que suportam a principal função destas células enquanto sensores de homeostasia do parênquima cerebral. Estas alterações foram observadas imediatamente após o nascimento e persistiram até à idade adulta. Os estudos in vitro *e* ex vivo *mostraram que, paralelamente a estas alterações, a DEX afetou o sistema adenosinérgico, importante regulador da morfologia e função da microglia, também envolvido na fisiopatologia de doenças neuropsiquiátricas, nomeadamente a ansiedade e a depressão.*

Palavras-chave: *microglia, neurodesenvolvimento, corticosteróides, adenosina*



1. INTRODUCTION

On the front page:

Schematic representation of a microglial cell manually reconstructed using Neurolucida software. The tridimensional image used for the reconstruction was acquired in the prefrontal cortex region of a young adult female Wistar rat (post-natal day 90) that received a unique dose *in utero* of dexamethasone (1 mg kg^{-1}).

1. Introduction

Microglia are supporting cells of the central nervous system (CNS) with innate immunity competences. In physiological conditions, microglia exert important functions as sensors of the brain parenchyma homeostasis (Davalos *et al.,* 2005; Nimmerjahn *et al.,* 2005), in order to assess deviations from normality eventually requiring a rectifier intervention. Microglial cells regulate neuronal activity, by interacting with neuronal cellular compartments, namely the synapse (for a review see, e.g. Kettenmann *et al.,* 2013). In non-physiological conditions, microglial cells are known to respond to an insult/damage, by changing cell shape (e.g. Gyoneva *et al.,* 2014), migrating to the affected area (Duan *et al.,* 2009), phagocytosing dead or dying neurons, cell debris and extracellular components (Brown and Neher, 2014), as well as secreting inflammatory mediators (e.g. Frank *et al.,* 2007). Reestablished brain homeostasis, microglia re-acquire the original ramified morphology and survey the brain parenchyma by expanding and retracting processes (reviewed in Tremblay *et al.,* 2011). Of note, the dynamic of extension and retraction of processes is under the control of purines, in particular adenosine (Gyoneva et al., 2009; 2014; Orr et al., 2009).

1.1. Microglia

Microglia are the resident immune cells of the CNS and play an important role during neurodevelopment and in adulthood. These cells were first distinguished from other CNS cells by Ramón y Cajal, that classified microglia as the 'third element' due to the morphological differences when compared with neurons and astrocytes (Cajal, 1913). However, the term 'microglia' was first used by del Rio Hortega, a student of Ramón y Cajal, around 1920. Del Rio Hortega distinguished microglia from oligodendrocytes and characterized their response and morphology in brain lesions. Much of what we know about microglia is due to del Rio Hortega and, for this reason, he can be considered the 'father of microglia' (Del Rio Hortega, 1937; Kettenmann *et al.,* 2011).

In contrast to the other CNS cells, microglia are from a hematopoietic origin and colonize the brain during the embrionary period (Dalmau *et al.,* 1997). During brain development, which is a period of remarkable plasticity with the formation of new synapses, microglia exert important functions in the elimination of supra-numerary or unwanted synapses, but also in the formation and maturation of new synapses (Kettenmann *et al.,* 2013).

1.1.1. Microglial brain colonization and relevant morphological and physiological characteristics during neurodevelopment

a) Colonization

Microglial cells are derived from mesoderm (primary germ layer localized between ectoderm and endoderm) and take up residence in the brain during early fetal development (Kaur *et al.,* 2001). These cells share similar properties with macrophages, namely the haematopoietic origin (derived from mesoderm) and the expression of macrophage-associated markers (e.g. ionized calcium binding adaptor molecule 1 (Iba1) and cluster of differentiation molecule 11b (CD11b); Saijo and Glass, 2011).

The initial colonization of the CNS by microglia is related with the development of the vascular brain system, following a caudal-cephalic gradient (for review, see Harry and Kraft, 2012). Furthermore, there is evidence that microglia can also entry in the brain by alternative routes, such as the brain ventricles and meninges (Dalmau *et al.,* 2003). In humans, brain colonization by microglia starts between 13-24 weeks of gestation (gestation period: 36 weeks), which is more or less equivalent to mice, where it occurs around the gestational day 9.5 (GD 9.5). In rats, migration starts slightly later, around GD 15-16 (gestation period: 21 days), and microglia acquire a more ramified and differentiated phenotype (GD 18-19) earlier than in humans and mice (Harry and Kraft, 2012).

Microglial amoeboid cell precursors migrate through the developing brain, proliferate and become ramified, originating mature microglia, as present in the adult brain. Only a percentage of microglial cell precursors persist and differentiate into ramified microglia, indicating that a large number of primitive microglial cells die during the process of colonization (Chan *et al.,* 2007). Quantitative studies have shown that there is a significant expansion of the microglial cell population from PND 6 to PND 9 in the CNS, resultant from the proliferation of amoeboid microglia and primitive ramified cells. Microglial proliferation peaks coincide with the period of maximal dendritic growth, synapse formation and myelination. On the other hand, the reduction of microglia observed between PND 9 and PND 18 coincides with a period of maturation of the cytoarchitecture of most brain regions. Taking out these periods of high proliferation and subsequent decrease, microglia maintain a relatively constant density during development (**Figure 1.1.**; Dalmau *et al.,* 2003).

The colonization and maturation of microglia during development is gender-dependent; hormonal secretion in the neonatal brain interferes with microglia and there are differences in

the number and in the function of microglia between males and females during development (Schwarz *et al.,* 2012b). These differences disappear at PND 17 in all brain regions analyzed (Schwarz *et al.,* 2012a,b).

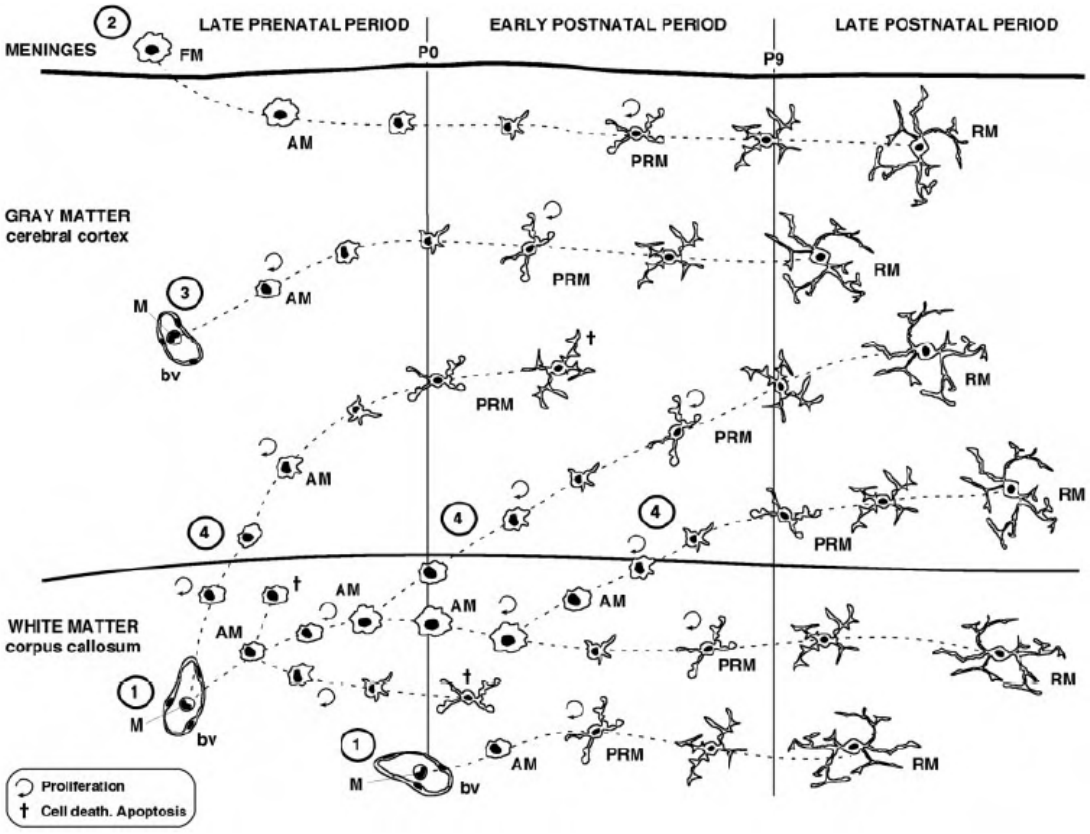


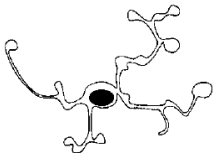
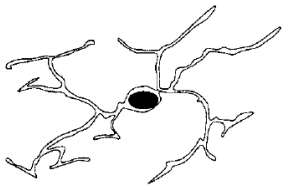
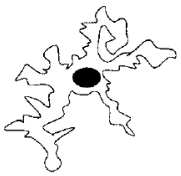
Figure 1.1| Microglial population growth in the developing brain. After colonization, primitive microglia migrate through the brain and proliferate. Therefore, not only the entrance via blood vessels, ventricles and meninges are important for the establishment of mature resident microglia, but also cell division during pre-natal and post-natal periods account for this stability. Meanwhile, many of these cells die by apoptosis. AM, amoeboid microglia; PRM, primitive ramified microglia; RM, resting microglia; bv, blood vessels; CNS, central nervous system (Dalmau *et al.,* 2003).

b) Morphology

The morphology of microglia during development is different from the adult healthy brain. Microglial cells in the developing brain are round and slightly ramified, more similar to adult amoeboid microglia, typically associated with an 'activated' profile, in which cells appear more round-shaped and with small processes. The diverse morphological phenotypes that can be

found in the developing brain, as well as the respective ages where they are present, is summarized in **Table I.i** (Dalmau *et al.,* 1998a).

Table I.i| Classification of the morphological types of microglial cells in the post-natal rat hippocampus. AM, amoeboid microglia; PND, postnatal day (Dalmau *et al*., 1998a).

TYPE OF CELL	SHAPE	CELL PROCESSES	DIAMETER	TIME COURSE OF APPEARANCE	CELL MORPHOLOGY
AM type 2	Round	None occasional filopodia	15-20 µm	PND0-PND9, scarcely at PND12	
AM type 3	Pleomorphic	Filopodia and/or pseudopodia	15-50 µm	PND0-PND9, some at PND15	
Primitive ramified microglia	Oval to slightly elongated	Scantly developed processes showing a beaded shape	50-75/80 µm	PND0-PND12, some at PND15 and rarely at PND18	
'Resting' microglia	Oval to roundish	Fully developed processes	85-100 µm	Some at PND12, PND15-PND18	
Reactive-like microglia	Large, plump, round to oval	Retracted, coarse processes	40/50-80 µm	Mainly from PND9 to PND18	

Morphological maturation of microglia starts during the period of spine formation, suggesting that these cells may be actively involved in synaptogenesis, which in turn may also influence the arrangement of microglial cells in different sub-regions (Dalmau *et al.,* 1998a). Differentiation processes are also accompanied by changes in the expression of purine-related enzymes in microglia, namely 5'Nase and PNPase (Dalmau *et al.,* 1998b).

Microglial precursor cells colonize the brain presenting a round shape, without cytoplasmatic projections with the form of little spikes. Later in development, amoeboid microglia start to acquire filopodia and pseudopodia (temporary cytoplasmatic projections),

gradually assuming the so-called primitive ramified microglia (PRM) profile, that represent the intermediate form of the differentiation process between amoeboid and ramified, mature microglia (Dalmau *et al.,* 2003).

c) Function

During development, microglia is mainly located within the neuropil layers, which are regions enriched in synaptic elements and with low number of cell bodies (e.g. neocortex and olfactory bulb) (Dalmau *et al.,* 1997). This suggests that these cells, alone or in coordination with other glial cells, namely astrocytes, may play a role in synaptogenesis. Indeed, it was recently shown that microglia, besides the known role in developmental synapse phagocytosis, also contribute to the formation/maturation of new synapses (Cristóvão *et al.*, 2014; Lim *et al.*, 2013; Parkhurst *et al.,* 2013). Studies performed by Paolicelli and collaborators (2011), have shown that microglia can engulf and eliminate synapses during normal brain development, by a process called synaptic pruning, and deficient synaptic pruning results in an excess of dendritic spines and increase of immature synapses. Synaptic pruning is a regulatory process that facilitates structural changes in neurons and synapses, and occurs during late development until sexual differentiation in humans (Iglesias *et al.,* 2004).

In summary, microglia have an important role in the support of neurons and formation/maintenance or elimination of synapses during development. Thus, any interference with microglia during neurodevelopment is prone to impact on brain functioning and health, consequences that may last throughout life.

1.2. Corticosteroids

Corticosteroids are hormones naturally produced in our body and induce a variety of cellular and organic responses (e.g. immune response, energy metabolism, behavior). However, in some situations, such as at risk of premature delivery, exogenous corticosteroids can be prescribed to promote lung maturation of the fetus. Exogenous corticosteroids negatively impact on the developing brain, and may have implications for the newborn at school age, such as higher susceptibility for depression and pathological anxiety (Yeh *et al.,* 2004).

Corticosteroids are divided in two groups: glucocorticoids (GC) and mineralocorticoids (MC), which act selectively through the activation of receptors, glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), respectively. These receptors are mainly localized at the

cytoplasm, and their activation requires the access of the hormone to the intracellular milieu. In the brain, GR are widely distributed, being more abundant in hypothalamic neurons and pituitary cells that produce melanocyte-stimulating hormone, adrenocorticotropic hormone (ACTH) and lipotropin. MR are not so spread in the brain as GR; however, they can be found in higher concentrations in the hippocampus and brain stem (De Kloet *et al.,* 1998).

The affinity for MR and GR varies according to the circulating levels of corticosteroids, being MR preferentially activated in basal conditions, while both MR and GR are activated in situations characterized by an increase corticosteroids levels, such as stress conditions.

Stress is a state that results from an adverse or demanding circumstance. In stress conditions, occurs the release of GC (in humans, cortisol) from the adrenal gland, subsequent to the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Briefly, the cascade of events begins with the release of the corticotrophic releasing hormone (CRH) in the paraventricular nuclei of the hypothalamus. This hormone acts on the anterior pituitary, stimulating the release of adrenocorticotrophic hormone (ACTH) into the circulatory system. ACTH then stimulates the biosynthesis and release of GC, namely cortisol. In order to prevent deleterious effects of chronic exposure to GC, HPA axis is protected by a negative feedback loop whereby cortisol binds to receptors in the pituitary gland and hypothalamus, as well as in the hippocampus and in the prefrontal cortex, inhibiting or turning of HPA axis response (**Figure 1.2**; Waffarn *et al.,* 2012).

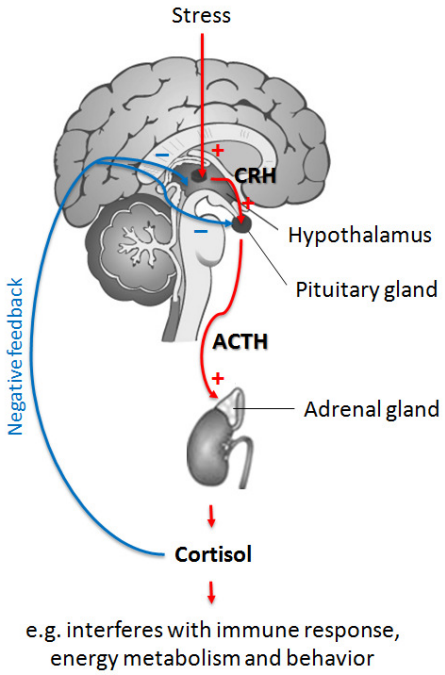
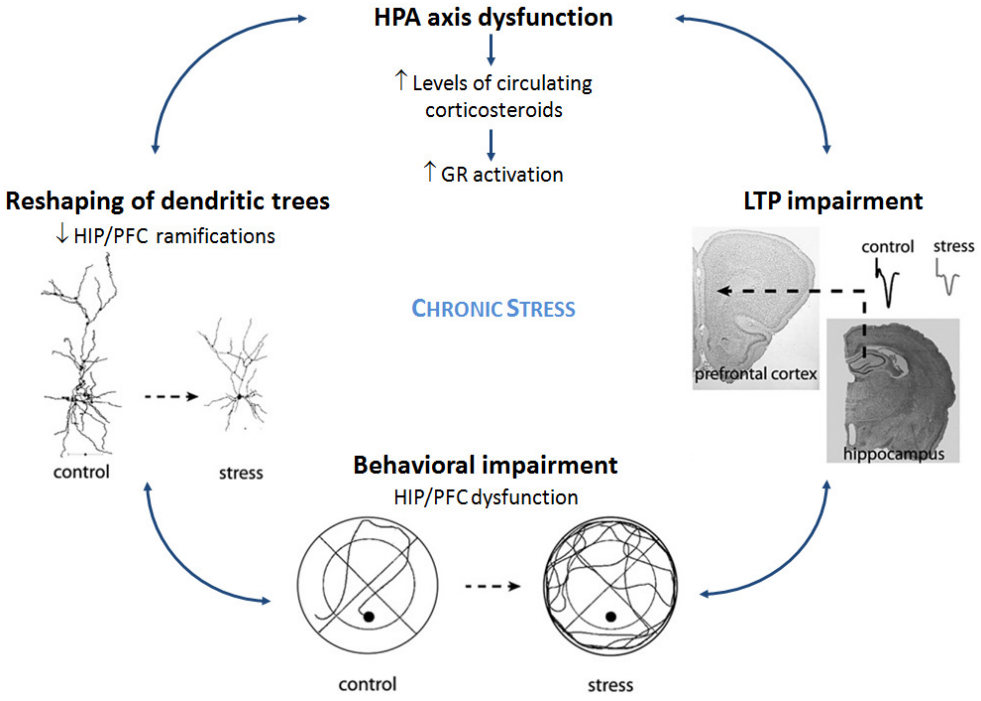


Figure 1.2| Hypothalamic–pituitary–adrenal axis. In stress conditions, the hypothalamus releases CRH, which stimulate the anterior pituitary gland to secrete ACTH. This hormone will enter in the bloodstream and induce the release of cortisol by the adrenal glands. CRH, corticotrophic releasing hormone; ACTH, adrenocorticotrophic hormone (Waffarn *et al.,* 2012).

In situations of chronic stress, there is a disruption of the HPA axis. The increase of GC leads to an increase of GR activation, with consequences for brain structure and function (McArthur *et al.,* 2005). Higher activation of GR affects the induction of long-term potentiation (LTP), impairs cognitive performance and causes atrophy of neuronal dendrites, which results in the reduction of hippocampal and prefrontal cortex (PFC) volume (**Figure 1.3.**). Hippocampus (HIP) is a critical brain region for learning and memory, while prefrontal cortex is involved in anxiety, mood, cognitive function and behavioral control (Cerqueira *et al.,* 2005; Cerqueira *et al.,* 2007; Cerqueira *et al.,* 2008). The connection between HIP and PFC occurs by pyramidal cells of the subiculum and ventral CA1 regions of the hippocampus that travel through the fimbria fornix system until the prefrontal cortex, where they establish glutamatergic contacts with pyramidal cells and interneurons. This connection has particular importance in cognition, as well as in the regulation of HPA axis (Sousa *et al.,* 2008). The activity and plasticity of these two regions, in particular, may have a role in the physiology and behavior in situations of chronic stress (Sousa *et al.,* 2008; Oliveira *et al.,* 2013). Chronic stress may progress to a more severe condition, resulting in psychiatric disorders, such as depression and pathological anxiety.



←Figure 1.3| Impact of chronic stress in the hippocampus and prefrontal cortex. Changes in GR activation will trigger cellular and molecular changes in these regions, and leading to behavioral impairment. HPA axis, hypothalamic–pituitary–adrenal axis; GR, glucocorticoid receptors; LTP, long-term potentiation; HIP, hippocampus; PFC, prefrontal cortex (Sousa *et al*., 2008).

1.2.1. Impact of the administration of exogenous corticosteroids for brain wiring and function

Exogenous corticosteroids are usually prescribed to pregnant women in late gestation at risk of preterm delivery to help pulmonary maturation of newborns, reducing the risk of morbidity and mortality by respiratory distress syndrome (Brownfoot *et al.,* 2013). One of the corticosteroids prescribed is dexamethasone (DEX; Romagnoli *et al*., 1999), a synthetic glucocorticoid with anti-inflammatory and immunosuppressant properties. Despite the benefits for the newborn in terms of respiratory function, DEX has also demonstrated unwanted side effects for the newborn. Children exposed in early stages of development, have increased susceptibility to develop cardiovascular, metabolic and auto-immune disorders, as well as neuropsychiatric abnormalities, such as depression and pathological anxiety (Yeh *et al*., 2004; Nagano *et al.,* 2008; Purdy *et al.,* 2013).

DEX has affinity for glucocorticoid receptors, rather than for mineralocorticoid receptor (Sorrels *et al.,* 2009). Most negative brain effects of DEX have been attributed to the selective activation of GR (Mesquita *et al*., 2009; Yu *et al*., 2010). Their small size and high lipophilicity allow them to cross the placenta and easily access the brain, increasing glucocorticoid levels, which impact on the developing brain (Mesquita *et al.,* 2009). The impact of these insults during development and the persistence throughout life is largely influenced by the embryonic stage where DEX is administrated, and also by the number and interval between treatments (Rice *et al.,* 2000). On the other hand, sex steroids, such as testosterone, also have an impact in the developing brain: males are more susceptible to more severe neuropsychiatric conditions in a more premature phase, while females are more likely to be diagnosed with disorders, typically later in life (Schwarz *et al.,* 2012b).

Studies using pregnant Wistar rats that received a single dose of DEX in the last third of pregnancy have shown that male progeny display an anxious phenotype and signs of impaired GC negative feedback in adulthood (Oliveira *et al.,* 2006; Mesquita *et al*., 2009). In addition, prenatal DEX treatment does not affect the litter size or the sex of the progeny that received *in utero* DEX (Oliveira *et al.,* 2006; Roque *et al.,* 2011).

At cellular and molecular levels, animals prenatally treated with DEX also have a significant reduction in the volume and number of cells in the nucleus accumbens (Nacc), a component of the mesolimbic reward circuit, including a reduction of dopaminergic enervation (Leão *et al.,* 2007; Oliveira *et al.,* 2012). It was also observed that glucocorticoids have an impact upon neuronal differentiation and migration during critical phases of neurodevelopment (Fukumoto *et al.,* 2009). In the hippocampus, the adult progeny have a significant impairment in the spatial learning and long-term potentiation (LTP) and in the volume and number of cells and synaptic

contacts. Prenatal treatment with DEX also impairs PFC (Diaz *et al.,* 2010). In addition, the HIP-PFC pathway is also affected by chronic stress exposure (Sousa *et al.,* 2008). Furthermore, it was also reported that antenatal exposure to GC reduces the expression of the serotonergic receptor 5-HT_{1A}, with impairment for the cognitive, learning and memory behaviors (Van den Hove *et al*., 2006), indicating that a variety of neurotransmitter systems and signaling cascades are affected by DEX.

1.2.2. Impact of corticosteroids upon microglia

Microglia are immunocompetent cells of the CNS, with an important function in synapse formation and/or removal, as previously stated, particularly during development. On the other hand, corticosteroids are well known for their anti-inflammatory and immunosupressive properties. However, the role of corticosteroids in inflammatory processes in the brain is not consensual; corticosteroids can increase or decrease neuroinflammation (Sorrells *et al.,* 2009; Carrillo de Sauvage *et al.,* 2013).

One of the factors that may determine the response of microglia is the origin of the corticosteroids, i.e., if they are naturally produced or synthetic. This distinction is crucial because natural and synthetic GC have different receptor binding affinities. Natural GC, such as cortisol and corticosterone, have high affinity to corticosterone-binding globulin (CBG) and only a small percentage of unbound GC cross the blood-brain barrier (BBB) and cell membranes. Once in the cytoplasm, natural GC can bind not only to the GR but also to the MR, which affinity is higher and the effects are not so severe as compared with synthetic compounds. Synthetic GC, namely DEX, do not bind to CBG and MR, and have a stronger affinity to GR. Thus, the effects of synthetic GC are much stronger than natural GC. Both activated MR and GR cross the nucleus, where they mediate changes in gene transcription. In combination, they can produce an 'inverse-U' pattern, where the effect produced is the opposite between basal and elevated GC levels (Sorrells *et al.,* 2009).

Another factor that can interfere with the inflammatory response to DEX are brain regional differences in GR and MR expression. For example, GR activation during chronic stress increases TNF-α, IL-1β and iNOS expression in the HIP and frontal cortex upon bacterial lipopolysaccharide (LPS) administration, while in the hypothalamus there is a decrease of these factors (Munhoz e*t al.,* 2006). In the particular case of frontal cortex, GR signaling seems to be essential for chronic stress, as demonstrated by the administration of GR inhibitors during chronic stress (De Pablos *et al.,* 2006).

Inflammatory responses can also be influenced by the duration of the exposure to the GC (acute, subacute or chronic), by the administered dose and by the exposure time. Finally, the nature of the inflammatory response triggered by DEX may also be affected by factors such as species, strain, gender, age, circadian rhythm, immune challenge used and the outcome measured (Sorrells *et al.,* 2009).

In stress conditions, microglia exhibit a pro-inflammatory profile, becoming 'activated', i.e., retracts their processes and acquire an amoeboid shape. They also release pro-inflammatory cytokines, such as interleukin-1β (Nair *et al.,* 2006; Frank *et al.,* 2007). Moreover, another study indicated that GC inhibit reactive oxygen species (ROS) production, as well as nitric oxide (NO) species in microglial activated by LPS (Huo *et al*., 2011). However, corticosterone exposure, a nonselective corticosteroid, after a stress condition reverses the pro-inflammatory profile of microglia (Sugama *et al.,* 2013).

1.3. The adenosinergic system

Purines, namely ATP and adenosine, are molecules that can act in the brain as neurotransmitters or neuromodulators, through the activation of purinergic receptors. Adenosine is an endogenous compound, widely present in the brain, which belongs to the purinergic family. It is an important neuromodulator that regulates neuronal functions (Dias *et al.,* 2013), as well as microglial responses (Haskó *et al.,* 2005). To date, four subtypes of adenosine receptors were identified and cloned: A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R, with distinct pharmacological and functional properties (Fredholm *et al.,* 2001; Pedata *et al.,* 2001).

The formation of adenosine in the brain occur both intracellularly, being released by bidirectional nucleoside transporters (Cunha, 2008; Latini and Pedata, 2001), as well as extracellularly, by nucleotides metabolism, through the activity of ecto-nucleotidases (Latini and Pedata, 2001). Adenosine can be degraded to adenosine monophosphate (AMP) by phosphorylation by adenosine kinase (AK), or to inosine, by adenosine deaminase (ADA). Adenosine can also be degraded through a minor pathway that corresponds to the reversible reaction catalysed by S-adenosyl homocysteine (SAH) hydrolase, originating SAH from L-homocysteine (Fredholm *et al.,* 2001).

Adenosine receptors have seven-transmembrane domains and are coupled to G-proteins (Stiles, 1992). They can be pharmacologically differentiated based on the respective signaling pathway: A_1R and A_3R are usually coupled to $G_{i/o}$ proteins, mediating the inhibition of adenylate cyclase, while $A_{2A}R$ and $A_{2B}R$ are typically coupled to $G_{s/o}$ proteins, stimulating adenylate cyclase

and increasing cyclic adenosine 5'-monophosphate (cAMP; Fredholm *et al.,* 2007; **Figure 1.4.**). The affinity of these receptors to adenosine is variable and the activation is determined by the concentration of adenosine, that varies under pathophysiological conditions and in general neuronal activity (Fredholm *et al.,* 2001). In addition, A_1R and $A_{2A}R$ are high affinity receptors; for this reason, are more relevant in physiological conditions in the brain.

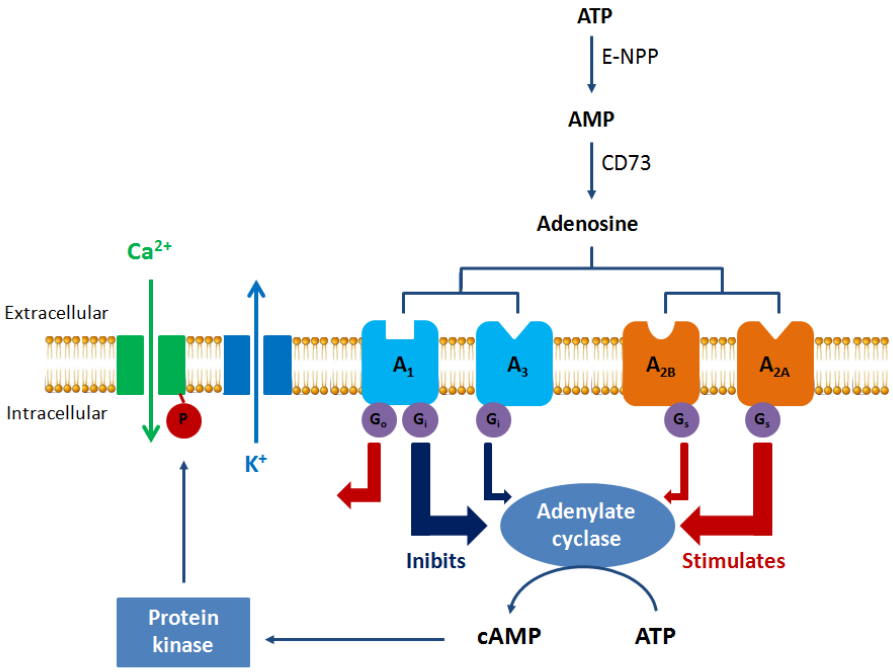


Figure 1.4| Adenosine formation and respective molecular pathways. A_1R and A_3R are coupled to G_i proteins, mainly performing an inhibitory function, while $A_{2A}R$ and $A_{2B}R$ are coupled to G_s stimulatory proteins. ATP, adenosine triphosphate; AMP, adenosine monophosphate; E-NPP, Ecto-nucleotide pyrophosphatase/ phosphodiesterase; CD73, cluster of differentiation 73 or ecto-5'-nucleotidase; A_1, adenosine A_1 receptor; A_{2A}, adenosine A_{2A} receptor; A_{2B}, adenosine A_{2B} receptor; A_3, adenosine A_3 receptor; G_i, guanosine nucleotide binding protein with inhibitory function; G_s, guanosine nucleotide binding protein with stimulatory function; cAMP, cyclic adenosine monophosphate; Ca^{2+}, calcium ion; K^+, potassium ion (adapted from Sperlágh *et al.*, 2007; Landolt *et al.*, 2012).

Among adenosine receptors, A_1R are the most abundant and widespread receptors in the adult rodent brain. They are not homogeneously distributed and their abundance depends on the brain region; it is highly expressed in the brain cortex, cerebellum, hippocampus, and dorsal horn of the spinal cord. $A_{2A}R$ are highly expressed in the striatum and olfactory bulb and less expressed in the other regions (Ribeiro *et al.,* 2003; **Figure 1.5.**). In addition to neurons, in particular nerve terminals, both A_1R and $A_{2A}R$ can also be found in other CNS cells, namely microglia and astrocytes.

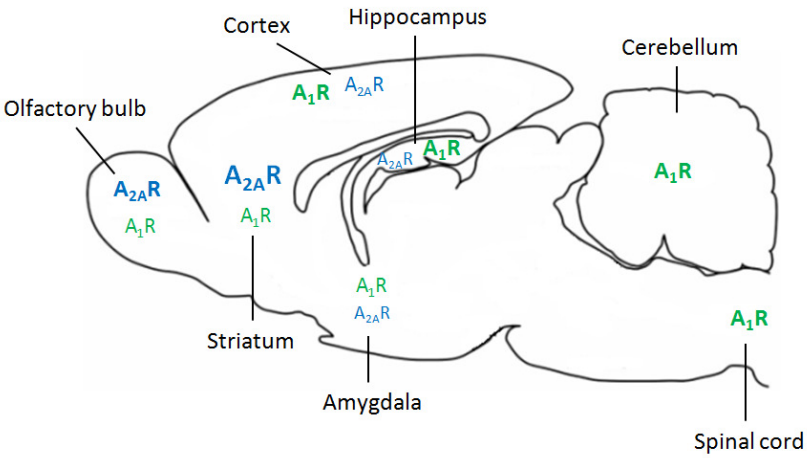


Figure 1.5| Distribution of adenosine A_1 and A_{2A} receptors in the adult rat brain (sagital view). Depending on the brain region, high levels of adenosine A_1R and $A_{2A}R$ are indicated by bigger alphabets, while low levels are indicated with smaller alphabets. A_1R, adenosine A_1 receptor; $A_{2A}R$, adenosine A_{2A} receptor (adapted from Ribeiro *et al*., 2003).

Microglia are equipped with all adenosine receptor subtypes and their functions are mainly related with the control of the innate immune response (Daré *et al.,* 2006).

$A_{2A}R$ control the synthesis and release of different inflammatory mediators: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF; Gomes *et al.,* 2013), cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2) and nitric oxide (NO; Saura *et al.,* 2005). Furthermore, A_{2A} receptors are responsible for the retraction of the microglial processes in chronic inflammation underlying pathological conditions (Orr *et al.,* 2009; Gyoneva *et al*., 2009, 2014).

Activation of $A_{2A}R$ by adenosine or by an agonist (e.g. CGS21680) regulates the transcription and *de novo* synthesis of diverse subtypes K^+ channels, via cAMP and protein kinase C (PKC) pathways, and the expression of the subtype Kv1.3 of K^+ channels. This mechanism participates in the transition of the 'resting' state to the 'active' form on microglia (Kettenmann *et al*., 2011; Saijo and Glass, 2011).

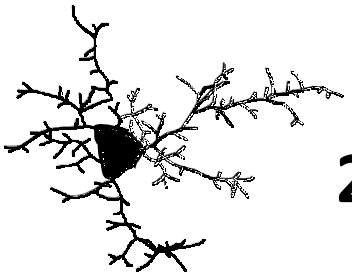
In addiction, is also known that the activation of $A_{2A}R$ can be dependent on glutamate levels, going from an anti-inflammatory to a pro-inflammatory action (Dai *et al*., 2010).

Main functions of microglial adenosine receptors are listed in **Table I.ii** (Domercq *et al.,* 2013).

Table I.ii| Function of microglial adenosine receptors. A_1R, adenosine A_1 receptor; $A_{2A}R$, adenosine A_{2A} receptor; $A_{2B}R$, adenosine A_{2B} receptor; A_3R, adenosine A_3 receptor.

RECEPTOR	FUNCTION	REFERENCES
A_1R	Anti-inflammatory properties	Haselkorn *et al*., 2010
	Prevention of neuropathic pain	Luongo *et al*., 2012
$A_{2A}R$	Process retraction	Orr *et al*., 2009
	Microglial activation	Yao *et al*., 2012
$A_{2B}R$	Anti-inflammatory properties; release of IL-10	Koscsó *et al.,* 2012
A_3R	Process extension and migration	Ohsawa *et al.,* 2012

The presence and expression of A_1R and $A_{2A}R$ in the neonatal brain was already studied. Experiments carried out by Weaver in the 1990s, have shown that A_1R and $A_{2A}R$ are expressed at GD 14, soon after the first sets of neurons complete neurogenesis (Weaver, 1993 and 1996).



2. RATIONALE AND AIMS OF THE STUDY

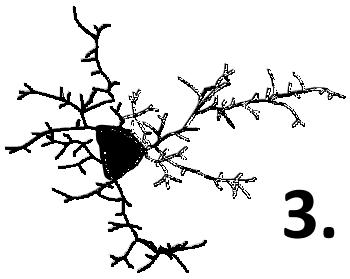
2. Rationale and aims of the study

Dexamethasone (DEX) is a synthetic GC clinically used in neonatal care to prevent respiratory distress in pregnancies at risk of preterm delivery. Despite this benefit for the newborn, DEX also causes unwanted effects in CNS, namely neuropsychiatric disorders (e.g. anxiety and higher susceptibility to depression; Roque *et al.,* 2011). The cellular and molecular mechanism by which DEX induces these neuropsychiatric abnormalities is not already known. A recent study from Rodrigues and collaborators (Rodrigues *et al.,* 2012), indicates that these neuropsychiatric phenotypes are related with structural changes in neurons, namely an increase in the number of immature synapses. Microglial cells, which are key players in CNS inflammatory events, also have the potential to interfere with synapse formation when primed by immunomodulators (Cristóvão *et al.,* 2014).

Based on these evidences, the main goal of the present study is to understand whether microglia of the progeny is affected by DEX administered during gestation, and if this could be paralleled by an anxious phenotype later in life. Considering the described crosstalk between corticosteroids and adenosine, the ability of adenosine receptors to control microglial functions and the ability of adenosine receptors modulation to interfere with neuropsychiatric disorders (Gomes *et al*. 2011), another goal of the present work is to verify if DEX alters A_1R and $A_{2A}R$ density in different regions of the brain.

To address these questions, I will use an *in vitro* model, in order to analyze the ability of DEX to selectively interfere with microglia, namely in the density of microglial adenosine and glucocorticoid receptors. After clarifying the ability of DEX to modulate microglial $A_{2A}R$, which are known to regulate microglial processes dynamic (Orr *et al.,* 2009), I will switch to an *ex vivo* model to analyze the impact of DEX upon microglia morphology, with particular focus on the morphometric analysis of cellular processes. This analysis will be performed at different ages, culminating at adulthood, where DEX-induced neuropsychiatric consequences are studied by behavioral analysis.

Although A_1R and $A_{2A}R$ mapping will be performed in different brain areas, the main focus will be given to the PFC, which is critically involved in stress, anxiety and depression.



3. EXPERIMENTAL PROCEDURES

3. Experimental Procedures

3.1. Cell cultures and pharmacological treatment

An immortalized mouse microglial cell line, N9 (a kindly gift from Professor Claudia Verderio, National Research Council, Neuroscience Institute, Cellular and Molecular Pharmacology, Milan, Italy), was used to test the impact of dexamethasone (DEX) in the corticosteroid and adenosinergic systems of microglia. This cell line was left to grow in Roswell Park Memorial Institute (RPMI) medium, pH 7.2, supplemented with 5% Fetal Bovine Serum (FBS) heat-inactivated, 1% streptomycin and penicillin (GIBCO, Porto, Portugal), 23.8 mM sodium bicarbonate buffer and 30 mM glucose (Sigma, Sintra, Portugal), and maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2) and 95% oxygen (O_2; Gomes *et al*., 2013). Once reached the adequate confluence (70-80% of the total area of the culture flask), N9 cells were detached from the culture flasks (75 cm^2, Corning, USA) by trypsinization (0.12% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS), pH 7.4) followed by a step of trypsin inactivation by the action of serum included in the culture medium. Then, the number of cells in suspension was estimated by using a hemocytometer, which required previous cell staining with the vital dye trypan blue (Sigma, Portugal). Cells were cultured in 6-well plates in the density of $2.5x10^5$ cells per well in a final volume of 1.5 mL of RPMI medium. After a 24h period of cell stabilization, different concentrations of DEX (0.1 µM, 1 µM and 10 µM; see section **3.1.5.**) were added to the culture medium for different periods (3, 6, 24 and 48 hours). Completed the incubation time, cells were lysed with radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM sodium chloride (NaCl), 1% IGEPAL (NP-40; v/v), 0.5% sodium deoxycholate (w/v), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS; w/v)) supplemented with protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg mL^{-1} CLAP, 1 mM sodium ortovanadate and 1 mM sodium fluoride (NaF), and total extracts were collected and stored at -20°C.

3.1.1. Tetrazolium viability assay

The metabolic activity of N9 cells exposed to DEX (0.1 µM, 1 µM and 10 µM) during 24h was evaluated by the quantification of the enzymatic reduction of tetrazolium salt (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Mosmann, 1983). Cells were washed with Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM MgCl2.6H2O, 1 mM $CaCl_2.2H_2O$, 6 mM D-glucose, 10 mM HEPES, 10 mM NaHCl3, pH 7.4) previously heated at 37°C, and

incubated with MTT solution (0.5 mg/mL diluted in Krebs solution; Sigma, Portugal) during 45 min at 37°C in a humidified atmosphere containing 5% CO_2 and 95% O_2. The reduction reaction resulted in the formation of purple formazan crystals, that were dissolved in 0.04 M HCl (in absolute isopropanol). Cell viability was obtained by the difference between the absorbance of viable cells that absorbed at 570 nm and non-viable cells at 620 nm.

3.1.2. Bicinchoninic acid protein assay

Total protein content present in the sample solutions was determined by the bicinchoninic acid (BCA) protein assay. Total protein concentration in the solution is inferred from the quantifiable color change from light green to purple, proportional to protein concentration and resultant from the reduction of Cu^{2+} ions to Cu^{+} and the subsequent chelatation of Cu^{+} by bicinchoninic acid (Smith *et al*., 1985), which results in the formation of the purple product that strongly absorbs at 570 nm. A standard concentration curve of bovine serum albumin (BSA; Sigma, Portugal) was prepared by serial dilutions (0 µg μL^{-1}; 0.0625 µg μL^{-1}; 0.125 µg μL^{-1}; 0.25 µg μL^{-1}; 0.5 µg μL^{-1}; 1 µg μL^{-1}; 2 µg μL^{-1}; 4 µg μL^{-1}) in milli-Q water. Samples and lysis buffer (RIPA with protease inhibitors) were also diluted (5-10x) in milli-Q water in order to be within the concentration curve. Standard concentration curve and diluted samples were applied in triplicate in a 96-well plate. Diluted lysis buffer was added to the concentration curve and milli-Q water to the samples. The plate was then incubated with the BCA reagent (A:B=50:1; Pierce, USA) at 37°C during 30 min, and the absorbance measured at 570 nm.

3.1.3. Western blotting

After determining total protein concentration, each sample solution was diluted in 1 volume of sample buffer 6x (500 mM Tris.Cl pH 6.8, 30% glycerol (v/v), 10% SDS (w/v), 600 mM dithiotreitol (DTT) and 0,024% bromophenol blue (w/v)) and in milli-Q water (volume obtained by the subtraction of the total volumes of sample and sample buffer 6x), in order to get a normalized amount of total protein among samples. After equalizing total protein, samples were denatured (heated in a digital thermoblock at 70°C (ideal temperature for heptaspan membrane receptors) during 7-8 min to enable the access of the antibody to the portion of the protein of interest (epitope). Samples were then separated according to the molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using a 10% resolving gel (H_2O milli-Q, 1.5 M Tris pH 8.8, 10% acrylamide (v/v), 1% SDS (w/v), 2%

ammonium persulfate (APS; w/v) and tetramethylethylenediamine (TEMED)) with a 4% stacking gel (milli-Q water, 0.5 M Tris pH 6.8, 4% acrylamide (v/v), 1% SDS (w/v), 2% APS (w/v) and TEMED) under reducing conditions (192 mM bicine, 25 mM Tris, 0.1% SDS, pH 8.3), at 120 V during approximately 60 min at room temperature (RT). Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Portugal) previously activated in 100% methanol (30 s), immersed in ultra-pure water to remove the excess of methanol (2 min) and in transfer buffer (10 mM CAPS pH 11 and 10% methanol (v/v) during 5 min. Electrotransference occurred at 1A during 2 hours at 4°C and moderate agitation to maintain the solution homogeneity. Membranes were blocked to prevent unspecific binding of the antibody with 5% non-fat dry milk diluted in 0.1% TBS-T (Tris buffered saline solution, 20 mM Tris, 1.5 M NaCl, pH 7.6 with 0.1% Tween-20 (v/v)) during 1 hour at RT, and incubated overnight at 4°C in the diluted primary antibody in 1% non-fat dry milk (in 0.1% TBS-T). Dilutions used for the primary antibodies are summarized in the **Table III.i**. Membranes were washed in 0.1% TBS-T (3 x 15 min), and incubated with the correspondent secondary antibody diluted in 1% non-fat dry milk (in 0.1% TBS-T; **Table III.i**) during 2 hours at RT. After a final washing step in 0.1% TBS-T (3 x 15 min), membranes were incubated with ECF, a fluorescent substrate for alkaline phosphatase-based detection (30s to 2 min; GE Healthcare, Portugal). The chemoluminescent reaction product was detected in a VersaDoc Imaging System (Bio-Rad Laboratories, Portugal) connected to Quantity One software. Membranes were always re-probed to confirm the amount of loaded protein by measuring the immunoreactivity against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; enzyme involved in the glycolytic pathway) or β-actin (cytoskeletal protein), that were not affected by the pharmacological treatment. Briefly, the membranes were first submerged in 40% methanol, during 30 min at RT with moderate agitation, to remove the ECF reaction product and, after washed in 0.1% TBS-T (3 x 10 min), antibodies were removed using stripping solution (200 mM glicine, 10% SDS (w/v), 0.1% Tween 20 (v/v), pH 2.2). Then, membranes were washed again (2 x 10 min), blocked to prevent unspecific binding and re-incubated with the respective primary and secondary antibodies, as previously described. The bands obtained in the Western blot procedure were quantified by using Image Lab 4.1 software (Bio-Rad Laboratories) and normalized to the correspondent GAPDH or β-actin protein band density.

Table III.i| Primary and secondary antibodies used for western blotting.

PROTEIN	LOADING PROTEIN (µg)	PRIMARY ANTIBODY	HOST	TYPE	DILUTION	SECONDARY ANTIBODY	HOST	TYPE	DILUTION
$A_{2A}R$	25-50	*Anti-$A_{2A}R$* Santa Cruz Biotechnology (sc-7504)	Goat	Polyclonal (R-18)	1:500	*Anti-goat* Santa Cruz Biotechnology (sc-2771)	Rabbit	IgG	1:5000
A_1R	25	*Anti-A_1R* Thermo Scientific (PA1-041A)	Rabbit	Polyclonal	1:5000	*Anti-rabbit* GE Healthcare (NIF1317)	Goat	IgG	1:20000
GR	25	*Anti-GR* Santa Cruz Biotechnology (sc-1004)	Rabbit	Polyclonal (M-20)	1:1000				
GAPDH	-	*Anti-GAPDH* Abcam (ab9485)	Rabbit	Polyclonal	1:1000				
β-actin	-	*Anti- β-actin* Sigma (A5316)	Mouse	Monoclonal (AC-74)	1:20000	*Anti-mouse* GE Healthcare (NIF1316)	Goat	Ig+IgM	1:20000

3.1.4. Immunocytochemistry

Treated cells were fixed in 4% paraformaldehyde (PFA) during 30 min and washed with PBS (3 x 10 min) at 4°C. Then, fixed cells were blocked to reduce the unspecific antibody binding and permeabilized to allow the access of the antibody to the specific epitope, in a solution with 5% BSA and 0.1% Triton X-100 during 2 hours at RT with moderate agitation. Cells were incubated with primary antibodies: ionized calcium binding adaptor molecule 1 (Iba1) antibody, a specific marker of microglia, and adenosine A_{2A} receptor ($A_{2A}R$) antibody (**Table III.ii**) diluted in the blocking solution and incubated overnight at 4°C with moderate agitation. Some slides were incubated in the absence of primary antibodies (negative controls) to confirm the specificity of the fluorescent staining. Finished the incubation time, cells were washed with PBS (3 x 10 min) and incubated with the secondary antibodies (**Table III.ii**) during 2 hours at RT. Finally, cells were washed again with PBS (3 x 10 min), nuclei were stained with the dye 4',6-diamidino-2-phenylindole (DAPI; 1:5000) during 10 min and coverslips were mounted in microscope slides with glycergel (Dako, Portugal) and left to dry overnight at 4°C.

Table III.ii| Primary and secondary antibodies used for immunocytochemistry.

Antibody	Supplier	Host	Type	Dilution
Anti-Iba1	WAKO (019-19741)	Rabbit	Polyclonal	1:1000
Anti-$A_{2A}R$	Santa Cruz Biotechnology (sc-7504)	Goat	Polyclonal (R-18)	1:200
Anti-rabbit Alexa Fluor 488	Invitrogen (A21206)	Donkey	IgG (H+L)	1:1000
Anti-goat Alexa Flour 594	Invitrogen (A11058)	Donkey	IgG (H+L)	1:1000

3.1.5. Drugs and reagents

Dexamethasone was purchased from Acros Organics, Geel, Belgium. Dexamethasone 1 mM (stock solution) was prepared in ultra-pure water, aliquoted and stored at -20°C. Different concentrations (0.1 µM, 1 µM and 10 µM) were tested *in vitro*.

Table III.iii| Drugs.

DRUG	LOT	CODE	SUPPLIER	PORTUGUESE DISTRIBUTOR
Dexamethasone, 96%	A0319607	230302500	Acros Organics, Geel, Belgium	José Manuel Gomes dos Santos, LDA

3.2. Animal handling and pharmacological treatment

Drug administration and animal care were performed in ICVS, University of Minho. Pregnant female Wistar rats (Charles-River Laboratories, Barcelona, Spain) received subcutaneous injections of dexamethasone (1 mg kg^{-1}) or saline (Sal) at days 18 or 19 of gestation. Newborns were sacrificed one day after birth, at post-natal day (PND) 1, to analyze microglial morphology and proteomic changes immediately after birth; at PND 7, an important timepoint for the ontogeny of adenosine receptors (Silva *et al.,* 2014) and at adulthood (PND 90), in order to clarify if eventual changes in microglia morphology are transient or persist at adulthood, where neuropsychiatric changes are reported (Roque *et al.,* 2011). Studies performed at 3 months of age required the separation of one group of animals at PND 21; these animals were housed according to the prenatal treatment, in groups of two to three animals per cage until behavior tasks and/or sacrifice, at PND 90. The animals were housed in an animal facility at 22°C, relative humidity of 55%, in a 12 hours light/12 hours dark cycle, with food and sterile tap water available *ad libitum.* The care and handling of the animals were in accordance with the local animal ethical committee.

3.2.1. Behavioral analysis

3.2.1.1. Elevated plus maze test

Anxiety-like behavior was accessed by the elevated plus maze (EPM) test, which is based on the higher avoidance of open spaces by anxious rodents. The reduction of the anxiety is indicated by the increase of time spent in the open arms of the maze consisting of two open arms (50.8 x 10.2 cm) and two closed arms (50.8 x 10.2 x 40.6 cm; see **Figure 3.1**) connected to each other at the center and elevated 72.4 cm from the floor (ENV-560; MedAssociates Inc, USA). Each animal was placed in the center of the maze, so that it could observe both open and closed arms and the time spent in both open and closed arms was recorded during 5 minutes using a video camera. Video analysis was blindly performed using Observador software.

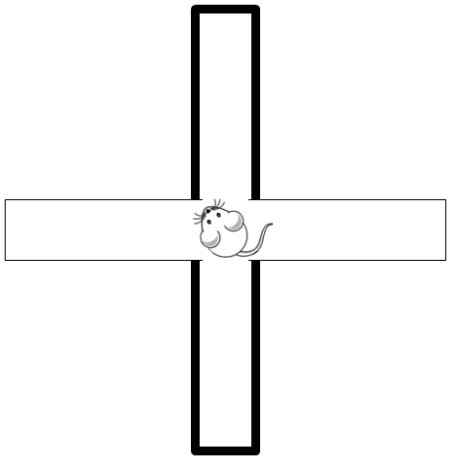


Figure 3.1| Schematic representation of the elevated plus maze test. Open arms are represented by a thin line, while closed arms by a bolder line. Wistar rats were placed in the center, where all arms are connected.

3.2.2. Brain dissection and tissue processing

3.2.2.1. Whole tissue lysates for western blotting

Animals were anesthetized with sodium pentobarbital (Eutasil, 60 mg kg-1 i.p.; Ceva Saúde Animal, Portugal) and transcardially perfused through the left ventricle with saline. Right auricle was open in order to create an open system for blood exit. Then, decapitated heads were rapidly frozen in liquid nitrogen during 5 seconds and brains were removed from the cavity. Brain regions of interest (prefrontal cortex, dorsal striatum, nucleus accumbens, hippocampus and amygdala) were isolated (a courtesy by Dr. Luísa Pinto, ICVS, University of Minho). Proteomic analysis was performed in the referred brain regions at PND1 and PND7 to have a more precise and complete characterization of the possible changes caused by DEX administration. However, further studies were performed in the prefrontal cortex, a core brain region implicated in depression and anxiety disorders (Miller *et al*., 2001).

Isolated brain areas were carefully kept in dry ice until freezing at -80ºC. For western blot analysis, total extracts were digested by adding RIPA buffer supplemented with protease inhibitors, 10 µM DTT and 5 µM PMSF, and homogenized using a tissue grinder (Size 0, Thomas Scientific, USA). Homogenates were then centrifuged at 400 x g for 10 min at 4°C, and the supernatants collected and stored at -20°C until further processing. Quantification of total extracts was performed by BCA method, as described in section **3.1.2.** and western blot analysis was performed, as described in section **3.1.3.**, with the same loading protein, as well as the respective antibodies and dilutions.

3.2.2.2. Fixation and cryosectioning for immunohistochemistry

Perfusion and fixation protocols were performed in ICVS, University of Minho. Briefly, animals were anesthetized with sodium pentobarbital and transcardially perfused with saline and 4% PFA. Brains were removed from the cavity, fixed in 4% PFA during 6 hours at 4°C and transferred to 30% sucrose (in PBS; w/v) overnight at 4°C. After fixation, brains were stored at -80°C until cryosectioning.

Brain sections were obtained using a cryostat (Leica, Germany), whose chamber temperature (CT) was at -21°C and the object (OT) at -19°C. Adult rat brains involved in optimum cutting temperature (OCT) compound (Tissue Tek, The Netherlands) were aligned according to the stereotactic coordinates of Paxinos book (1998) and neonatal rat brains also involved in OCT compound, were aligned according to Ramachandra *et al*., 2011. Coronal sections obtained from adult brains (50 µm thickness), were collected to 24-well plates previously filled with cryoprotection solution (0.1 M phosphate buffer, pH 7.2, 0.876 M sucrose, 30% ethylene glycol (v/v)), and neonatal coronal brain sections (40 µm thickness) were collected to gelatinized (Fluka, Portugal) microscope slides (Menzel-Gläser, Germany) and stored at -20°C.

3.2.3. Immunohistochemistry

Immunohistochemistry of adult brain sections was performed in free floating, while the sections of neonatal brains were handled in gelatinized microscope slides, where they have been collected (see section **3.2.2.2.**). In this particular case, a hydrophobic pen (Dako, Portugal) was used to provide a barrier and avoid the spillover of the solutions applied. The sections were washed in PBS (3 x 10 min) and then blocked and permeabilized with 5% BSA and 0.1% Triton X-100 for 2 hours at RT with mild agitation. Then, sections were incubated with the primary antibody (**Table III.iv**) diluted in blocking solution, and incubated 48 hours at 4°C with mild agitation. Negative controls remained with the same blocking solution. Sections were then washed in PBS (3 x 10 min), and incubated with the secondary antibody (**Table III.iv**) for 2 hours at RT with moderate agitation. Then, sections were washed with PBS (3 x 10 min) and incubated 10 min with DAPI (1:5000), and finally washed with PBS (3 x 5 min). Sections from adult brains were carefully mounted in gelatinized microscope slides with DAKO mounting medium (Dako, Portugal), with coverslips (Menzel-Gläser, Germany) and left to dry overnight. Regarding neonatal sections, DAKO mounting medium was used and the coverslips added; in the next day, the slides were sealed with nail polish.

Table III.iv| Primary and secondary antibodies used for immunohistochemistry.

Antibody	Supplier	Host	Type	Dilution
Anti-Iba1	WAKO (019-19741)	Rabbit	Polyclonal	1:1000
Anti-rabbit Alexa Fluor 488	Invitrogen (A21206)	Donkey	IgG (H+L)	1:1000

3.2.4. Image Acquisition

Images from microglia Iba1 immunoreactivity (IR) were acquired in the prefrontal cortex region. Ten z-stack fluorescent images were blindly acquired in the prefrontal cortex of each section using a confocal microscope (Observer.Z1, Zeiss, Germany), with LSM T-PMT camera, and connected to ZEN 2009 software (Carl Zeiss Imaging Systems). Neonatal sections were acquired using a 40x objective (EC Plan-Neofluar 40x/1.30 Oil DIC M27), because cells in this developmental stage are less complex and a higher resolution was not necessary. In the case of adult sections, it was possible to see all ramifications of microglia with a 63x objective (Plan-Apochromat 63x/1.40 Oil DIC M27) and this was more adequate and easier for the tridimensional reconstruction of microglial cells. Exposure and acquisition times were maintained between experiments. To have a general perspective of the analyzed region, some images were acquired with a 20x objective (Plan- Apochromat 20x/0.8 M27).

3.2.5. Morphometric analysis of microglia

Microglial cells were manually drawn in several planes of the same image using Neurolucida software, in order to reconstruct cells at the tridimensional level. For each neonatal section were drawn 20 microglial cells, while in adult brain sections 10 microglial cells were drawn per section. The results from the quantification of morphologic characteristics of microglial cells were obtained using Neurolucida Explorer, an extension of Neurolucida software. In this work, we focused in the perimeter, area, diameter and roundness of the cell body and in the number and length of the ramifications of microglia (for further review Beynon *et al.,* 2012; Pinto *et al.,* 2012).

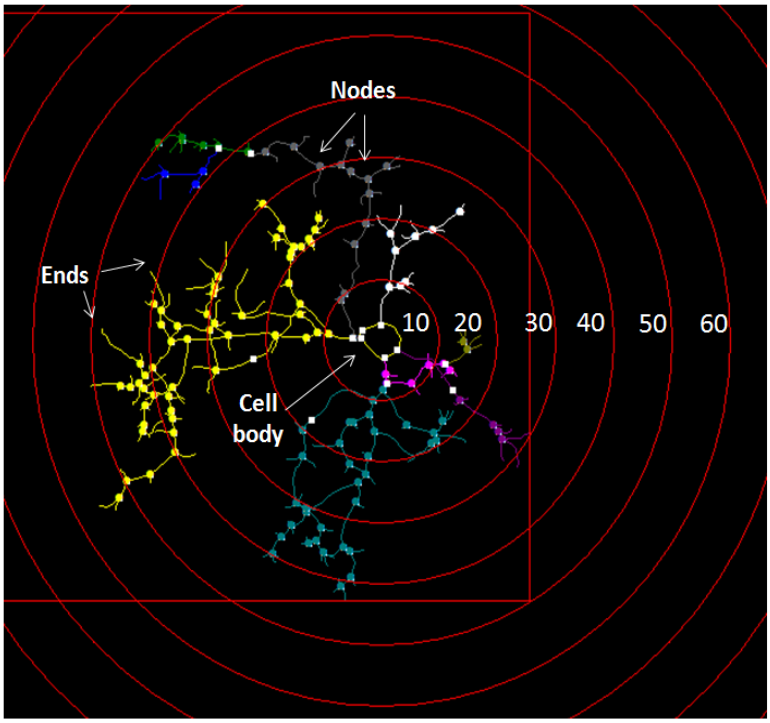


Figure 3.2| Representative scheme showing a tridimensional reconstruction of an adult microglial cell analyzed by sholl method (by radius). Localization of nodes and ends of microglia are also indicated in the figure.

3.3. Statistical analysis

Statistical analysis was performed in GraphPad Prism version 6.01 software. Quantitative data are expressed as mean ± SEM (standard error of the mean) of *n* experiments. Replicates were used for each experiment. Differences across experimental groups were obtained using Student's t test for independent means or by a one-way ANOVA followed by a Newman-Keuls post hoc test, for absolute values, which were considered significant for (*) $p<0.05$ or (**) $p<0.01$. Statistical analysis was performed in all experimental conditions with the absolute values obtained for each experiment and compared with the respective control conditions. However, graphic representations are expressed as % effect in the case of the western blot experiments.

4. RESULTS

4. Results

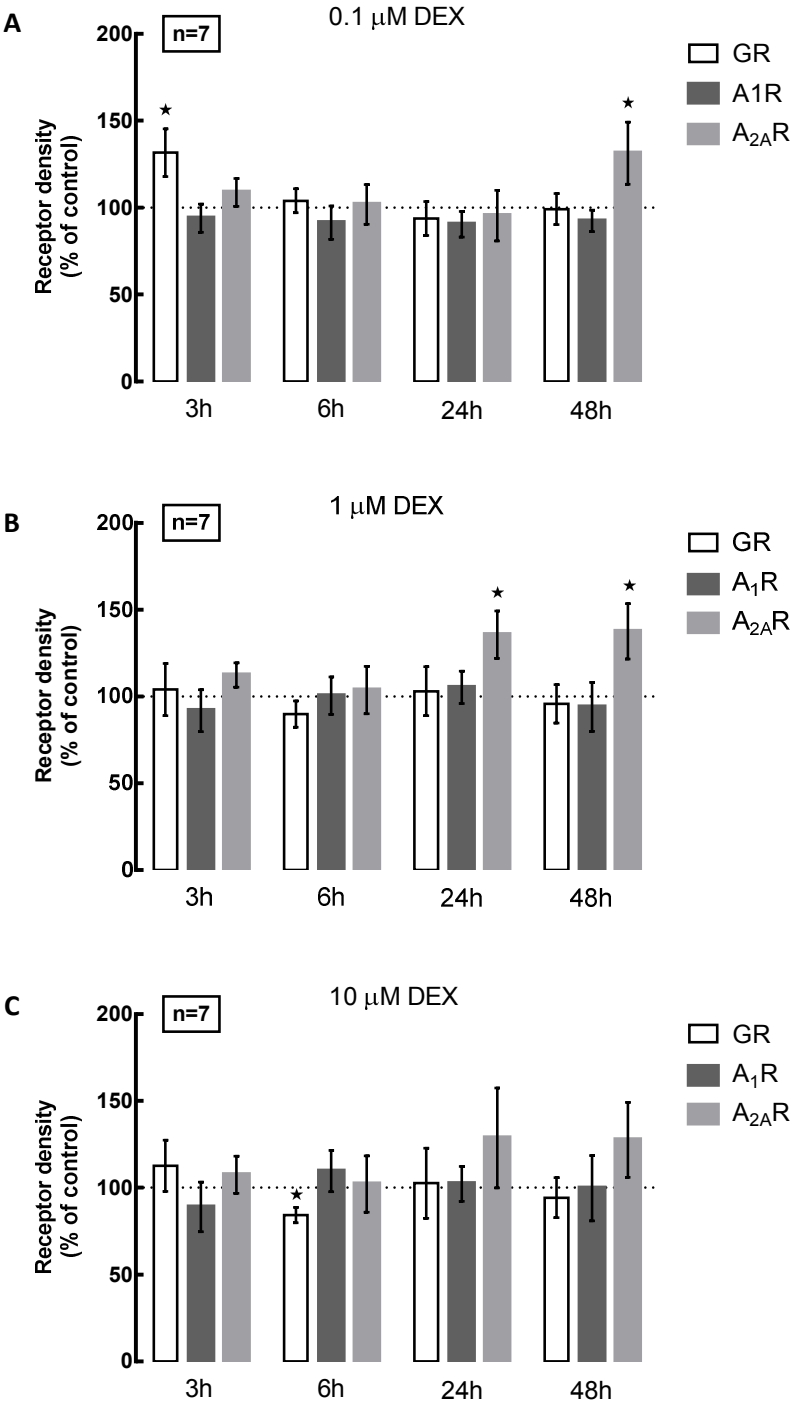
4.1. *In vitro* analysis of dexamethasone impact upon microglial adenosine receptors

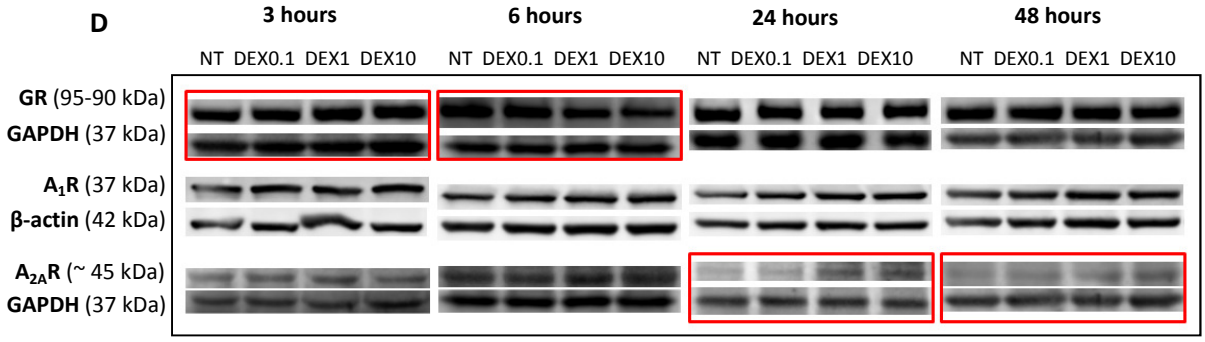
Dexamethasone is, as stated above, an anti-inflammatory, immunosuppressive drug. Before evaluating if *in utero* administration of DEX is able to change any morphological feature of microglial cells, it is important to check if the drug affects microglial cell viability. This issue is more adequately evaluated by using a cell line, without the presence and influence of other cells of the nervous system. Indeed, it is important to recall the main goal of the present work: to disentangle if DEX effect upon neuronal structure (formation of aberrant, immature synapses) and neuropsychiatric impact at adulthood could be paralleled by changes in microglial cells. The other main goal of this thesis is to quantify eventual changes in microglial adenosine receptors, under the exposure to DEX, considering that these receptors, in particular $A_{2A}R$ control the dynamics of microglial cell processes. Regarding this question, it is important to mention that the quality of the antibodies against $A_{2A}R$ is not adequate to accurately evaluate changes in the density of microglial receptors. This technical limitation led us to address the question by performing *in vitro* studies in a cell line. Although the nature of the information is different, it helps strengthening the main working hypothesis of the thesis.

4.1.1. Evaluation of the density of adenosine and corticosteroid receptors in microglial cells in the presence of dexamethasone: Effect of concentration and exposure time

The interaction between adenosine system and corticosteroids is already described (Seasholtz *et al.,* 1988). This interaction, together with the ability of both adenosine and glucocorticoids to control microglial functions, led us to test the ability of DEX to alter the density of adenosine receptors. Keeping in mind that several therapeutic regimens (doses and intervals between doses) are used clinically, it was considered of importance to analyze the impact of concentration and time of exposure upon the density of different receptors. These experiments are also important in the sense it will help defining experimental conditions to be performed in future experiments.

To address this question, N9 cells were incubated with different concentrations of DEX (0.1, 1 and 10 µM during 3, 6, 24 or 48 hours) and the density of GR, adenosine A_1R and $A_{2A}R$ was determined by western blot analysis of cell lysates.


A
0.1 μM DEX
n=7
Receptor density (% of control)
200
150
100
50
0
3h
6h
24h
48h
GR
A1R
A2AR
B
1 μM DEX
n=7
Receptor density (% of control)
GR
A1R
A2AR
C
10 μM DEX
n=7
Receptor density (% of control)
GR
A1R
A2AR


D
3 hours
6 hours
24 hours
48 hours
NT DEX0.1 DEX1 DEX10
GR (95-90 kDa)
GAPDH (37 kDa)
A1R (37 kDa)
β-actin (42 kDa)
A2AR (~ 45 kDa)
GAPDH (37 kDa)

← Figure 4.1| Glucocorticoid, adenosine A_1 and A_{2A} receptor levels in microglial cells treated with dexamethasone. N9 cells were incubated with different concentrations of DEX (0.1 µM 1 µM and 10 µM) during 3, 6, 24 and 48 hours. The density of GR, adenosine A_1R and $A_{2A}R$ was determined by western blot analysis of cell lysates. Receptor levels for the concentration 0.1 µM DEX are present in **(A)**, 1 µM DEX in **(B)** and 10 µM DEX in **(C)**. Representative images from western blot analysis are shown in **(D)** taking into account time and concentration of DEX. Results are expressed as mean ± SEM of 7 independent experiments performed in triplicate (*p<0.05, **p<0.01, compared with control conditions, Student's t test). SEM, standard error of the mean; DEX, dexamethasone; NT, non-treated; GR, glucocorticoid receptor; A_1R, adenosine A_1 receptor; $A_{2A}R$, adenosine A_{2A} receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

3 hours of exposure to the lower concentration of DEX (0.1 µM) resulted in an increase of GR density (131.6 ± 13.6%; n=7; p=0.0169, compared with non-treated cells). On the other hand, at 6 hours and for the highest concentration of DEX, a decrease in the density of GR was observed (84.27 ± 4.4%; n=7; p=0.0169, compared with non-treated cells), which was transient and not observed at 24 or 48 hours. On the other hand, at 24 hours there was an increase in the density of $A_{2A}R$ in the presence of DEX 1 µM (135.6 ± 13.6%; n=7; p=0.0343, compared with non-treated cells), that remained at 48 hours (131.3 ± 15.9%; n=7; p=0.0203, compared with non-treated cells). Concerning A_1R, no significant changes were observed in microglial cells *in vitro*; however, a tendency for a slight decrease was observed at 24 hours in the presence of the lower concentration of DEX (90.46 ± 7.4%; n=7; p=0.1789, compared with non-treated cells).

Table IV.i| Summary of the results obtained for the density of corticosteroid and adenosine receptors in function of the exposure time and concentration of DEX.

		Concentration		
		DEX 0.1 µM	**DEX 1 µM**	**DEX 10 µM**
Time	**3 hours**	↑GR	-	-
	6 hours	-	-	↓GR
	24 hours	-	↑$A_{2A}R$	-
	48 hours	↑$A_{2A}R$	↑$A_{2A}R$	-

In summary, these results indicate that DEX alters the density of corticosteroid and adenosine receptors, in a concentration- and time of exposure-dependent manner, which is particularly important considering that different therapeutic regimens with different doses and intervals between doses are clinically used.

Figure 4.2 shows a representative image of an immunocytochemistry performed in N9 cells, suggestive of an altered morphological phenotype, characterized by process retraction and increased density of $A_{2A}R$. Note that this analysis, although not quantitative, corroborates the observed increase in the density of $A_{2A}R$ in the presence of DEX and is highly suggestive of DEX-induced morphological changes, observations in line with the reported ability of $A_{2A}R$ to regulate the dynamics of microglial processes (Orr *et al.,* 2009).

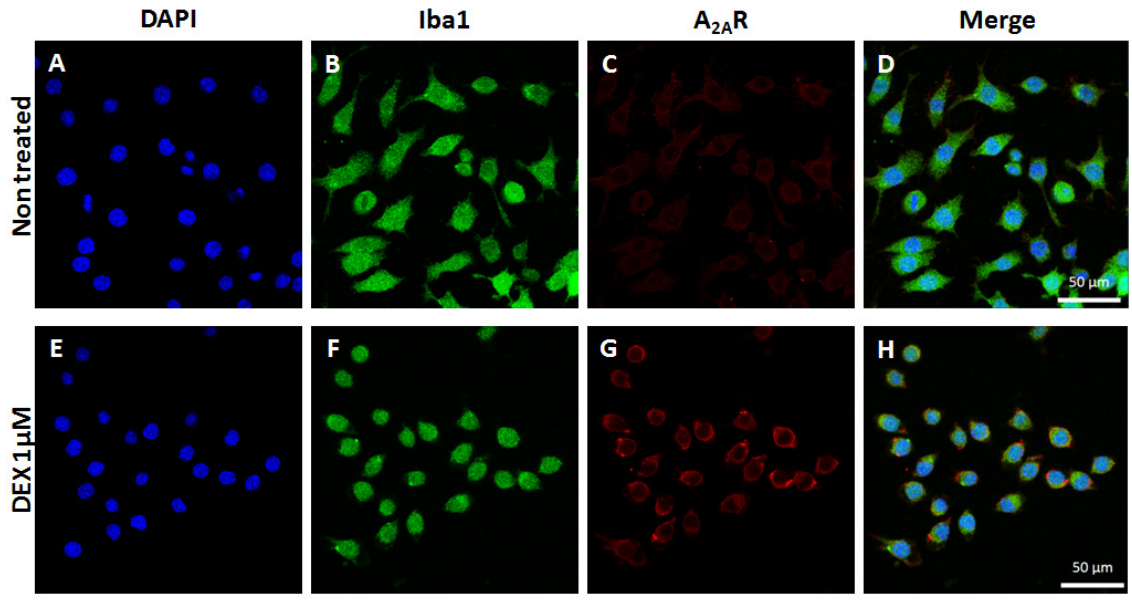


Figure 4.2| Representative image of the morphological aspect of microglia and $A_{2A}R$ labelling in the presence of DEX. Cells were incubated with DEX 1 µM for 48 hours and then fixed and stained with the microglial marker Iba1 (green) and $A_{2A}R$ (red), and with the dye DAPI (blue) for the nucleus. Images were acquired in fluorescence at 40x. **(A-D)** are represented the images for non-treated microglial cells, and in **(E-H)** the respective images for cells treated with DEX. DEX, dexamethasone; DAPI, 4',6-diamidino-2-phenylindole; Iba1, ionized calcium binding adaptor molecule 1; $A_{2A}R$, adenosine A_{2A} receptor. Scale bar: 50 µm.

4.1.2. Effect of dexamethasone upon microglial cell viability

N9 cell viability was assessed by performing the viability assay MTT, as described in the **Experimental procedures** section. N9 cells were exposed to DEX in different concentrations (0.1, 1 and 10 µM) for 24h, the time point considered of interest for further in vitro studies, considering DEX-induced changes in $A_{2A}R$ density (see section **4.1.1.**). Importantly, 24h in the presence of DEX, even at the highest concentration tested, did not decrease cell viability. **Figure 4.3** shows preliminary data indicating that DEX 0.1 µM (122.8 ± 13.5%; n=2), 1 µM (134.8 ± 14.7%; n=2) and 10 µM (140.2 ± 0.1%; n=2) does not decrease N9 viability, when compared with non-treated cells.

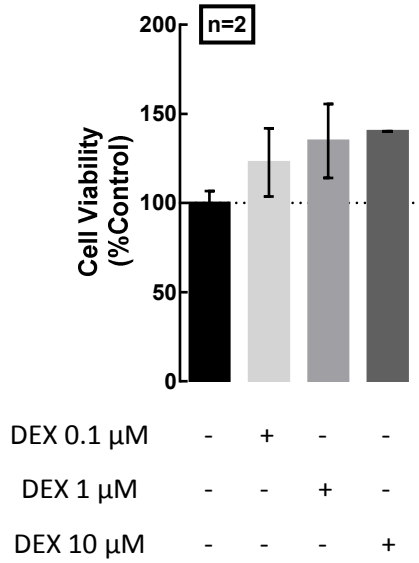


Figure 4.3| Microglial cell viability after DEX treatment, as assessed by MTT assay. N9 cells were exposed to different concentrations of DEX (0.1, 1 and 10 µM) for 24 hours and cell viability was calculated by the quantification of the metabolic reduction of tetrazolium salt (MTT). Results are expressed as mean ± SEM of 2 independent experiments performed in duplicate. SEM, standard error of the mean; DEX, dexamethasone.

4.2. *In vivo* behavioral analysis of anxiety of adult Wistar females treated with *in utero* dexamethasone

The behavioral effect of *in utero* administration of DEX, although already explored in male Wistar rats, was not studied in females. Considering the gender influence in the colonization of the brain by microglia and the sexual dimorphic susceptibility to anxiety and depression, it was considered of relevance to test if females exposed to DEX in utero also exhibit an hyper-anxious phenotype, as previously described for males (Roque *et al.,* 2011; Rodrigues *et al.,* 2012).

The anxiety-like profile in prenatal DEX treated females at PND 90, adulthood, where the neuropsychiatry changes were observed in the male progeny, was accessed by the elevated plus maze; the reduction of anxiety correlates with the increase of time spent in open arms. It was observed that prenatal DEX also induces an anxious phenotype in the female progeny at adulthood (time spent in the open arms: 0.1930 ± 0.03 sec; n=8; p=0.0140, compared with control conditions; **Figure 4.4**).

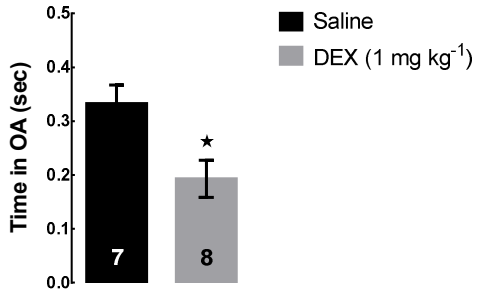


Figure 4.4|Impact of pre-natal DEX on the anxiety-like behavior of Wistar females at PND 90. Pregnant Wistar rats received 1mg kg^{-1} DEX at 18-19 gestation days and anxiety-like behavior of the female progeny was assessed by the elevated plus maze test at PND 90. Results are expressed as mean ± SEM from 7 to 8 animals per group (*p<0.05, compared with control conditions, Student's t test). SEM, standard error of the mean; DEX, dexamethasone; PND, postnatal day.

4.3. *Ex vivo* evaluation of the consequences of *in utero* administration of dexamethasone on adenosine receptors density and microglia morphological features

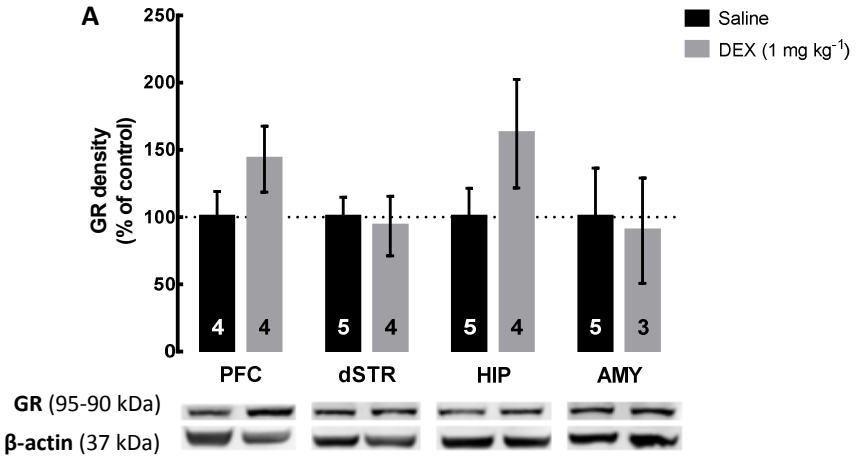
In order to clarify which regions were affected by DEX, diverse brain regions (prefrontal cortex, dorsal striatum, nucleus accumbens, hippocampus and amygdala) of animals treated *in utero* with DEX were analyzed by western blot to screen eventual changes in the density of adenosine and glucocorticoid receptors. This screening throughout the brain will be the basis for future studies; the main focus of the present thesis in terms of microglial characterization will be the prefrontal cortex, which is particularly involved in the regulation of the HPA axis and GC-induced behavioral changes. Thus, the morphometric analysis of microglia was only performed in the prefrontal cortex, aiming at characterizing short- (PND 1 and 7) and long-term (PND day 90) effects of DEX upon microglia morphology.

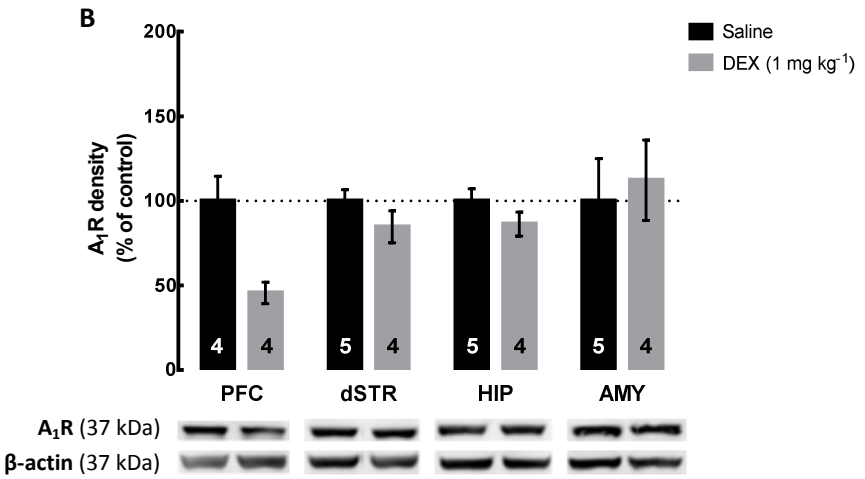
4.3.1. Impact of pre-natal dexamethasone treatment on adenosine receptors density in the brain

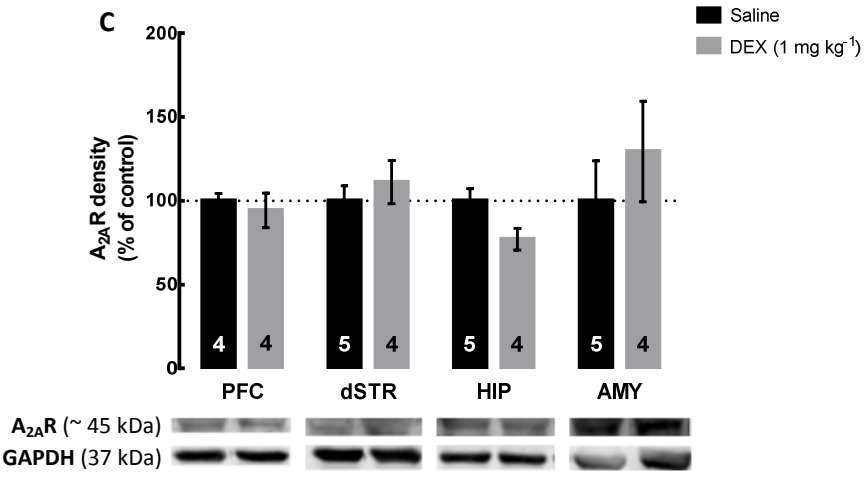
The density of adenosine A_1 and A_{2A} receptors, as well as glucocorticoids receptors was analyzed by western blot in total extracts of isolated brain regions (PFC, prefrontal cortex; dSTR, dorsal striatum; NAcc, nucleus accumbens; HIP, hippocampus; AMY, amygdala) from Wistar rats, treated *in utero* with DEX. The analysis was performed at PND 1 and 7 (limitations of time did not allow the evaluation of samples from PND90).

a) Postnatal day 1

In general, at PND 1 no significant differences in receptor density were detected. However, in PFC we could observe a trend for a decrease in the density of A_1R (45.56 ± 6.4%; n=3; p=0.0507, compared with control conditions).



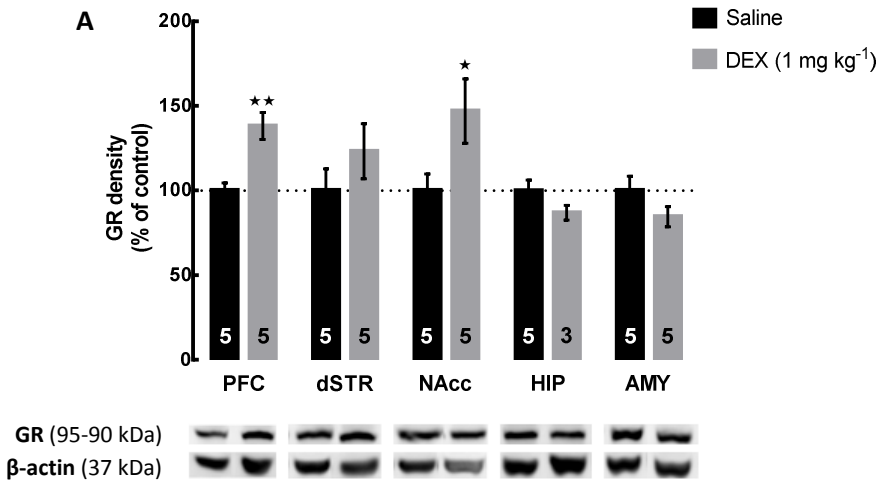




←Figure 4.5| Density of corticosteroid and adenosine receptors at PND 1. Pregnant Wistar rats received 1mg kg^{-1} DEX at 18 or 19 gestation day and the density of receptors was analyzed by western blot from total extracts obtained from the progeny at PND 1. Graphs and representative images of western blot analysis are shown in **(A)** for the density of GR, **(B)** for A_1R density and **(C)** for $A_{2A}R$ density. Results are expressed as mean ± SEM of 5 animals (*p<0.05, **p<0.01, compared with control conditions, Student's t test). SEM, standard error of the mean; DEX, dexamethasone; GR, glucocorticoid receptor; A_1R, adenosine A_1 receptor; $A_{2A}R$, adenosine A_{2A} receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

b) Postnatal day 7

PND 7 is period of important synapse formation and with the significant expansion of microglia, as reported by Dalmau and colleagues (2003). At this endpoint, we observed a significant increase of GR density in the PFC (138.2 ± 7.9%; n=5; p=0.0070, compared with control conditions) and in the nucleus accumbens (147.0 ± 19.1%; n=5; p=0.0104, compared with control conditions). These effects were paralleled by a significant decrease of A_1R density in the PFC (77.88 ± 8.7%; n=5; p=0.0133, compared with control conditions) and a significant increase in amygdala (121.7 ± 8.3; n=5; p=0.0164, compared with control conditions). Regarding $A_{2A}R$, a significant increase (145.7 ± 10.2; n=5; p=0.0322, compared with control conditions) was observed in the dorsal striatum. The density of $A_{2A}R$ in the hippocampus at PND 7 was not clear in the western blot membrane; for this reason, $A_{2A}R$ density quantification was not performed.



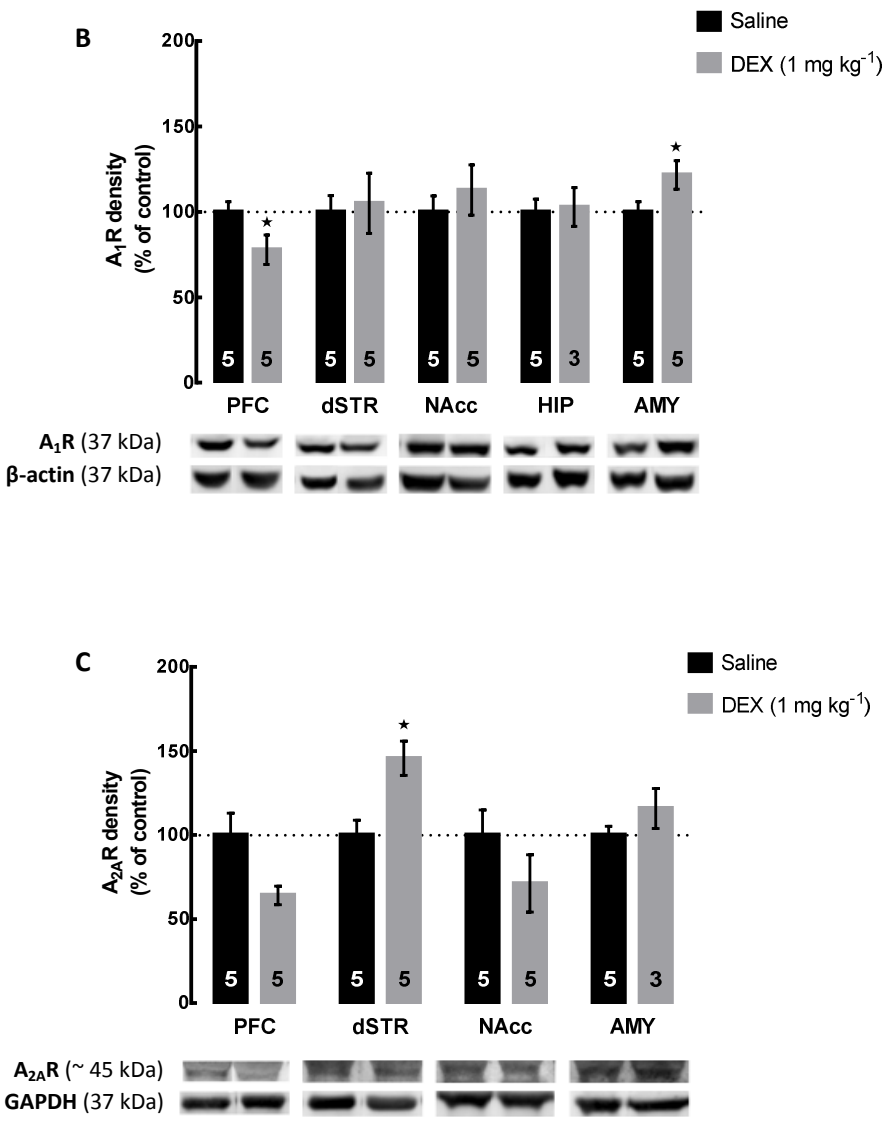


Figure 4.6| Density of corticosteroid and adenosine receptors at PND 7. Pregnant Wistar rats received 1mg kg^{-1} DEX at 18 or 19 gestation day and the density of receptors was analyzed by western blot of total extracts obtained from the progeny at PND 7. Graphs and representative images obtained from western blot analysis are shown in **(A)** for the density of GR, **(B)** for A_1R density and **(C)** for $A_{2A}R$ density. Results are expressed as mean ± SEM of 5 animals (*p<0.05, **p<0.01, compared with control conditions, Student's t test). SEM, standard error of the mean; DEX, dexamethasone; GR, glucocorticoid receptor; A_1R, adenosine A_1 receptor; $A_{2A}R$, adenosine A_{2A} receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table IV.ii summarizes the results obtained from the analysis of the density of corticosteroid and adenosine receptors in animals treated *in utero* with DEX.

Table IV.ii| Summary of changes in the density of corticosteroid and adenosine receptors with the pre-natal DEX treatment per brain region and post-natal age.

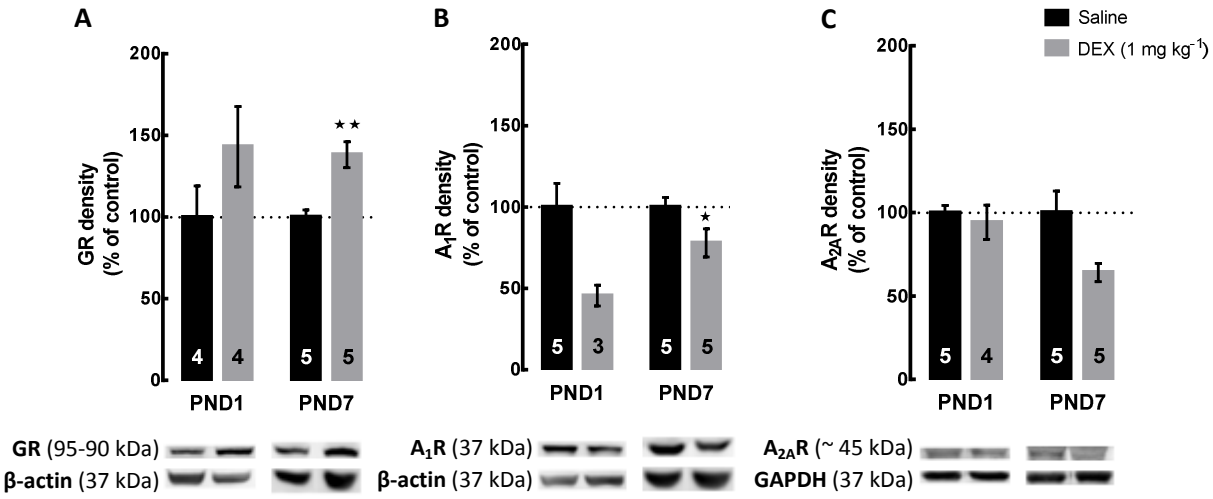
		Age	
		PND1	**PND7**
Brain region	**PFC**	-	↑GR ↓A_1R
	dSTR	-	↑A_{2A}R
	NAcc	Not analyzed	↑GR
	HIP	-	-
	AMY	-	↑A_1R

In summary, DEX affects the density of corticosteroid and adenosine receptors, an affect that is dependent of the age and the brain region of the animals treated *in utero* with dexamethasone.

4.3.2. Impact of pre-natal dexamethasone treatment on adenosine receptors density and microglial morphology in the prefrontal cortex

4.3.2.1. Adenosine receptors density in the prefrontal cortex after prenatal dexamethasone treatment

As referred above, the density of GR and A_1R was affected in the PFC, and no changes were observed for A_{2A}R in this region at PND 1 and 7. **Figure 4.7** shows a selection of data relative to PFC analysis, already presented in this thesis, for the sake of clarity.



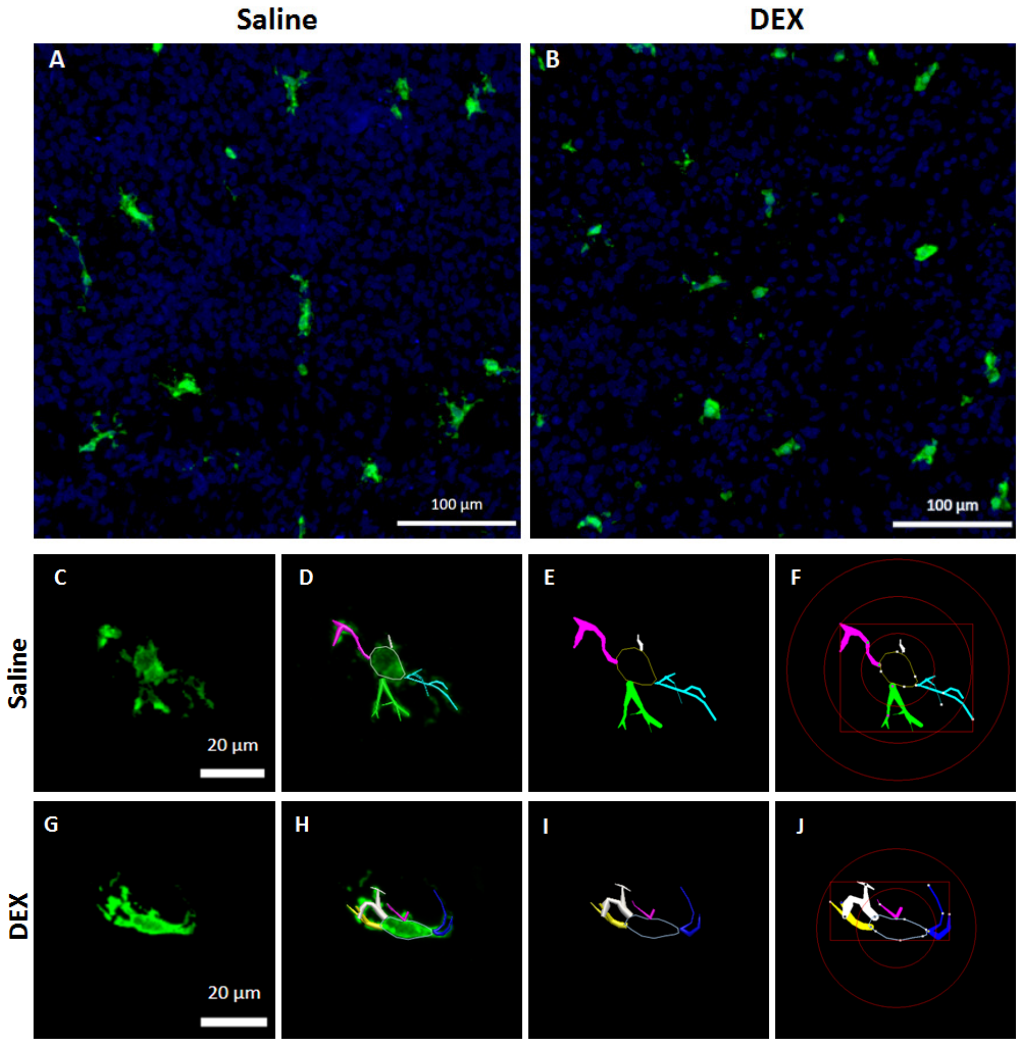
←Figure 4.7| Density of corticosteroid and adenosine receptors in the PFC after the pre-natal DEX treatment. Pregnant Wistar rats received 1mg kg^{-1} DEX at 18 or 19 gestation day and the density of receptors was analyzed by western blot of total extracts obtained from the progeny at PND 1 and 7. Graphs and representative images obtained from western blot analysis are shown in **(A)** for the density of GR, **(B)** for A_1R density and **(C)** for $A_{2A}R$ density. Results are expressed as mean ± SEM of 3 to 5 biological samples (*p<0.05, **p<0.01, compared with control conditions, Student's t test). SEM, standard error of the mean; DEX, dexamethasone; GR, glucocorticoid receptor; A_1R, adenosine A_1 receptor; $A_{2A}R$, adenosine A_{2A} receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

4.3.2.2. Morphology of microglia in the prefrontal cortex after prenatal dexamethasone treatment

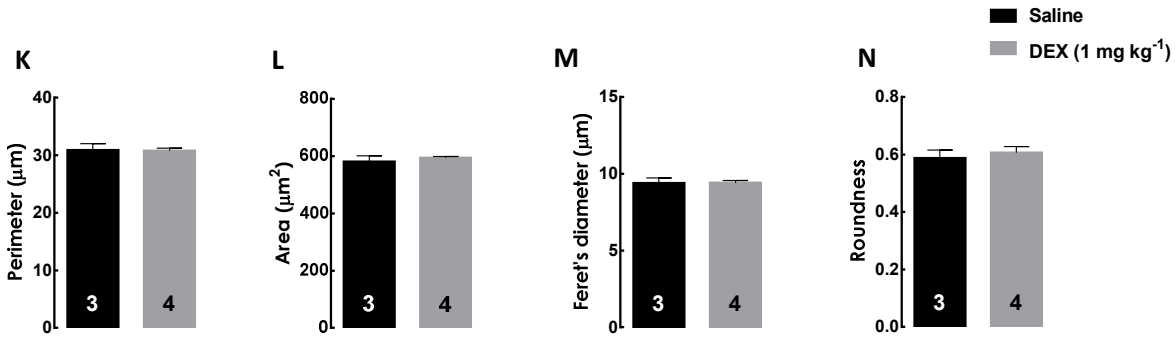
In order to analyze if prenatal DEX impacts on microglia morpholog at short- and long-term, we quantified particular morphological features (morphometry) of microglia in PFC at PND 1, 7 and 90 after birth. Microglia was identified by Iba1 staining and reconstructed at the tridimensional level by using Neurolucida software. The 3D-reconstruction was manual because the automatic tool was not accurate enough to reconstruct microglia. Reconstructed microglial cells were analyzed taking into account diverse features of the cell body and processes. In the cell body it was analysed the perimeter, area, feret's diameter and roundness. Regarding microglial processes, it was analyzed the number and the diameter of the processes by branch order and by radius (sholl analysis), as well as the total length and volume of the processes.

a) Postnatal day 1

One day after birth microglial cells are still colonizing and migrating along the brain. In PFC, it was observed that some cells start to differentiate and become slightly ramified, while others were still small and round. Other cells exhibit small pseudopodia at the tip of small branches, suggestive of migrating processes. For the morphometric analysis, migration regions were avoided and 20 cells were blindly acquired in PFC for each codified slice. The region chosen was the medial PFC with an interaural between 12.70 and 12.20 mm, and bregma between 3.70 and 3.20 mm (sagital view; according to the rat brain atlas of Paxinos, 1998).


Saline
DEX
A
B
100 µm
100 µm
C
D
E
F
Saline
20 µm
G
H
I
J
DEX
20 µm

Cell body


Saline
DEX (1 mg kg-1)
K
Perimeter (µm)
40
30
20
10
0
3
4
L
Area (µm2)
800
600
400
200
0
3
4
M
Feret's diameter (µm)
15
10
5
0
3
4
N
Roundness
0.8
0.6
0.4
0.2
0.0
3
4

Cell processes

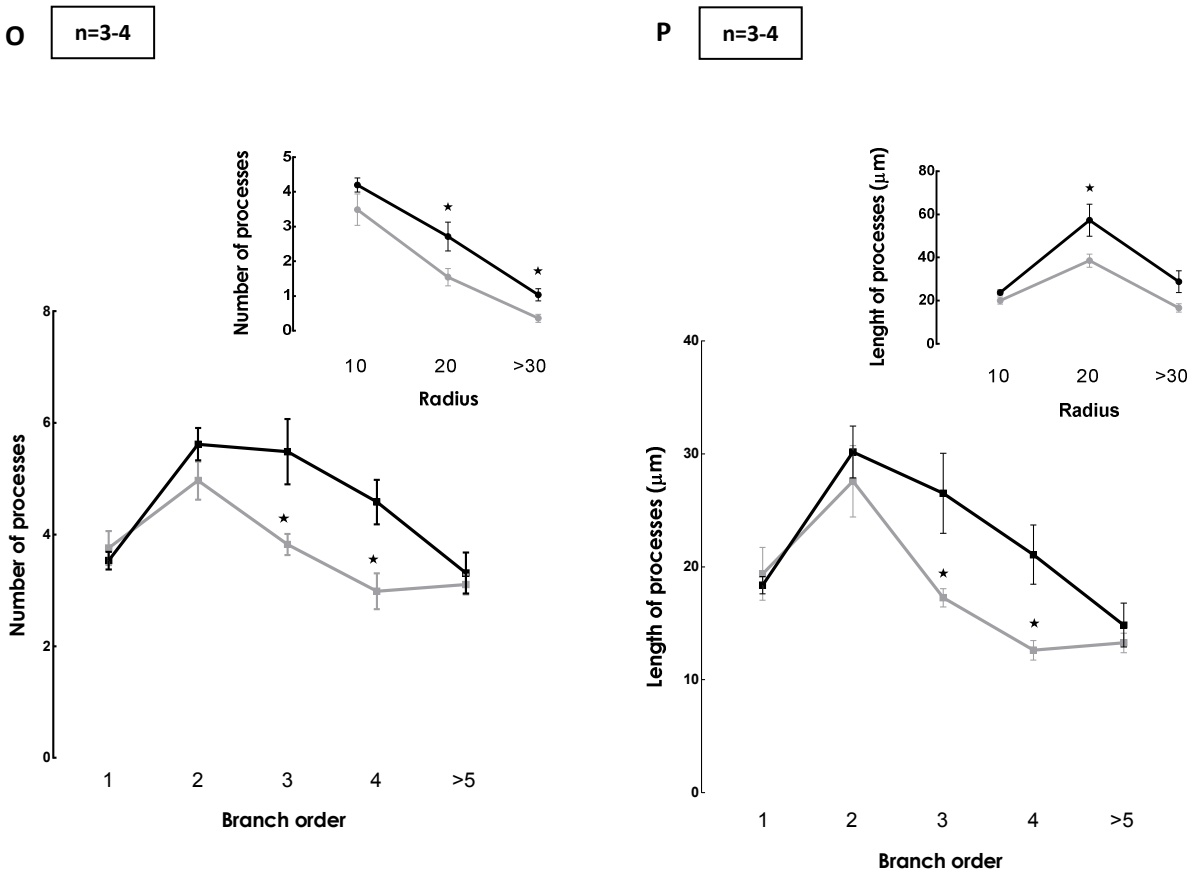


Figure 4.8| Effect of prenatal DEX treatment in the number and length of processes, ends, nodes and volume of microglia in the PFC at PND 1. Pregnant Wistar rats received 1mg kg^{-1} DEX at 18 or 19 gestation day, microglial cells of neonatal brains were stained with Iba1 at PND 1 and 3D reconstructions were performed using Neurolucida software. In **(A-J)** are shown representative images obtained from the Iba1 staining of microglia; **(K-N)** graphs from perimeter, area, feret's diameter and roundness from the cell body analysis; and **(O and P)** graphs from the analysis of the number and length of processes by order and by radius. Results are expressed as mean ± SEM of 3-4 biological samples (*p<0.05, **p<0.01, compared with control conditions, Student's t test). SEM, standard error of the mean; DEX, dexamethasone.

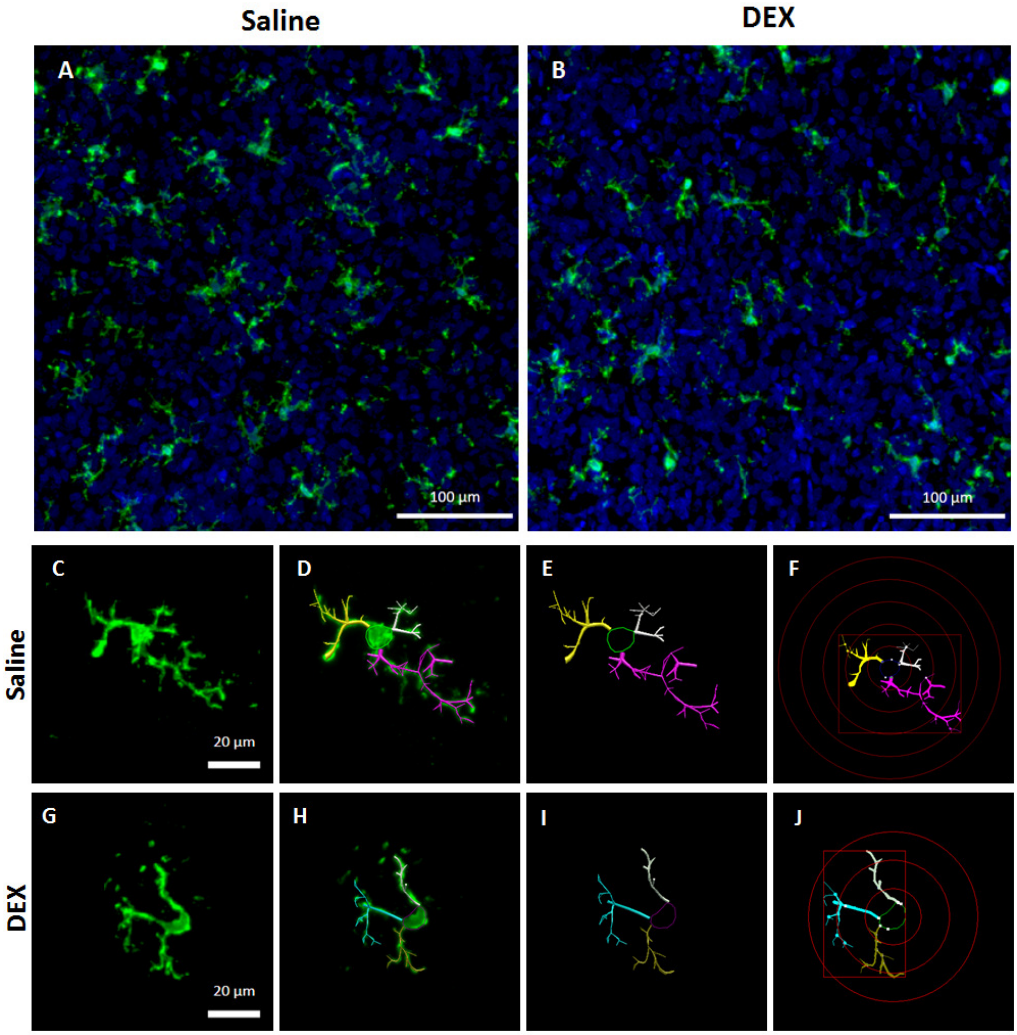
In general, the morphometric analysis of the main features of the cell body (perimeter, area, feret's diameter and roundness) did not show any particular impact of antenatal DEX at PND1, as shown in **Figure 4.8 (K-N)**.

By the analysis of processes, significant changes were already detected at PND7 at the level of the number and the length of the processes. A significant decrease in the number and length of processes of order 3 and 4 was observed at this post-natal age (number of processes of order 3: 3.82 ± 0.2; n=4; p=0.0272, compared with control conditions; and order 4: 2.98 ± 0.3; n=4; p=0.0251, compared with control conditions; length of processes of order 3: 17.26 ± 0.8 µm; n=4; p= 0.0313, compared with control conditions; order 4: 12.61 ± 0.9 µm; n=4; p=0.0173, compared with control conditions). In the analysis of processes by radius (more general and less

specific method), differences were also found in the number of processes at radius 20 (distance from cell body; 1.55 ± 0.2; n=4; p=0.0500, compared with control conditions) and radius 30 (0.36 ± 0.1; n=4; p=0.0205, compared with control conditions). In the length of processes there was a decrease at radius 20 (38.57 ± 3.0 µm; n=4; p=0.0466, compared with control conditions).

b) Postnatal day 7

At PND 7, microglial cells were, in general, more ramified in the PFC when compared with the same region at PND 1.



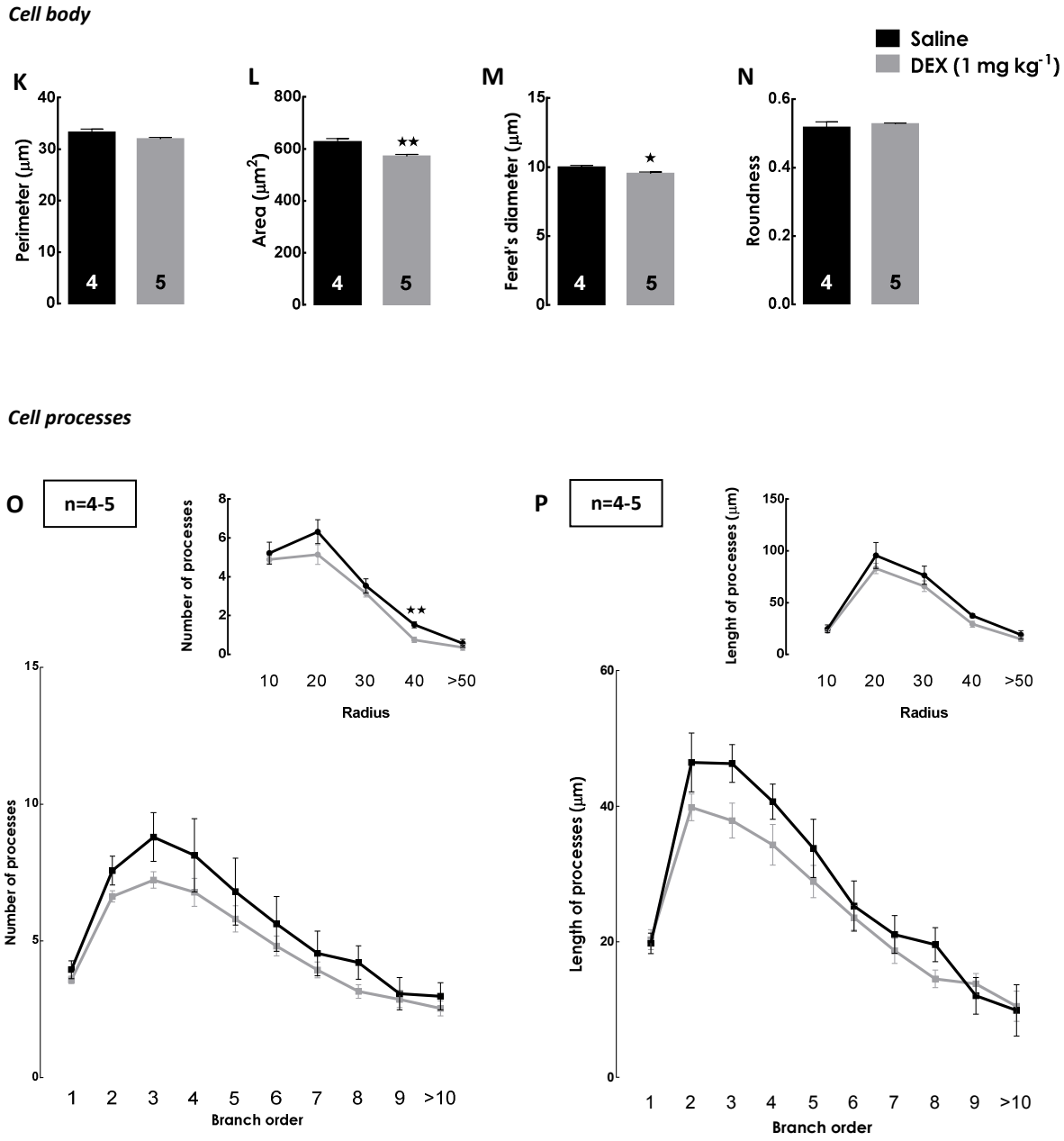


Figure 4.9| Effect of prenatal dexamethasone treatment in the number and length of processes, ends, nodes and volume of microglia in the prefrontal cortex at post-natal day 7. Pregnant Wistar rats received 1mg kg^{-1} DEX at 18-19 gestation days, microglial cells of neonatal brains were stained with Iba1 at postnatal day 7 and tridimensional reconstructions were performed using Neurolucida software. In **(A-J)** are shown representative images obtained from the Iba1 staining of microglia; **(K-N)** graphs from perimeter, area, feret's diameter and roundness from the cell body analysis; and **(O and P)** graphs from the analysis of the number and length of processes by order and by radius. Results are expressed as mean ± SEM of 3-4 biological samples (*p<0.05, **p<0.01, compared with control conditions, Student's t test). SEM, standard error of the mean; DEX, dexamethasone.

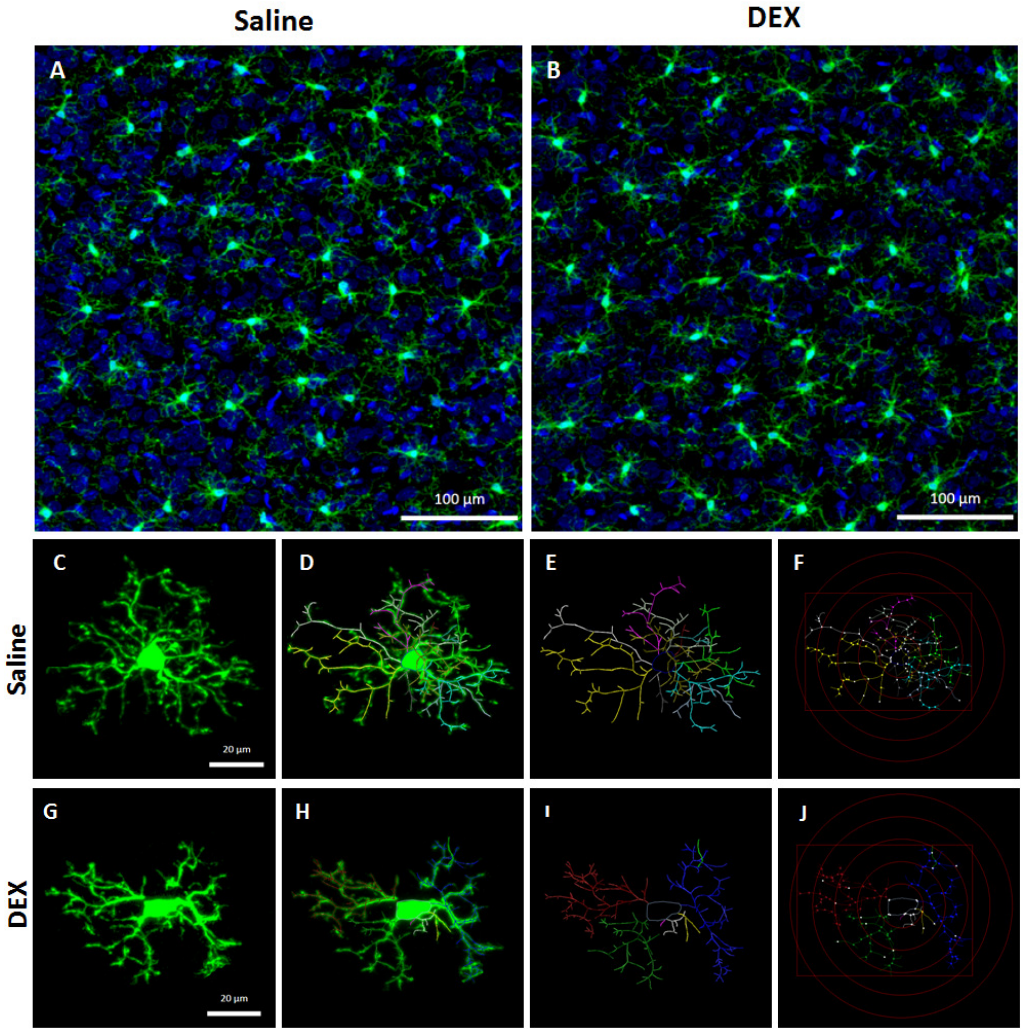
Interestingly, and in contrast to PND 1, at this developmental stage differences were found between microglial cell bodies of treated animals. The cell body area was affected by

antenatal DEX treatment (571.0 ± 7.9 μm^2; n=5; p=0.0069, compared with control conditions) as well as the feret's diameter (9.53 ± 0.1 µm; n=5; p=0.0410, compared with control conditions).

Regarding microglial processes, a tendency for a decrease was observed in the number of processes of third order (7.23 ± 0.3; n=5; p=0.1080, compared with control conditions) and the respective length (37.88 ± 2.6 µm; n=5; p=0.0618, compared with control conditions), although without statistical significance. Sholl analysis points towards a decrease in the number of processes for radius 40 (0.75 ± 0.1; n=5; p=0.0069, compared with control conditions).

c) Adulthood

At PND 90 (3 months), microglial cells are fully differentiated. Microglial cells are equipped with long, thin and highly branched processes and a small cell body. No amoeboid microglia was detected in the brain, and in particular in the PFC. Microglia were scattered throughout all brain in a more or less homogeneous way. Morphometric analysis of microglia was performed in the PFC of adult female progeny that received *in utero* DEX.



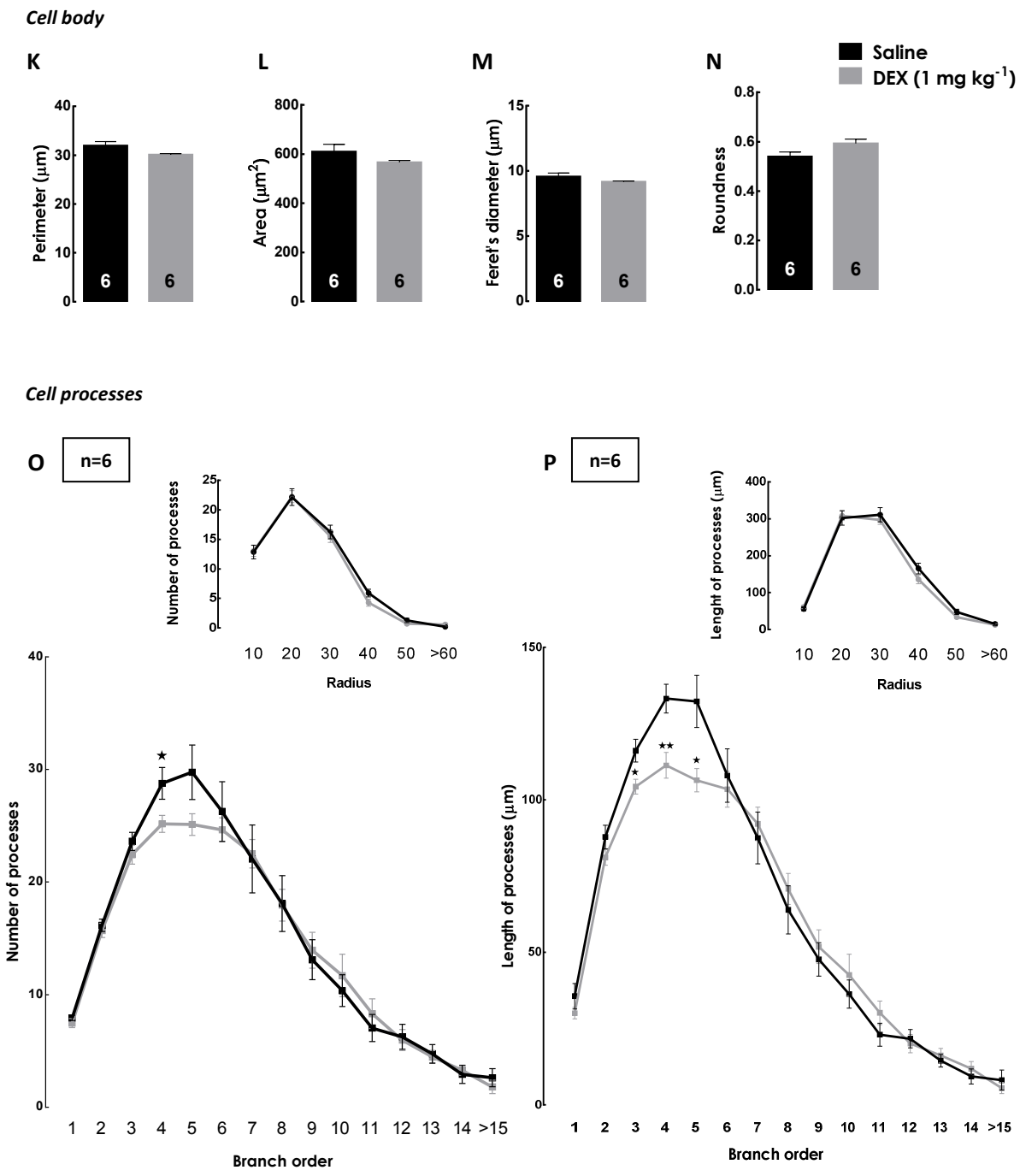
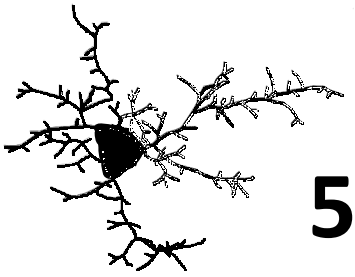


Figure 4.10| Effect of prenatal dexamethasone treatment in the number and length of processes, ends, nodes and volume of microglia in the prefrontal cortex at postnatal day 90. Pregnant Wistar rats received 1mg kg^{-1} DEX at 18-19 gestation days, microglial cells of brains were stained with Iba1 at postnatal day 90 and tridimensional reconstructions were performed using Neurolucida software. In **(A-J)** are shown representative images obtained from the Iba1 staining of microglia; **(K-N)** graphs from perimeter, area, feret's diameter and roundness from the cell body analysis; and **(O and P)** graphs from the analysis of the number and length of processes by order and by radius. Results are expressed as mean ± SEM of 3-4 biological samples (*p<0.05, **p<0.01, compared with control conditions, Student's t test). SEM, standard error of the mean; DEX, dexamethasone.

In adulthood, no differences were detected in the perimeter, area, feret's diameter and roundness of the cell body. Thus, the differences in area and feret's diameter observed at postnatal day 7 were transient and not remained in the adult age. So, dexamethasone does not impact upon cell body of microglia.

In terms of number of processes, there was a decrease in number of processes of order 4 (25.17 ± 0.7632; n=6; p=0.0478, compared with control conditions) and a tendency to decrease at order 5 (25.12 ± 0.9659; n=6; p=0.1068, compared with control conditions). The length of processes was also decreased at order 3 (104.3 ± 2.428 µm; n=6; p=0.0241, compared with control conditions), order 4 (111.3 ± 4.232; n=6; p=0.0061 µm, compared with control conditions), and order 5 (106.5 ± 3.816 µm; n=6; p=0.0202, compared with control conditions).



5. DISCUSSION

5. Discussion

Dexamethasone (DEX) is a synthetic drug widely used in neonatal care in order to reduce neonatal complications associated with premature newborns, such as respiratory distress syndrome. This drug is selective for glucocorticoid receptors, and its administration negatively impacts on neurodevelopment.

The main focus of the present work was on the impact of DEX upon microglial morphology, as well as on changes at the level of the adenosinergic system. The major findings that conduced to the present thesis are: (1) young adult Wistar females exposed *in utero* to DEX, exhibited an anxious phenotype at adulthood; (2) the density of microglial adenosine A_{2A} receptors was altered by DEX *in vitro*, an effect that dependent on the concentration and time of exposure to the drug; (3) the density of adenosine receptors evaluated *ex vivo* in total extracts of different brain regions was affected by antenatal DEX, an effect region-specific and dependent on the post-natal age studied; (4) microglia morphology in PFC was affected by prenatal DEX treatment, an effect that persisted throughout life. This study provides the first evidence that the adenosinergic system, which is a key modulator of microglial function in the mature brain, is affected by DEX during early phases of neurodevelopment. It was also showed, for the first time, that antenatal DEX triggers a microglial plasticity process that lasts until adulthood and parallels the previously described morphological reorganization of neurons and abnormal behavior.

5.1. Microglial adenosine receptors density after dexamethasone treatment

In order to clarify if the adenosinergic system of microglia is directly affected by DEX it was considered important to perform *in vitro* studies in a pure microglial cell line (N9 cells). This was due to technical limitations associated with the fluorescent labeling of $A_{2A}R$ by using commercially available antibodies. Thus, the use of a microglial cell line was a complementary strategy to confirm the ability of DEX to exert a direct effect upon the microglial adenosinergic system. These *in vitro* studies allowed to conclude that DEX directly interferes with the density of GR and $A_{2A}R$, and that this effect depends on the concentration and on the time of incubation with DEX. Although aware of the differences between clinics and fundamental science, it is considered of relevance this dependence of DEX effects on the concentration and time of exposure, considering different therapeutic regimens used and taking into consideration the results of the present thesis. A_1R were not altered in the conditions tested in this work. Interestingly, while GR variations seem to occur for shorter incubation times (3 and 6 hours), changes at the level of $A_{2A}R$ were observed at later timepoints (24 and 48 hours), suggesting that

a transient change of GR precedes a later and long-lasting change in $A_{2A}R$ density. The proteomic analysis of $A_{2A}R$ density, as assessed by western blot assays, was further supported by the qualitative analysis of immunocytochemistry data suggesting DEX-induced morphological changes temporally correlated with changes in the density of $A_{2A}R$. This qualitative analysis, which pointed towards a retraction of microglial processes, is in line with the ability of $A_{2A}R$ to control process dynamics of microglial cells, as described by Orr and coworkers (2009) and Gyoneva et al. (2014). Preliminary results on the viability of microglial cells exposed to DEX seem to indicate that the drug does not interfere with cell viability, suggesting that DEX-induced microglial atrophy is apparently not related with a decrease in viability.

This *in vitro* pilot study was the basis for the subsequent *ex vivo* study of DEX impact on microglia morphology.

5.2. Morphology of microglia in the prefrontal cortex after *in utero* dexamethasone treatment

The animal model of antenatal DEX administration used in the present work was already characterized in terms of neuronal morphologic features and behavior; it was showed that this administration regimen is associated with a phenomenon of spine reorganization correlated with neuropsychiatric-like abnormalities (depression and anxiety; Rodrigues *et al.,* 2012) in males. Considering that microglia is involved in synapse formation and that microglia treatment with immunomodulators (e.g. LPS) increases the density of synaptic proteins associated with synapse formation (Cristóvão *et al.,* 2014), it was hypothesized that DEX-induced changes in microglial cells could mediate neuronal effects. The main goal of the present thesis was to characterize microglia morphology in the previously described animal model. The working hypothesis was confirmed by tridimensional reconstruction of Iba1 stained microglia in the cerebral parenchyma of animals exposed *in utero* to DEX. DEX is a small and hydrophobic molecule, which easily crosses the placenta and the blood-brain barrier and, in consequence, impacts on the developing brain. To the mothers, there is no evidence of possible side effects caused by DEX administration (Royal College of Obstetricians and Gynaecologists, 2010); thus, they were not considered in the present study. However, in future work it would be interesting to study the effect of DEX in the maternal brain, namely in terms of microglia morphology and density of $A_{2A}R$.

Although several regions are related with depression and anxiety-like phenotypes, for the present thesis it was only considered the PFC, affected in stress conditions, in which

corticosteroids are main effectors. Regarding the progeny, tridimensional reconstructions of microglial cells in the PFC revealed DEX-induced changes in microglia morphology at PND 1 and PND 7, alterations that persisted until adulthood (PND 90). One of the main goals of the present work was to clarify if microglia morphology was only affected during the postnatal period, presenting a transient phenotype absent in the adulthood, or if changes triggered by DEX were irreversible. Surprinsingly, microglial morphology changes remained throughout life, at least until the latest age analyzed (PND 90).

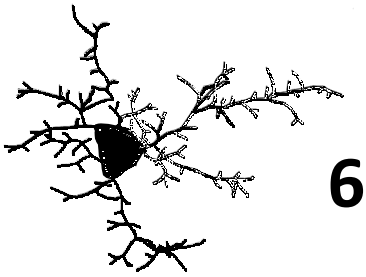
Changes in microglia morphology were mainly detected at the level of cellular processes, without significant morphological changes in cell body features, except at PND 7 where a transient decrease in the area and feret's diameter of the cell body was observed. Microglial processes dynamically survey the brain parenchyma by the constant extension and retraction of thin, long and ramified processes in the mature and healthy brain, a function essential to their main function (surveillance) in physiologic conditions. The present results suggest that this microglial function may be compromised in DEX-treated animals and that this malfunction may be the underlying cause of behavioral abnormalities. Further studies on the analysis of microglial reactivity an insult (e.g. stressor) may help clarify if the sensor ability of microglia is associated with changes in morphology and correlate with behavioral changes.

5.3. Adenosine receptors after *in utero* dexamethasone treatment

Microglial dynamics is controlled by adenosine A_{2A} receptors (Orr *et al.,* 2009; Gyoneva *et al.*, 2012), as well as other microglial functions. $A_{2A}R$ are also involved in the pathophysiology of depression and anxiety (for a review see, e.g. Gomes *et al.*, 2011). For this reason, and considering the results of the present thesis, $A_{2A}R$ will be preferentially studied as main pharmacological targets in future work.

In the present work, it was observed that the density of adenosine and glucocorticoid receptors are affected by prenatal DEX exposure, an effect that dependent on the age and brain region. Of note, a certain liability was observed in the profile of adenosinergic receptors throughout the brain during early phases of post-natal development, and it is considered of importance to screen the density of adenosine receptors at adulthood, where behavioral changes are observed. Further studies are needed in order to confirm the direct involvement of adenosine receptors in microglial morphological adaptation and anxious profile subsequent to DEX treatment.

These observations about the adenosinergic system may also be important for future experiments designed to modulate microglia reactivity, as well as to prevent or treat depression and anxiety in this particular model of DEX administration. For example, the administration of selective antagonists, such as SCH 58261 emerges as a candidate strategy to control microglia plasticity and, eventually, the pathophysiologic process of depression and anxiety.



6. CONCLUSIONS AND FUTURE DIRECTIONS

6. Conclusions and future directions

Prenatal treatment with DEX impacts on microglial cell morphology, as well as on the adenosinergic system. These findings may be linked to depression and anxious-like phenotype observed later in life, and modulation of the adenosinergic system may constitute a new approach for the treatment and/or prevention of the pathophysiology of mood disorders.

In the future, it would be important to perform some experiments using selective antagonists of adenosine A_{2A} receptors, such as SCH58261, in animals that received *in utero* DEX, since $A_{2A}R$ antagonists have already proven to be effective in the treatment of neuropsychiatric disorders. On the other hand, it would also be interesting to perform a similar study with caffeine, a non-selective antagonist of $A_{2A}R$, given in the drinking water of prenatal treated animals, in order to have an epidemiological correlate of the caffeine consumption impact upon depression and anxiety-like behavior. An important control study to be performed in parallel would be the use of antidepressants with proved efficacy, such as fluoxetine and imipramine.

Regarding microglia, it would be important to observe processes dynamics in the brain parenchyma during development, as well as in adulthood of animals treated with DEX. In future work, it would be desirable to perform some experiments using two-photon microscopy for *in vivo a*nd real time monitoring of process dynamics. Using this technique, it would be possible to observe *in vivo* the dynamics of microglial processes in the parenchyma, i.e., if processes were more or less dynamic by prenatal DEX treatment, as well as the respective microglial interactions with synapses.

It would be also important to clarify if the process of colonization by microglia during neurodevelopment was or not affected by DEX treatment.

In the absence of microglia, it would also be important to study the impact of DEX upon synapses and neurons. Selective depletion of microglia, could be performed *ex vivo* using chemical tools and/or *in vivo* by injection of a pharmacological compound able to selectively eliminate myeloid cells, including microglia from the brain (e.g. clodronate liposomes). After that, it would be necessary to evaluate *in vivo* or *ex vivo*, if synaptic transmission is or not affected by DEX in the absence of microglia; morphology of neurons and synapses should be also observed in those conditions. Ideally, the *in vivo* model should be preferable since it is also important to perform behavior studies in order to evaluate their depressive and anxious-like profile in the absence of microglia.

Finally, it would be important to observe the morphology of microglia and synaptic transmission in the PFC, in knock-out animals that do not express glucocorticoid receptors in microglia, and perform behavior studies in order to evaluate the neuropsychiatric profile both in the postnatal period and in adulthood.

7. REFERENCES

7. References

Beynon, S.B., Walker, F.R. **Microglial activation in the injured and healthy brain: What are we really talking about? Practical and theoretical issues associated with the measurement of changes in microglial morphology.** Neuroscience 225, 162-171 (2012).

Brown, G. C., Neher, J. J. **Microglial phagocytosis of live neurons.** Nature Reviews Neuroscience, 15(4), 209-216 (2014).

Brownfoot, F.C., Gagliardi, D.I., Bain, E., Middleton, P., Crowther, C.A. **Different corticosteroids and regimens for accelerating fetal lung maturation for women at risk of preterm birth.** Cochrane Database of Systematic Reviews (2013).

Cajal, R. **Contribucion al conocimiento de la neuroglia del cerebro humano.** Trab Lab Invest Biol (1913).

Carrillo de Sauvage, M.A., Maatouk, L., Arnoux, I., Pasco, M., Sanz Diez, A., Delahaye, M., Herrero, M.T., Newman, T.A., Calvo, C.F., Audinat, E., Tronche, F., Vyas S. **Potent and multiple regulatory actions of microglial glucocorticoid receptors during CNS inflammation.** Cell death and differentiation 20, 1546-1557 (2013).

Cerqueira, J.J., Almeida, O.F.X., Sousa, N. **The stressed prefrontal cortex. Left? Right!** Brain Behavior and Immunity 22, 630-638 (2008).

Cerqueira, J.J., Pêgo, J.M., Taipa, R., Bessa, J.M., Almeida, O.F.X., Sousa, N. **Morphological correlates of corticosteroid-induced changes in prefrontal cortex-dependent behaviors.** Journal of Neuroscience 25, 7792-7800 (2005).

Cerqueira, J.J., Taipa, R., Uylings, H.B.M., Almeida, O.F.X., Sousa, N. **Specific configuration of dendritic degeneration in pyramidal neurons of the medial prefrontal cortex induced by differing corticosteroid regimens.** Cerebral Cortex 17, 1998-2006 (2007).

Chan, W.Y., Kohsaka, S., Rezaie, P. **The origin and cell lineage of microglia - New concepts.** Brain Research Reviews 53, 344-354 (2007).

Cristóvão, G., Pinto, M.J., Cunha, R.A., Almeida, R.D., Gomes, C.A. **Activation of microglia bolsters synapse formation.** Frontiers in Cellular Neuroscience 8 (2014).

Cunha, R.A. **Different cellular sources and different roles of adenosine: A_1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A_{2A} receptor-mediated facilitation of plasticity.** Neurochemistry International 52, 65-72 (2008).

Dai, S.S., Zhou, Y.G., Li, W., An, J.H., Li, P., Yang, N., Chen, X.W., Xiong, R.P., Liu, P., Zhao, Y., Shen, H.Y., Zhu, P.F., Chen, J.F. **Local glutamate level dictates adenosine A_{2A} receptor regulation of neuroinflammation and traumatic brain injury.** Journal of Neuroscience 30, 5802-5810 (2010).

Dalmau, I., Finsen, B., Tonder, N., Zimmer, J., Gonzalez, B., Castellano, B. **Development of microglia in the prenatal rat hippocampus.** Journal of Comparative Neurology 377, 70-84 (1997).

Dalmau, I., Finsen, B., Zimmer, J., Gonzalez, B., Castellano, B. **Development of microglia in the postnatal rat hippocampus.** Hippocampus 8, 458-474 (1998a).

Dalmau, I., Vela, J.M., Gonzalez, B., Castellano, B. **Expression of purine metabolism-related enzymes by microglial cells in the developing rat brain.** Journal of Comparative Neurology 398, 333-346 (1998b).

Dalmau, I., Vela, J.M., Gonzalez, B., Finsen, B., Castellano, B. **Dynamics of microglia in the developing rat brain.** Journal of Comparative Neurology 458, 144-157 (2003).

Daré, E., Schulte, G., Karovic, O., Hammarberg, C., Fredholm, B. B. **Modulation of glial cell functions by adenosine receptors.** Physiology & behavior, 92(1), 15-20 (2006).

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L., Gan, W. B. **ATP mediates rapid microglial response to local brain injury *in vivo*.** Nature neuroscience 8(6) 752-758 (2005).

De Kloet, E.R., Vreugdenhil, E., Oitzl, M.S., Joels, M. **Brain corticosteroid receptor balance in health and disease.** Endocrine Reviews 19, 269-301 (1998).

De Pablos, R.M., Villarán, R.F., Argüelles, S., Herrera, A.J., Venero, J.L., Ayala, A., Cano, J., Machado, A. **Stress increases vulnerability to inflammation in the rat prefrontal cortex.** Journal of Neuroscience 26, 5709-5719 (2006).

Del Rio-Hortega, P. **Microglia.** Penfield W. New York: Hoeber Cytology and Cellular Pathology of the Nervous System. 482-534 (1932).

Dias, R. B., Rombo, D. M., Ribeiro, J. A., Henley, J. M., Sebastião, A. M. **Adenosine: setting the stage for plasticity.** Trends in neurosciences,36(4), 248-257 (2013).

Diaz Heijtz, R., Fuchs, E., Feldon, J., Pryce, C.R., Forssberg, H. **Effects of antenatal dexamethasone treatment on glucocorticoid receptor and calcyon gene expression in the prefrontal cortex of neonatal and adult common marmoset monkeys.** Behavioral and brain functions : BBF 6, 18-18 (2010).

Domercq, M., Vazquez-Villoldo, N., Matute, C. **Neurotransmitter signaling in the pathophysiology of microglia.** Frontiers in Cellular Neuroscience 7, 49 (2013).

Duan, Y., Sahley, C. L., Muller, K. J. **ATP and NO dually control migration of microglia to nerve lesions.** Developmental neurobiology, 69(1), 60-72 (2009).

Frank, M. G., Baratta, M. V., Sprunger, D. B., Watkins, L. R., Maier, S. F. **Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses.** Brain, behavior, and immunity, 21(1), 47-59 (2007).

Frank, M.G., Baratta, M.V., Sprunger, D.B., Watkins, L.R., Maier, S.F. **Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses.** Brain Behavior and Immunity 21, 47-59 (2007).

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K.N., Linden, J. **International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors.** Pharmacological Reviews 53, 527-552 (2001).

Fukumoto, K., Morita, T., Mayanagi, T., Tanokashira, D., Yoshida, T., Sakai, A., Sobue, K. **Detrimental effects of glucocorticoids on neuronal migration during brain development.** Molecular Psychiatry 14, 1119-1131 (2009).

Gomes, C.V., Ferreira, R., George, J., Sanches, R., Rodrigues, D.I., Gonçalves, N., Cunha, R.A. **Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A_{2A} receptor-dependent manner: A_{2A} receptor blockade prevents BDNF release and proliferation of microglia.** Journal of Neuroinflammation 10 (2013).

Gomes, C.V., Kaster, M.P., Tome, A.R., Agostinho, P.M., Cunha, R.A. **Adenosine receptors and brain diseases: Neuroprotection and neurodegeneration.** Biochimica Et Biophysica Acta-Biomembranes 1808, 1380-1399 (2011).

Gyoneva, S., Davalos, D., Biswas, D., Swanger, S. A., Garnier-Amblard, E., Loth, F., Akassoglou, K., Traynelis, S. F. **Systemic inflammation regulates microglial responses to tissue damage *in vivo*.** Glia 62(8) 1345-1360 (2014).

Gyoneva, S., Orr, A.G., Traynelis, S.F. **Differential regulation of microglial motility by ATP/ADP and adenosine.** Parkinsonism & related disorders 15 Suppl 3, S195-199 (2009).

Harry, G.J., Kraft, A.D. **Microglia in the developing brain: A potential target with lifetime effects.** Neurotoxicology 33, 191-206 (2012).

Haskó, G., Pacher, P., Sylvester Vizi, E., Illes, P. **Adenosine receptor signaling in the brain immune system.** Trends in pharmacological sciences, 26(10), 511-516 (2005).

Huo, Y., Rangarajan, P., Ling, E.A., Dheen, S.T. **Dexamethasone inhibits the Nox-dependent ROS production via suppression of MKP-1-dependent MAPK pathways in activated microglia.** Bmc Neuroscience 12 (2011).

Iglesias, J., Eriksson, J., Grize, F., Tomassini, M., Villa, A. **Dynamics of pruning in simulated large-scale spiking neural networks.** BioSystems 79: 11–20 (2005).

Kaur, C., Hao, A.J., Wu, C.H., Ling, E.A. **Origin of microglia.** Microscopy Research and Technique 54, 2-9 (2001).

Kettenmann, H., Hanisch, U.K., Noda, M., Verkhratsky, A. **Physiology of Microglia.** Physiological Reviews 91 (2011).

Kettenmann, H., Kirchhoff, F., Verkhratsky, A. **Microglia: New Roles for the Synaptic Stripper.** Neuron 77, 10-18 (2013).

Kettenmann, H., Kirchhoff, F., Verkhratsky, A. **Microglia: new roles for the synaptic stripper.** Neuron, 77(1), 10-18 (2013).

Landolt, H.P., Retey, J.V., Adam, M. **Reduced neurobehavioral impairment from sleep deprivation in older adults: contribution of adenosinergic mechanisms.** Frontiers in neurology 3, 62-62 (2012).

Latini, S., Pedata, F. **Adenosine in the central nervous system: release mechanisms and extracellular concentrations.** Journal of neurochemistry, 79(3), 463-484 (2001).

Leão, P., Sousa, J.C., Oliveira, M., Silva, R., Almeida, O.F.X., Sousa, N. **Programming effects of antenatal dexamethasone in the developing mesolimbic pathways.** Synapse 61, 40-49 (2007).

Lim, S.H., Park, E., You, B., Jung, Y., Park, A.R., Park, S.G., Lee, J.R. **Neuronal synapse formation induced by microglia and interleukin 10.** Plos One 8 (2013).

McArthur, S., McHale, E., Dalley, J.W., Buckingham, J.C., Gillies, G.E. **Altered mesencephalic dopaminergic populations in adulthood as a consequence of brief perinatal glucocorticoid exposure.** Journal of Neuroendocrinology 17, 475-482 (2005).

Mesquita, A.R., Wegerich, Y., Patchev, A.V., Oliveira, M., Leão, P., Sousa, N., Almeida, O.F.X. **Glucocorticoids and neuro- and behavioural development.** Seminars in Fetal & Neonatal Medicine 14, 130-135 (2009).

Miller EK, Cohen JD. **An integrative theory of prefrontal cortex function.** Annual Review of Neuroscience. 24:167-202 (2001).

Mosmann, T. **Rapid colorimetric assay for cellular growth and survival – application to proliferation and cyto-toxicity assays.** Journal of Immunological Methods 65, 55-63 (1983).

Munhoz, C.D., Lepsch, L.B., Kawamoto, E.M., Malta, M.B., Lima, L.S., Avellar, M.C., Sapolsky, R.M., Scavone, C. **Chronic unpredictable stress exacerbates lipopolysaccharide-induced activation of nuclear factor-kappa B in the frontal cortex and hippocampus via glucocorticoid secretion.** Journal of Neuroscience 26, 3813-3820 (2006).

Nagano, M., Ozawa, H., Suzuki, H. **Prenatal dexamethasone exposure affects anxiety-like behaviour and neuroendocrine systems in an age-dependent manner.** Neuroscience Research 60, 364-371 (2008).

Nair, A., Bonneau, R.H. **Stress-induced elevation of glucocorticoids increases microglia proliferation through NMDA receptor activation.** Journal of Neuroimmunology 171, 72-85 (2006).

Nimmerjahn, A., Kirchhoff, F., Helmchen, F. **Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*.** Science 308(5726) 1314-1318 (2005).

Oliveira, J.F., Dias, N.S., Correia, M., Gama-Pereira, F., Sardinha, V.M., Lima, A., Oliveira, A.F., Jacinto, L.R., Ferreira, D.S., Silva, A.M., Reis, J.S., Cerqueira, J.J., Sousa, N. **Chronic stress disrupts neural coherence between cortico-limbic structures.** Frontiers in Neural Circuits 7 (2013).

Oliveira, M., Bessa, J.M., Mesquita, A., Tavares, H., Carvalho, A., Silva, R., Pêgo, J.M., Cerqueira, J.J., Palha, J.A., Almeida, O.F.X., Sousa, N. **Induction of a hyperanxious state by antenatal dexamethasone: A case for less detrimental natural corticosteroids.** Biological Psychiatry 59, 844-852 (2006).

Oliveira, M., Rodrigues, A.J., Leão, P., Cardona, D., Pêgo, J.M., Sousa, N. **The bed nucleus of stria terminalis and the amygdala as targets of antenatal glucocorticoids: implications for fear and anxiety responses.** Psychopharmacology 220, 443-453 (2012).

Orr, A.G., Orr, A.L., Li, X.-J., Gross, R.E., Traynelis, S.F. **Adenosine A_{2A} receptor mediates microglial process retraction.** Nature Neuroscience 12, 872-U884 (2009).

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L., Ragozzino, D., Gross, C.T. **Synaptic pruning by microglia is necessary for normal brain development.** Science 333, 1456-1458 (2011).

Parkhurst, C.N., Yang, G., Ninan, I., Savas, J.N., Yates, J.R., Lafaille, J.J., Hempstead, B.L., Littman, D.R., Gan, W.B. **Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor.** Cell 155, 1596-1609 (2013).

Paxinos, G., Watson, C. **The rat brain in stereotaxic coordinates.** Academic Press (1998).

Pedata, F., Corsi, C., Melani, A., Bordoni, F., Latini, S. **Adenosine extracellular brain concentrations and role of A_{2A} receptors in ischemia.** Neuroprotective Agents 939, 74-84 (2001).

Pinto, L., Mateus-Pinheiro, A., Morais, M., Bessa, J.M., Sousa, N. **Immuno-golgi as a tool for analyzing neuronal 3D-dendritic structure in phenotypically characterized neurons.** Plos One 7 (2012).

Ramachandra, R., Subramanian, T. **Atlas of the neonatal rat brain.** CRC Press Taylor & Francis Group (2011).

Ribeiro, J.A., Sebastião, A.M., Mendonça, A. **Adenosine receptors in the nervous system: pathophysiological implications.** Progress in Neurobiology 68, 377-392 (2003).

Rice, D., Barone, S. **Critical periods of vulnerability for the developing nervous system: Evidence from humans and animal models.** Environmental Health Perspectives 108, 511-533 (2000).

Rodrigues, A.J., Leão, P., Pêgo, J.M., Cardona, D., Carvalho, M.M., Oliveira, M., Costa, B.M., Carvalho, A.F., Morgado, P., Araújo, D., Palha, J.A., Almeida, O.F.X., Sousa, N. **Mechanisms of initiation and reversal of drug-seeking behavior induced by prenatal exposure to glucocorticoids.** Molecular Psychiatry 17, 1295-1305 (2012).

Romagnoli, C., Zecca, E., Vento, G., De Carolis, M.P., Papacci, P., Tortorolo, G. **Early postnatal dexamethasone for the prevention of chronic lung disease in high-risk preterm infants.** Intensive Care Medicine 25, 717-721 (1999).

Roque, S., Oliveira, T.G., Nobrega, C., Barreira-Silva, P., Nunes-Alves, C., Sousa, N., Palha, J.A., Correia-Neves, M. **Interplay between depressive-like behavior and the immune system in an animal model of prenatal dexamethasone administration.** Frontiers in behavioral neuroscience 5, 4-4 (2011).

Royal College of Obstetricians and Gynaecologists. **Antenatal corticosteroids to prevent neonatal morbidity and mortality.** Green-top guideline no. 7 (2010).

Saijo, K., Glass, C.K. **Microglial cell origin and phenotypes in health and disease.** Nature Reviews Immunology 11 (2011).

Saura, J., Angulo, E., Ejarque, A., Casadó, V., Tusell, J.M., Moratalla, R., Chen, J.F., Schwarzschild, M.A., Lluis, C., Franco, R., Serratosa, J. **Adenosine A_{2A} receptor stimulation potentiates nitric oxide release by activated microglia.** Journal of Neurochemistry 95 (2005).

Schwarz, J.M., Bilbo, S.D. **Sex, glia, and development: Interactions in health and disease.** Hormones and Behavior 62, 243-253 (2012a).

Schwarz, J.M., Sholar, P.W., Bilbo, S.D. **Sex differences in microglial colonization of the developing rat brain.** Journal of Neurochemistry 120, 948-963 (2012b).

Seasholtz, A. F., Thompson, R. C., Douglass, J. O. (1988). **Identification of a cyclic adenosine monophosphate-responsive element in the rat corticotropin-releasing hormone gene.** Molecular Endocrinology, 2(12), 1311-1319.

Silva, C.G., Métin, C., Fazeli, W., Machado, N.J., Darmopil, S., Launay, P.S., Ghestem, A., Nesa, M.P., Bassot, E., Szabó, E., Baqi, Y., Müller, C.E., Tomé, A.R., Ivanov, A., Isbrandt, D., Zilberter, Y., Cunha, R.A., Esclapez, M., Bernard, C. **Adenosine receptor antagonists including caffeine alter fetal brain development in mice.** Science Translational Medicine 5 (2013).

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fijomoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. **Measurement of protein using bicinchoninic acid.** Analytical Biochemistry 150, 76-85 (1985).

Sorrells, S.F., Caso, J.R., Munhoz, C.D., Sapolsky, R.M. **The stressed CNS: When glucocorticoids aggravate inflammation.** Neuron 64, 33-39 (2009).

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

Sperlagh, B., Illes, P. **Purinergic modulation of microglial cell activation.** Purinergic Signalling 3 (2007).

Stiles, G.L. **Adenosine receptors.** The journal of biological chemistry, 267:10(5) 6451-6454 (1992).

Sugama, S., Takenouchi, T., Fujita, M., Kitani, H., Conti, B., Hashimoto, M. **Corticosteroids limit microglial activation occurring during acute stress.** Neuroscience 232, 13-20 (2013).

Tremblay, M.E., Stevens, B., Sierra, A., Wake, H., Bessis, A., Nimmerjahn, A. **The role of microglia in the healthy brain.** Journal of Neuroscience 31, 16064-16069 (2011).

Van den Hove, D.L.A., Lauder, J.M., Scheepens, A., Prickaerts, J., Blanco, C.E., Steinbusch, H.W.M. **Prenatal stress in the rat alters 5-HT_{1A} receptor binding in the ventral hippocampus**. Brain Research 1090, 29-34 (2006).

Waffarn, F., Davis, E.P. **Effects of antenatal corticosteroids on the hypothalamic-pituitary-adrenocortical axis of the fetus and newborn: experimental findings and clinical considerations.** American Journal of Obstetrics and Gynecology 207, 446-454 (2012).

Weaver, D.R. **A_1 adenosine receptor gene expression in fetal rat brain.** Brain research. Developmental brain research 94, 205-223 (1996).

Weaver, D.R. **A_{2A} adenosine receptor gene expression in developing rat brain.** Brain research. Molecular brain research 20, 313-327 (1993).

Yeh, T.F., Lin, Y.J., Lin, H.C., Huang, C.C., Hsieh, W.S., Lin, C.H., Tsai, C.H. **Outcomes at school age after postnatal dexamethasone therapy for lung disease of prematurity.** New England Journal of Medicine 350, 1304-1313 (2004).

Yu, S.Y., Patchev, A.V., Wu, Y., Lu, J., Holsboer, F., Zhang, J.Z., Sousa, N., Almeida, O.F.X. **Depletion of the neural precursor cell pool by glucocorticoids.** Annals of Neurology 67, 21-30 (2010).