Helena Alexandra Ribeiro de Carvalho Pinheiro

GLUCOCORTICOID EFFECTS IN THE DEVELOPING BRAIN: A ROAD TO NEUROPSYCHIATRIC DISORDERS

Dissertação de Mestrado em Investigação Biomédica, orientada por Doutora Catarina Gomes e co-orientada por Doutor António Francisco Ambrósio e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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Cover image: primary hippocampal neurons from rat brain cultured *in vitro* for 12 days (blue: nuclei; white: neurons' cytoskeleton).

Glucocorticoid effects in the developing brain:
a road to neuropsychiatric disorders

Helena Alexandra Ribeiro de Carvalho Pinheiro

Dissertation presented to the Faculty of Medicine of the University of Coimbra to fulfill the necessary requirements to obtain the Master degree in Biomedical Research. The work was performed in the Retinal Dysfunction and Neuroinflammation Lab from the Institute of Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, under the supervision of Doctor Catarina A. Reis Vale Gomes and co-supervision of Doctor António Francisco Rosa Gomes Ambrósio.

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Se podes olhar, vê. Se podes ver, repara.

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(Epígrafe do Ensaio Sobre a Cegueira, de José Saramago)

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ABBREVIATIONS LIST

A

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 Adenocorticotropic hormone

ATP Adenosine 5'-triphosphate

B

BDNF Brain-derived neurotrophic factor

BSA Bovine serum albumine

C

cAMP Cyclic adenosine monophosphate

CNS Central nervous system

CRH Corticotropin releasing hormone

D

DAPI 4',6-diamidino-2-phenylindole

DEX Dexamethasone

DIV# Days *in vitro* (number)

E

ED# Embryonic day (number)

F

FBS Fetal bovine serum

G

GC Glucocorticoid

GR Glucocorticoid receptor

H

HBSS Hank's balanced salt solution

HPA Hypothalamic-pituitary-adrenal

I

Iba1 Ionized calcium-binding adapter molecule 1

L

LPS Lipopolysaccharide

M

MIF Mifepristone

MR Mineralocorticoid receptor

N

NGF Nerve growth factor

NT Non-treated

O

OCT Optimal cutting temperature compound

P

PBS Phosphate buffer solution

PFC Prefrontal cortex

PND# Post natal day (number)

R

RT Room temperature

S

SCH SCH58261; selective $A_{2A}R$ antagonist

T

TrkB Tropomyosin receptor kinase B

TUJ1 Neuron-specific class III beta-tubulin

ABSTRACT

The exposure to high levels of glucocorticoids during brain development, either due to pharmacological therapies or stress, can have a long term impact in the brain cytoarchitecture and behaviour, increasing the susceptibility to neuropsychiatric disorders.

Given the immunomodulatory role of glucocorticoids and the immunological fingerprint found in animals prenatally exposed to these hormones, microglial cells are likely implicated in the pathophysiology of these disorders. Several studies, including previous work by our group, show that prenatal exposure of rats to dexamethasone, a glucocorticoid widely used in clinics, leads to an anxious-phenotype in adulthood. These behavioural alterations were correlated with an atrophy of microglia at early stages of life, which was maintained in adulthood, in females' prefrontal cortex, a region implicated in the response to stress. Adenosine A_{2A} receptors, as regulators of microglia and involved in the pathophysiology of anxiety, emerge as possible molecular mediators of this process. Indeed, the chronic treatment with a selective antagonist of adenosine A_{2A} receptors, which was already described as anxiolytic, was able to revert the anxious behaviour in males, but not in females.

In the present thesis, our main aim was to characterize microglia morphology in the prefrontal cortex of adult male rats prenatally exposed to dexamethasone, in order to shed some light on the gender-specific response to treatment. By manual three-dimensional reconstruction of microglia cells, we report that *in utero* exposure to dexamethasone induces a long-term hyper-ramification of microglia (increased number and length of processes), contrasting with the results previously obtained with females in the prefrontal cortex. The chronic blockade of adenosine A_{2A} receptors, which aggravated females' atrophy, was able to ameliorate microglia hypertrophy in males, showing a correlation between microglia morphology and behavioural improvement. Moreover, the relevance of adenosine A_{2A} receptors for the maintenance of microglia morphology in physiological conditions seems to be gender biased, once the chronic blockade of adenosine A_{2A} receptors *per se* had a mild effect on microglia morphology in males, whereas in females it induced a strong atrophy.

Considering the contribution of microglia during development to neuronal cytoarchitecture and formation of synaptic networks, it is essential not to discard those effects. We then initiated a project using an *in vitro* model aiming at understanding the alterations induced by dexamethasone in neuron-microglia crosstalk during development. We started by characterizing the direct effect of the exposure to

dexamethasone in hippocampal neurons, at early stages of polarization and dendritic development (two and five days in culture, respectively). We report that the exposure to dexamethasone induces dimorphic alterations in axon and dendrites, characterized by a hypertrophy in axon length (mediated by adenosine A_{2A} receptors) and atrophy in dendrites (mediated by glucocorticoids receptors). Further analysis also revealed that the tonic activation of adenosine A_{2A} receptors may play an important role in neuronal polarization.

Thus, this work calls the attention to the importance of fully understanding the deleterious effects of the exposure to high levels of glucocorticoids during brain development in the crosstalk between several cell types in the brain, and to urgently take notice of gender differences in response to insults and pharmacological treatments.

KEYWORDS: brain development, glucocorticoids, microglia, hippocampal neurons, adenosine A_{2A} receptors, gender specificity

RESUMO

A exposição a níveis elevados de glucocorticóides durante o desenvolvimento, na sequência de tratamentos farmacológicos ou *stress*, pode ter um impacto a longo prazo na citoarquitectura neuronal e no comportamento, conduzindo a uma maior suscetibilidade a doenças neuropsiquiátricas.

Tendo em consideração o papel imunomodulador dos glucocorticóides e as marcas imunológicas em animais expostos a estas hormonas durante o período pré-natal, o envolvimento das células da microglia na fisiopatologia destes distúrbios é expectável. O nosso grupo demonstrou previamente que a exposição pré-natal de ratos a dexametasona, um glucocorticóide sintético frequentemente utilizado em terapêutica, conduz a um fenótipo ansioso na idade adulta. Estas alterações comportamentais estão associadas a uma atrofia das células da microglia logo após o nascimento, que foi mantida na idade adulta, no córtex pré-frontal (região associada à resposta ao *stress*) de ratos fêmea. Os recetores A_{2A} de adenosina, como reguladores da microglia e estando envolvidos na fisiopatologia de distúrbios de ansiedade, surgem como candidatos a mediadores moleculares deste processo. De fato, o tratamento crónico com um antagonista seletivo dos recetores A_{2A} de adenosina, que já foi descrito como ansiolítico, reverteu o perfil ansioso em ratos macho; no entanto, não reverteu o perfil ansioso nas fêmeas.

Na presente tese, o objetivo principal consistiu na caracterização da morfologia da microglia do córtex pré-frontal de machos adultos expostos a dexametasona no período pré-natal, para clarificar as diferenças entre géneros na resposta ao tratamento. Através de reconstrução manual e tridimensional da morfologia da microglia, reporta-se que a exposição a dexametasona induz uma hiper-ramificação da microglia (aumento do número e comprimento dos processos), a longo prazo, contrastante com os resultados previamente obtidos no córtex pré-frontal de ratos fêmea. O bloqueio crónico dos recetores A_{2A} de adenosina, que no caso das fêmeas agravou o fenótipo, foi capaz de melhorar a hipertrofia da microglia nos machos, indicando uma correlação entre a morfologia da microglia e a recuperação no fénotipo comportamental. Adicionalmente, a relevância dos recetores A_{2A} de adenosina para a manutenção da morfologia da microglia em condições fisiológicas poderá depender do género, uma vez que o bloqueio crónico *per se* dos recetores A_{2A} de adenosina teve um efeito moderado na morfologia da microglia em ratos macho, enquanto que nas fêmeas induziu uma atrofia elevada.

Considerando a importância da microglia durante o neurodesenvolvimento na citoarquitetura neuronal e na formação de circuitos sinápticos, é essencial não

desprezar estes efeitos. Assim, inicou-se um projeto, utilizando um modelo *in vitro,* com o objetivo de clarificar as alterações induzidas pela exposição a dexametasona na comunicação entre microglia e neurónios durante o desenvolvimento. Começou-se pela caracterização dos efeitos diretos da exposição a dexametasona em neurónios do hipocampo, em momentos cruciais de polarização e desenvolvimento dendrítico (dois e cinco dias em cultura, respetivamente). A exposição a dexametasona induziu alterações dimórficas no axónio e nas dendrites, caracterizadas pela hipertrofia do comprimento do axónio (dependente dos recetores A_{2A} de adenosina) e atrofia das dendrites (dependente dos recetores de glucocorticóides). Além disso, os dados obtidos sugerem que a ativação tónica dos recetores A_{2A} de adenosina tem um papel importante na polarização neuronal.

Deste modo, este trabalho evidencia a importância de compreender integralmente os efeitos deletérios da exposição a níveis elevados de glucocorticóides, durante o desenvolvimento neuronal, na comunicação entre os diversos tipos celulares, assim como a necessidade urgente de considerar as diferenças entre géneros na resposta a insultos e tratamentos farmacológicos.

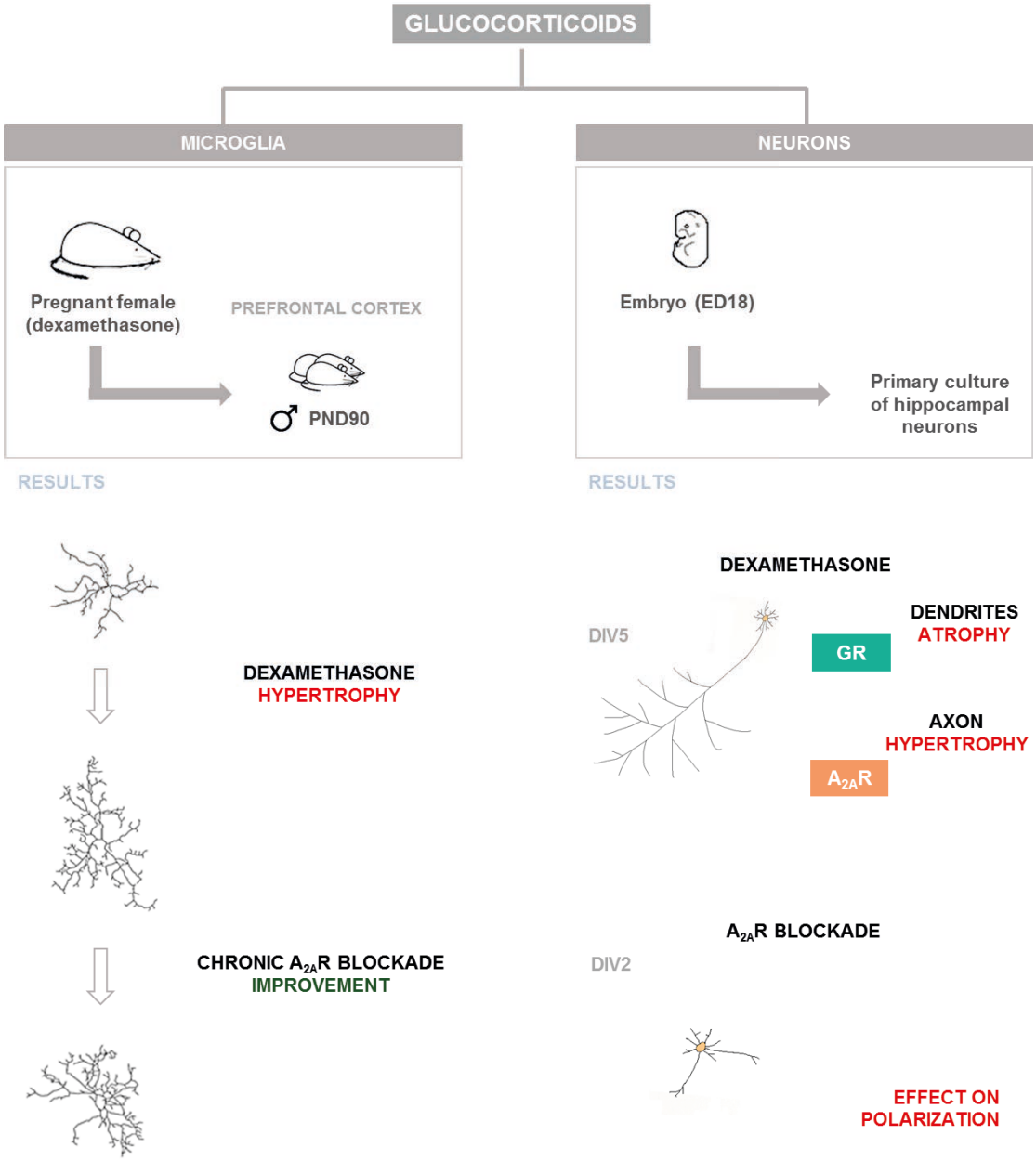
PALAVRAS-CHAVE: neurodesenvolvimento, glucocorticóides, microglia, neurónios do hipocampo, recetores A_{2A} de adenosina, especificidade de género

GRAPHICAL ABSTRACT

Graphical abstract summarizing the main results presented in this thesis, and the corresponding model.

In the left, the effect of dexamethasone and chronic treatment with $A_{2A}R$ selective antagonist in the morphology of microglia in the prefrontal cortex of adult male rats prenatally exposed to dexamethasone.

In the right, the effect of dexamethasone and $A_{2A}R$ selective antagonist exposure in hippocampal neurons primary cultures.



1. INTRODUCTION

1.1. OVERVIEW OF BRAIN DEVELOPMENT

The development of the human brain is a long and well regulated process initiated at the third week post conception, which occurs until late adolescence, arguably, throughout all lifespan. It's the outcome of a fine-tuned crosstalk between gene expression and environmental input (Stiles and Jernigan, 2010).

Brain development comprehends three major events: neuron production, neuron migration and neuron differentiation. In humans, neuron production begins on the embryonic day 42 (ED42) and is roughly complete by midgestation, except at very specific sites of neurogenesis, which remain active in adulthood (Ming and Song, 2011). After their production, neurons migrate to different brain areas and start to differentiate and form connections with other neurons, establishing the first neuronal networks (Stiles and Jernigan, 2010).

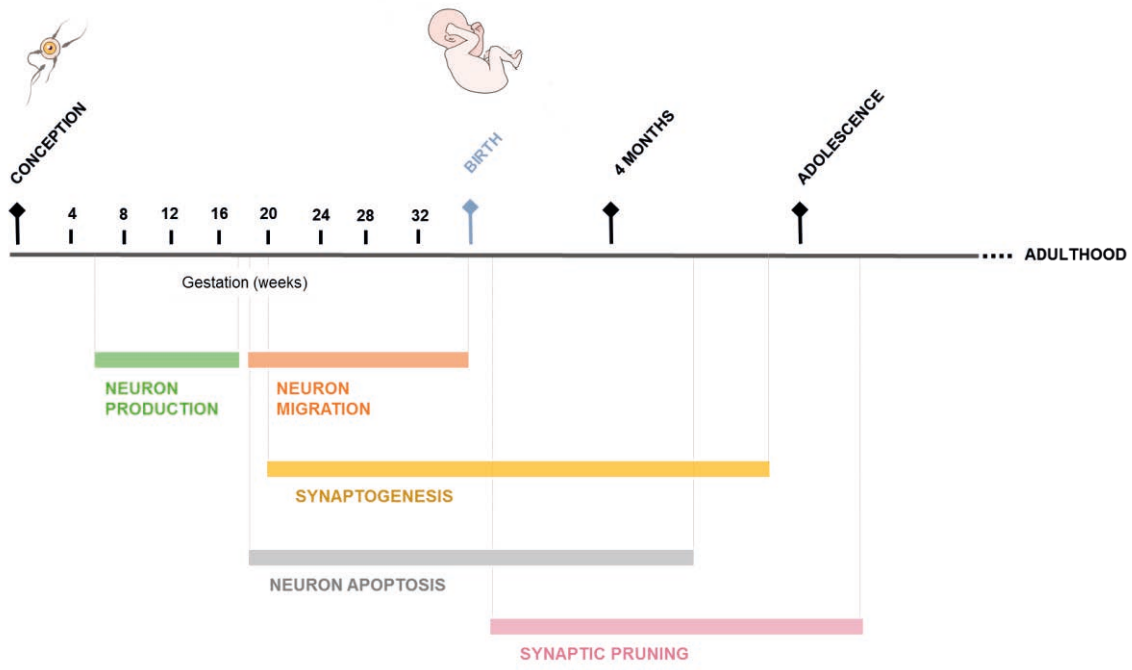


Figure 1| Timeline of brain development main events. Based on the reviews by (Stiles and Jernigan, 2010) and (Tau and Peterson, 2010).

1.1.1. Neuronal development

During the development of the central nervous system, neural stem cells generate neurons, as well as two types of glial cells: olygodendrocytes and astrocytes. The process responsible for the differentiation of these multipotent cells into neurons, and glia, is called neurogenesis and gliogenesis, respectively (Gotz and Huttner, 2005). The brain is also colonized by microglia; however, these cells derive from progenitors in the yolk-sac

(Ginhoux *et al.*, 2013). This topic will be further developed over the next section of this chapter.

After neurogenesis, neurons start migrating to their final location, differentiate, polarize, and establish synapses with other neurons, a process named synaptogenesis, which is the final aim of this process (Komuro and Rakic, 1998, Kriegstein and Noctor, 2004). Synaptogenesis is a long process which starts during pregnancy, and continues after birth (Huttenlocher and Dabholkar, 1997). With temporal heterogeneity between brain regions, the development of mature neuronal networks comprehends an exuberant formation of synapses and neurons, and their progressive elimination, by synaptic pruning and neuronal programmed cell death (Huttenlocher et al., 1982, Huttenlocher and Dabholkar, 1997). In this period of high developmental plasticity, the "best" neuronal circuits are being selected between the many competing inputs (Hensch, 2005). Thus, it is a critical period for the maturation of brain circuits, and any deleterious environmental stimuli can compromise their development, with negative repercussions in adult health (Groger et al., 2016).

1.1.2. Neuronal development in culture

In vitro, hippocampal neurons are also able to make synaptic contacts with one another, forming extensive networks, similar to the normal development of neuron networks in the brain (Bartlett and Banker, 1984b, Kaech and Banker, 2006).

The development of these cultures is widely characterized, and comprehends five major stages (Dotti et al., 1988, Kaech and Banker, 2006). In the first stage, right after the attachment of cells to the substrate, neurons develop highly motile lamellipodia (plate-like cytoskeletal extensions) around the cell body. In the second stage, lamellipodia are lost and there is an outgrowth of minor processes. These minor processes are undistinguishable from each other; however, in the third stage of development, one of these processes starts to elongate at a faster rate than the others, giving rise to the axon. This is the first evidence of neuronal polarity, and occurs between 12-24 h in culture. Stage 4, which begins in DIV (days *in vitro*) 3-4, corresponds to the elongation of the remaining minor processes, which become dendrites. By this stage, the polarization of the neuron is complete. Stage 5 corresponds to the maturation of the axonal and dendritic arborizations, forming the so-called neuronal network referred above (Bartlett and Banker, 1984b, Dotti et al., 1988).

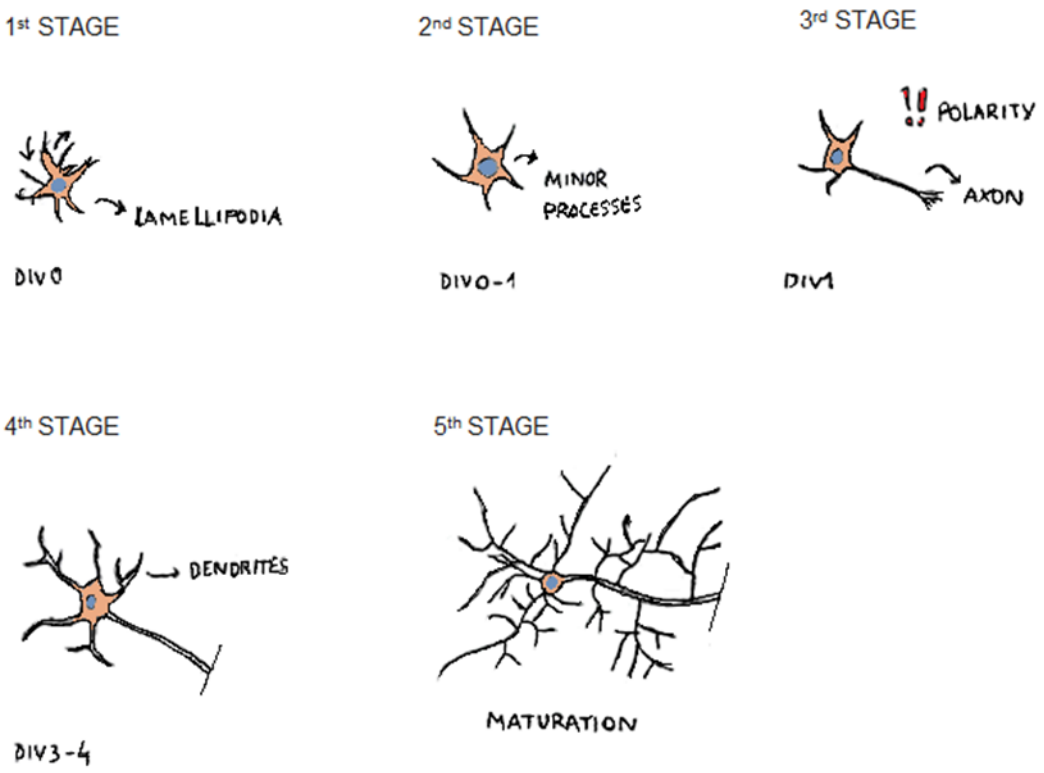


Figure 2| Scheme illustrating the five stages of embryonic hippocampal neurons development *in vitro*. Drawings based on the information from (Dotti et al., 1988).

In several aspects, the development of hippocampal neurons in culture mimics neuronal development *in situ*. The axons and dendrites in culture acquire the normal synaptic polarity, correspondent to the predominance of presynaptic axons and postsynaptic dendrites (Bartlett and Banker, 1984b). There is also a high similarity in gene expression in hippocampal neurons, *in vitro* and *in situ* (Dabrowski et al., 2003).

Dendrites and axons considerably differ in their morphology. While dendrites are thicker at the base and rapidly taper, axons are thinner next to the cell body, but maintain the diameter along their length. This allows an easy distinction between both (Bartlett and Banker, 1984a, Kaech and Banker, 2006).

Importantly, it was also shown that the stage of development of neurons *in situ*, does not compromise the normal progress in culture through the stages described above, once the neurons are reprogrammed when dissociated from the tissue (Fletcher and Banker, 1989). The combination of these aspects led to the wide use of embryonic hippocampal cultures (Goldin et al., 2001, Babu et al., 2009).

1.2. OVERVIEW OF MICROGLIA

In 1919, Pio del Rio-Hortega, distinguished microglia from oligodendrocytes for the first time. At the time, he was able to relate these cells in the brain to macrophages, given their migratory and phagocytic capacity (del Rio-Hortega 1919). In fact, for long, the role of microglia in the brain was solely associated to pathological conditions, and their inflammatory profile (van Rossum and Hanisch, 2004). However, this paradigm changed around 2005, by the observation that the "resting state" of microglia is in fact highly dynamic and motile in the healthy brain (Davalos et al., 2005, Nimmerjahn et al., 2005). This statement set the stage to study microglia in non-pathological conditions and their importance in neuronal connectivity.

1.2.1. From the embryonic to the adult brain

Microglia derive from monocyte/macrophages precursors, originated from primitive hematopoiesis in the yolk-sac (the main hematopoietic site in the early embryo) early in development (before ED8, in mice). They colonize the brain, actively proliferating during embryonic and postnatal development (Alliot et al., 1999, Ginhoux et al., 2013). The colonization of the CNS (central nervous system) by microglia is gender-specific. Males have higher number of microglia in early postnatal life, whereas females have a higher number later in development, and maintain it in adulthood (Schwarz et al., 2012). Microglia colonization is also accompanied by their differentiation. In the embryonic and early postnatal period, microglia have an amoeboid or primitive ramified morphology, and a higher capacity of proliferation. These microglia further differentiate into the ramified cells present in the adult brain (Dalmau et al., 2003).

In the human brain, a limited number of ameboid microglia are present at gestational week 4.5 (Andjelkovic et al., 1998), and accumulate in clusters. Fetal microglia are usually located at highly vascularized regions (Rezaie et al., 2005). From gestational weeks 9 to 14, the size and density of microglia clusters increase (Monier et al., 2006). As neurons start to populate and develop in the brain, there is a decrease in the necessity for clearance of neurons, and thus, a shift in microglia morphology occurs. From the middle of the second trimester, there is a decrease in amoeboid shaped microglia, accompanied by the increase in highly ramified microglia, bearing thin and long processes (Harry, 2013). After the gestational week 16, microglia are already distributed along the parenchyma and two weeks after, most cells already present the characteristic ramified morphology (Monier et al., 2006). Thus, the maturation of microglia morphology in the human brain comprehends the same stages as murine microglia.

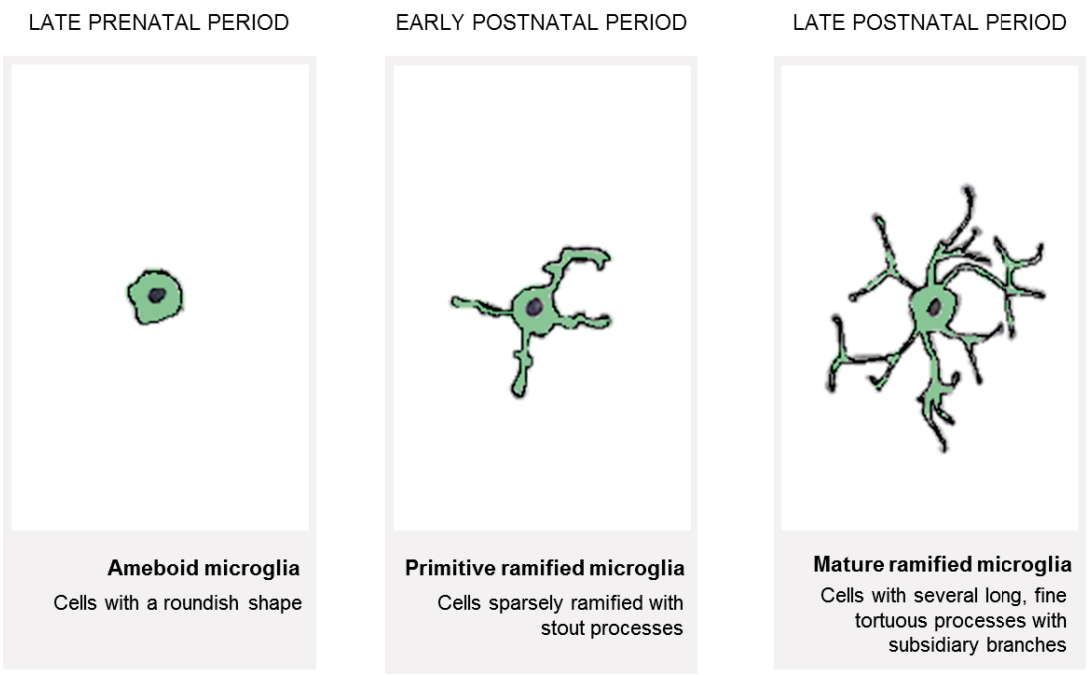


Figure 3| Main morphological stages of microglia during development. Drawings based on the representation of microglia morphologic development in the rat brain described in Dalmau *et al*., 2003.

By two-photon microscopy, the ramified morphology of adult microglia was shown to be highly dynamic under physiological conditions, with continuous extension and withdrawal of processes and motile protrusions (Nimmerjahn et al., 2005). Despite their high turnover, the number of protrusions in a cell is rather constant over time, as well as the overall length of the processes and the location of the soma. This structural organization of microglia allows the constant scanning of the brain, without disturbing the environment. It is estimated microglia are able to screen the complete brain parenchyma once every few hours (Nimmerjahn et al., 2005). Wake and colleagues also showed that microglia briefly contact with neuronal processes, around once every hour, which foresaw a role on the monitoring of normal neuronal function (Wake et al., 2009). The expression of receptors for all neurotransmitters (Kettenmann et al., 2011), further strengthens this hypothesis. Indeed, microglia contact with neuronal processes is directed to synapses and associated with increased neuronal activity (Wake et al., 2009). As a consequence of this interaction, it was described an impact on neuronal transmission, decreasing neuronal activity (Li et al., 2012).

Thus, the effective communication between microglia and neurons is essential for brain homeostasis. One of the most important forms of neuron-microglia crosstalk, in physiological and pathological conditions, is the expression of the receptor CX_3CR1 by microglia, and its interaction with its ligand - fractalkine - derived from neurons (Sheridan and Murphy, 2013).

The ramified morphology and constant structural changes are presumed to be important for their well described function of immune surveillance (Nimmerjahn et al., 2005). Microglia can respond rapidly to subtle changes in their environment, sensing them through a plethora of receptors, such as purinergic receptors (Davalos et al., 2005), fractalkine receptor (Pagani et al., 2015), receptors for complement proteins, cell adhesion molecules and inflammatory cytokines/chemokines (Rock et al., 2004); and can respond to these changes by the production and release of pro- and anti-inflammatory cytokines and neurotrophic factors, engulfment of cellular debris (Chen and Trapp, 2016) and displacement of synaptic terminals (Chen et al., 2014).

Although maintaining a proper identity, microglia in the adult brain are highly heterogeneous between region, with differences in gene expression and sensitivity to ageing (Doorn et al., 2015, Grabert et al., 2016), as well as in density and morphology (Lawson et al., 1990, Mittelbronn et al., 2001).

1.2.2. Role in the developing brain

The conservation, between different species, of the early colonization of the brain by microglia raises the idea of its involvement in the development of the CNS (Alliot et al., 1999, Cuadros and Navascues, 2001, Herbomel et al., 2001, Verney et al., 2010). Indeed, microglia intimately participate in several milestones of neuronal development.

Very early in development, during neurogenesis, microglia are able to phagocytose neuronal precursor cells, regulating their number (Cunningham et al., 2013). During programmed neuronal apoptosis, microglia induce neuronal cell death (Wakselman et al., 2008) and rapidly digest dying neurons, preventing the diffusion of degradation products (Peri and Nusslein-Volhard, 2008).

Importantly, the involvement of microglia in synaptic pruning was also described (Paolicelli et al., 2011). Microglia actively phagocytose synapses in a process dependent on neuron-microglia communication, once its disruption was demonstrated to compromise this process (in CX3CR1 KO mice), leading to an increase in the density of spines and immature synapses (Paolicelli et al., 2011). In a later study, the specific pruning of pre-synaptic terminals with lower neuronal activity was described (Schafer et al., 2012).

However the understanding of which synapses are eliminated is not clear; a widely accepted hypothesis defends that synaptic elimination results from competition between them. In the presence of stronger and more efficient neighbouring synapses, a weaker

synapse is eliminated. This process could be mediated by protection of stronger synapses and/or tagging of the weaker synapses for elimination (Stephan et al., 2012).

In what respects the recognition of synapses by microglia, the importance of the classic complement cascade was already observed (Stevens et al., 2007, Schafer et al., 2012). Stevens and colleagues showed that C1q, the initial protein of the complement cascade, is up-regulated in the CNS during development, and is localized at synapses. According with the hypothesis of tagging synapses for elimination, mice lacking C1q or the downstream protein C3 have deficiencies in synapse elimination (Stevens et al., 2007). The importance of C3 was also described for the engulfment of pre-synaptic terminals (Schafer et al., 2012).

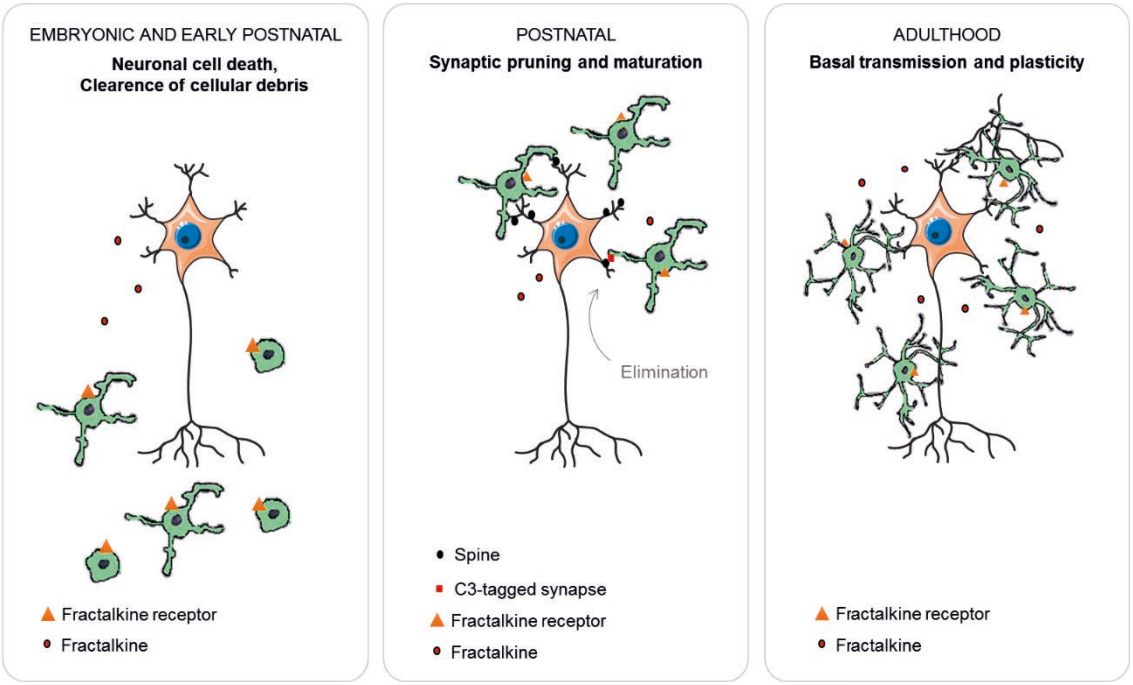


Figure 4| Representative summary of microglia main functions in the developing and mature CNS. Based on the review by (Schafer et al., 2013).

In a contrasting aspect in the regulation of synapses, our group demonstrated that microglia are also able to promote synapse formation, after priming with an inflammatory stimuli (Cristovao et al., 2014). In fact, microglia promotion of synapse formation was shown to be induced both through brain-derived neurotrophic factor (BDNF) (Parkhurst et al., 2013) and interleukin-10 (Lim et al., 2013).

These data suggest microglia have an essential role in the maturation of neuronal circuits. Ergo, during development, any alteration in the communication between microglia and neurons or in their ability to phagocytose can potentially have irreversible impacts on brain wiring, and lead to neuropsychiatric disorders (Paolicelli and Gross, 2011, Zhan et al., 2014).

1.3. DEVELOPMENTAL ORIGIN OF NEUROPSYCHIATRIC DISORDERS

The development and maturation of neuronal circuits can be affected by tenuous modulations in environmental factors, and any alteration during this important phase can have irreversible consequences (Furukawa et al., 1999).

Prenatal or early-life stressful experiences can lead to the activation of certain neuronal circuits and increase the susceptibility to neuropsychiatric disorders later in life (Sousa, 2016).

Structural abnormalities induced by stress are mainly observed in regions of the brain linked to emotional responses, such as the prefrontal cortex (PFC), hippocampus and amygdala (Shirazi et al., 2015). This provides a link between developmental adversity and emotional related disorders.

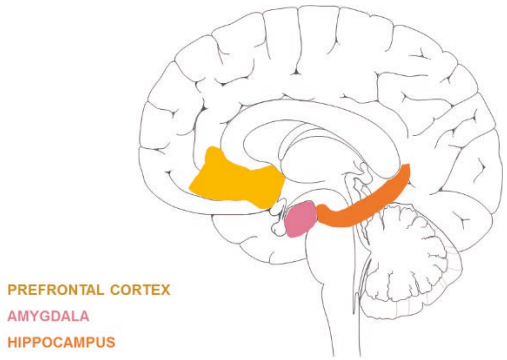


Figure 5| Location in the brain of the main regions associated to neuropsychiatric disorders: the prefrontal cortex (gold), the amygdala (purple) and the hippocampus (orange).

1.3.1. Regulation of stress: the HPA axis

Glucocorticoids (GC) are steroid hormones with a wide range of physiological effects, produced according to a circadian rhythm, and in stressful situations (Smith and Vale, 2006, Chung et al., 2011). Stress increases the production of GC, which in turn regulate a wide range of physiological changes, aiming to surpass the stressful event and restore homeostasis (Smith and Vale, 2006).

The levels of the endogenous glucocorticoid hormone, cortisol (in humans and non-human primates) and corticosterone (in rodends), are tightly regulated by the hypothalamic-pituitary-adrenocortical axis (HPA axis). Briefly, the cascade of events culminating in the production of GC by the adrenal glands begins with the release of corticotropin-releasing hormone (CRH) by the hypothalamus. CRH acts on the pituitary gland, stimulating the secretion of adenocorticotropic hormone (ACTH) into circulation. ACTH induces the

synthesis and release of GC by the adrenal glands. The regulation of the HPA axis is under a negative feedback regulation, in which high circulating levels of GC bind to receptors in the brain and normalize GC production (Waffarn and Davis, 2012).

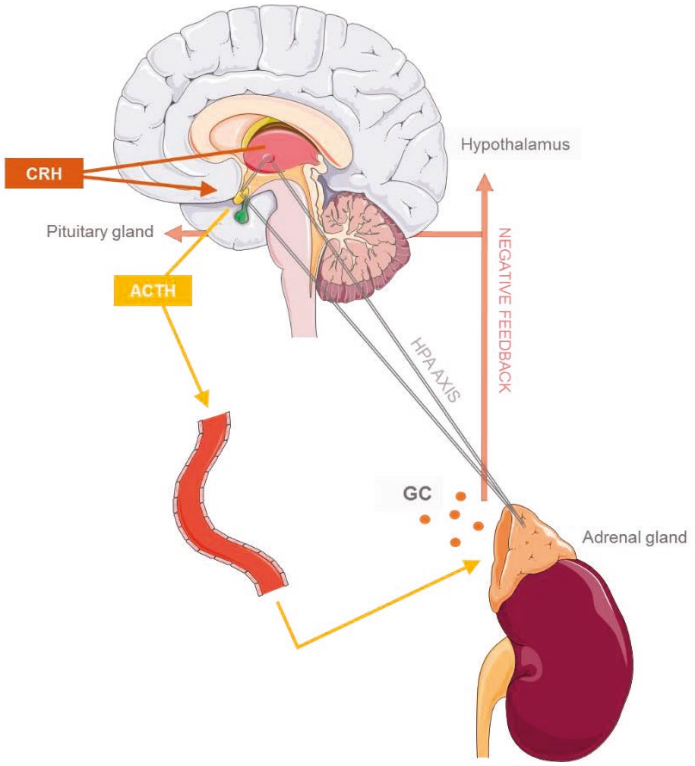


Figure 6| Regulation of the hypothalamic-pituitary-adrenocortical axis. The hypothalamus produces CRH when activated by the circadian rhythm or stress and stimulates the pituitary gland to produce ACTH. This culminates with GC production by the adrenal glands. Based on information from (Waffarn and Davis, 2012).

Once in the circulation, GC exert a plethora of effects at the peripheral level. In the brain, GC cross the blood-brain barrier and bind to mineralocorticoid (MR) and glucocorticoid (GR) receptors. Since endogenous GC have a higher affinity to MR, low levels of GC bind preferentially these receptors, which regulate responses to resting levels of hormone. Conversely, GR are only activated by higher levels of GC, hence are thought to mediate responses related to stress (Myers et al., 2014).

In the fetus, the regulation of GC levels is one of the major controlling mechanisms for the transition to the extra-uterine life. During pregnancy, the levels of GC in the fetus are much lower than the circulating levels in the mother, due to their conversion in inactive metabolites by the placental enzyme 11β-hydroxylase 2 (also present in the brain), whose expression decreases at the end of gestation (Mesquita et al., 2009). Towards the end of pregnancy, the intrauterine levels of GC rise, inducing fetal maturation (Thorburn et al., 1977). However, under stress conditions, the fetal HPA axis is activated in earlier stages of development,

and the levels of cortisol raise before the proper time. Consequently, the earlier induction of tissue differentiation can have detrimental effects in the organism later in life (Fowden et al., 1998).

Thus, the administration of GC during prenatal and early life development is especially concerning. However, synthetic GC, such as dexamethasone (DEX) are administrated in women at risk of preterm delivery, to accelerate fetal lung maturation, and are a crucial clinical tool to increase survival probability (Brownfoot et al., 2008). The current available synthetic GC, which usually are not substrates to 11β-hydroxylase 2, are up to 20 times more potent than endogenous GC and have higher affinity to GR (contrasting with endogenous GC), inducing different mechanisms likely implicated in their detrimental effects (Fowden and Forhead, 2015). The antenatal exposure to synthetic GC was shown, in humans and animal models, to have long-term effects on HPA axis regulation (Nagano et al., 2008), brain structure and behaviour, neurosensory, neuroendocrine and cardio-metabolic functions. Beyond that, epigenetic modifications due to prenatal synthetic GC can be inherited by the second and third-generations (Constantinof et al., 2016).

1.3.2. The effects of brain developmental exposure to high levels of glucocorticoids

1.3.2.1. IMPACT ON BRAIN CIRCUITS

Several regions in the brain were shown to be important in response to stress. Commonly, these regions are associated to express high levels of GR, as the hippocampus and the amygdala (Patel et al., 2000). The PFC, which expresses GR, also plays a central role in the regulation of the HPA axis, as it was shown by demonstrating that damage to the PFC resulted in a dampened HPA response (Sullivan and Gratton, 1999).

Alterations in the circuits associated to prenatal high levels of GC have already been reported. A brief antenatal exposure to DEX induces long-term behavioural alterations, such as decreased locomotor activity and exploratory behaviour, increased susceptibility to depressive-like behaviour (Oliveira et al., 2006), anxious-behaviour and altered fear-response in adulthood (Oliveira et al., 2012). The behavioural phenotype is accompanied by contrasting alterations in dendritic arborization in the bed nucleus of the stria terminals (also referred as the extended amygdala) and the amygdala. In the first, there is an increase in dendritic length, whereas the amygdala is characterized by dendritic atrophy (Oliveira et al., 2012). In the same model, changes were also observed in the nucleus accumbens, associated to the reward circuit, consisting in a decrease in cell density and in dopaminergic innervation (Leao et al., 2007), which is probably linked to drug-seeking behaviour (Rodrigues et al., 2012).

Antenatal exposure to GC affects the normal development of the hippocampus, leading to a decrease in the size and an increase in the number of apoptotic cells during early life, alterations which are not maintained until adulthood (Noorlander et al., 2014). Also, in a model of maternal separation, alterations in the hippocampal structure were reported, specifically an atrophy of mossy fiber density (Huot et al., 2002) and dendrites (Batalha et al., 2013).

The prenatal exposure to GC also induces disturbances in HPA axis regulation, decreasing the levels of GC in the plasma, in response to restrain stress, which preceded the behavioural phenotype (Nagano et al., 2008).

1.3.2.2. IMPACT ON IMMUNE RESPONSE

From an historical point of view, GC are strongly associated to their anti-inflammatory response. Currently, this is not so clear, since several reports have also demonstrated the influence of GC as a priming to neuroinflammatory response, sensitizing the system before immunological challenges (Sorrells and Sapolsky, 2007).

However, it is clear that stress/GC levels can affect microglia, once these cells are equipped with receptors for steroid hormones, namely GR and MR (Sierra et al., 2008). In fact, chronic stress was shown to lead to dynamic, bi-directional alterations in microglia, characterized by an initial phase of proliferation and a later phase of apoptosis and morphologic dystrophy (decrease in the soma area and length of processes) (Kreisel et al., 2014).

Also, an increase in the motility of microglia was reported in young adult male mice, in response to an early-life stressful stimulus, maternal deprivation (Takatsuru et al., 2015), showing that, despite their dynamic identity, alterations in microglia can be maintained for a long period.

Likewise, the inflammatory profile of microglia can also have long-term alterations. Diz-Chaves and colleagues reported that prenatal stress induces an increase in the expression of proinflammatory cytokines in response to an immune challenge in adulthood (Diz-Chaves et al., 2013).

Ultimately, it is already widely accepted that early-life adversities have permanent effects in the modulation of immune responses (Fagundes et al., 2013). Additionally, neuroinflammatory disturbances have been implicated in a range of neuropsychiatric disorders, such as depression, obsessive-compulsive disorder and schizophrenia (Ganguly and Brenhouse, 2015).

1.4. OVERVIEW OF THE ADENOSINERGIC SYSTEM

Besides their intracellular roles, purines such as adenosine 5'-triphosphate (ATP) act as extracellular signalling molecules, in both neuronal and non-neuronal cells. Adenosine can be synthesized, either in the cytoplasm or in the extracellular space, by the breakdown of ATP by ectonucleotidases. Additionally, it can also be synthesized from the aminoacid S-adenosyl-L-homocysteine. Adenosine is then transported by bidirectional nucleoside transporters between the intra and extracellular spaces (Figure 7) (Sitkovsky et al., 2004).

Three types of receptors are associated to the purinergic metabolism, P1, P2X and P2Y, each with several subtypes. Adenosine activates P1 receptors, which have four subtypes: A_1, A_{2A}, A_{2B} and A_3 (Burnstock, 2007).

1.4.1. Adenosine A_{2A} receptors in the brain

P1 receptors are coupled to G proteins, which can either activate or inactivate adenylate cyclase. The activation of A_1 and A_3 negatively regulates adenylate cyclase, whereas A_{2A} and A_{2B} are positively coupled to this enzyme (Fredholm, 1995).

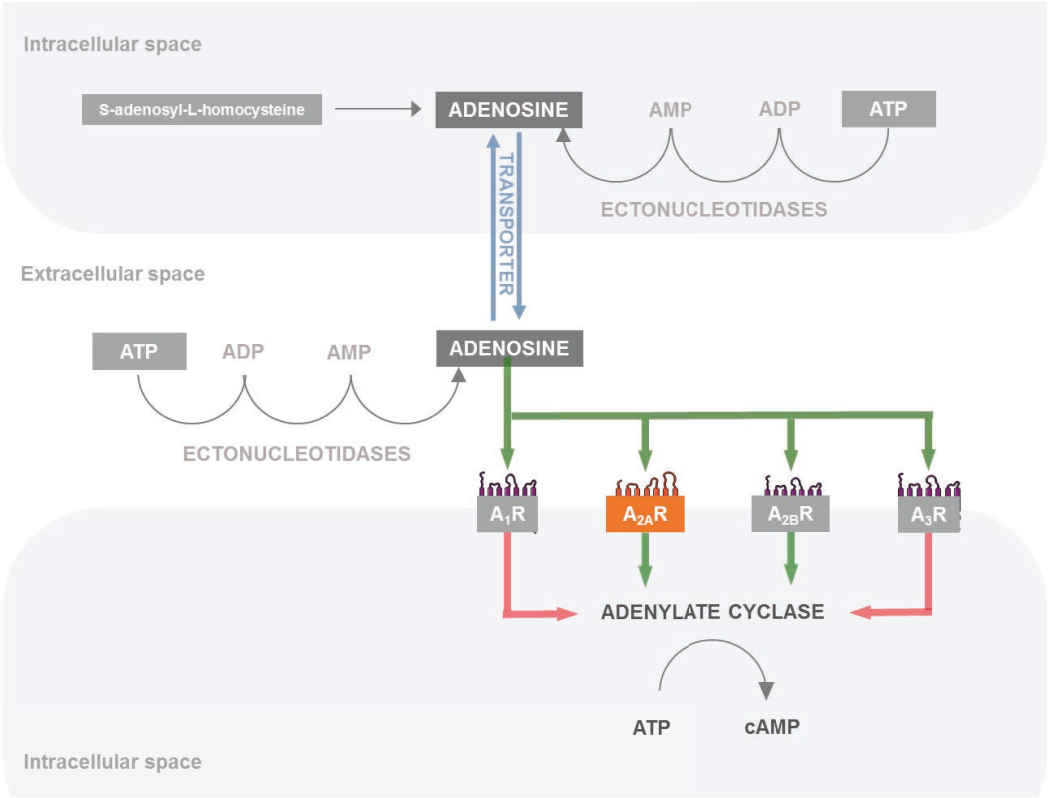


Figure 7| Adenosine synthesis and intracellular response of its binding to P1 receptors. Adenosine is produced from S-adenosyl-L-homocysteine, and ATP breakdown. It is transported by bidirectional nucleoside transporters and binds to A_1, A_{2A}, A_{2B} and A_3 receptors. A_1 and A_3 receptors inhibit the conversion of ATP into cyclic adenosine monophosphate (cAMP), whereas A_{2A} and A_{2B} receptors stimulate it.

The activation of $A_{2A}R$ in the brain is best known for its importance as a modulator of synaptic transmission and plasticity (Sebastiao and Ribeiro, 2009). However, the time-dependent variations of the expression of this receptor during embryonic and early postnatal life (Aden et al., 2000) indicates it also plays a role in development. Expression of $A_{2A}R$ mRNA in the rat brain was reported soon at embryonic day 14, and the levels rise during development (Weaver, 1993, Aden et al., 2000). In the early postnatal brain, the levels of $A_{2A}R$ expression are higher, decreasing after 21 days until adulthood, except in the caudate putamen, where the expression of $A_{2A}R$ remains high (Johansson et al., 1997).

In the case of neuropsychiatric disorders, the involvement of $A_{2A}R$ has also been pointed, as well as the potential of its inhibition as a therapeutic target (Cunha et al., 2008).

In fact, the genetic deletion or pharmacological blockade of $A_{2A}R$ were shown to be beneficial in a model of chronic stress, both as a preventive and a therapeutic tool (Kaster et al., 2015). Accordingly, both genetic and pharmacological inactivation of these receptors were reported to have antidepressant effect (El Yacoubi et al., 2001).

Yet, knock-out animals for $A_{2A}R$ present an anxious-behaviour (Kaster et al., 2015), what reinforces its importance during development.

1.4.1.1. EFFECTS ON NEURONAL MORPHOLOGY

The effect of adenosine on neuronal morphogenesis was reported for the first time in 1995, by Heilbronn and Zimmermann, in a study showing that increased levels of adenosine during development stimulates neurite outgrowth (Heilbronn and Zimmermann, 1995). Since this report, other studies in the neuronal differentiated PC12 cell line have demonstrated that $A_{2A}R$ activation contributes to the increase in the number and length of neurites (Cheng et al., 2002, Charles et al., 2003).

However, in *in vivo* models, animals treated during the first postnatal days with caffeine, a natural antagonist of $A_{2A}R$ and A_1R, showed an increase in dendritic length and branching, which was maintained until postnatal day 70 (PND70) (Juarez-Mendez et al., 2006). In the PFC, the treatment with an $A_{2A}R$ antagonist in adulthood was shown to increase dendritic branching in hippocampal pyramidal neurons (Batalha et al., 2013), in a model of maternal separation.

It was also observed that the treatment with caffeine during gestation led to a delay in the migration of neurons in the hippocampus (Silva et al., 2013), which could be explained by an impairment in axonal development. Indeed, *in vitro*, Ribeiro and colleagues reported, for the first time, that $A_{2A}R$ activation increases axonal elongation during neuronal

development, by stimulating microtubule instability and growth speed. In the same study, it was also shown that the activation of $A_{2A}R$ led to an increase in dendritic branching, in a mechanism dependent on BDNF (Ribeiro et al., 2016).

1.4.1.2. EFFECTS ON MICROGLIA

$A_{2A}R$ have also been shown to regulate several aspects of microglia function, including a modulation of their inflammatory profile. Rebola *et al*. showed that the blockade of $A_{2A}R$, by administration of an antagonist, is able to decrease the expression of activation markers in microglia in the hippocampus, in response to lipopolysaccharide (LPS) stimulus, indicating a role of this receptor in neuroinflammation mediated by microglia (Rebola et al., 2011). The activation of $A_{2A}R$ was also shown to potentiate the release of NO (Saura et al., 2005), cyclooxygenase-2 and prostaglandin E2 (Fiebich et al., 1996).

Not accordingly, in primary murine microglia, the activation of the receptor was reported to supress inflammatory stimuli-dependent production of TNF-α and CXCL10, both pro-inflammatory molecules (Newell et al., 2015). Thus, the role of the activation of $A_{2A}R$ on microglia cannot be simply categorized as pro- or anti-inflammatory. In fact, different danger signals have a differential impact on the modulation of the purinergic system, and therefore lead to contrasting responses by microglia (George et al., 2015).

The release of neurotrophic factors by microglia can also be under the regulation of $A_{2A}R$, as it was made evident by the stimulation of BDNF (Gomes et al., 2013) and nerve growth factor (NGF) release (Heese et al., 1997), upon $A_{2A}R$ activation.

In order to respond properly to stimuli, microglia are known to adapt their morphology. It was shown that, in response to inflammation, the retraction of microglia processes is also dependent on the activation of $A_{2A}R$ (Gyoneva et al., 2009, Orr et al., 2009, Gyoneva et al., 2014a, Gyoneva et al., 2014b).

These data indicate that several microglial cells responses and functions are modulated by the levels of adenosine.

2. RATIONALE AND AIMS

The exposure to high levels of GC during the development of neuronal circuits, both due to pharmacological treatments or stress, can have a permanent impact and lead to neuropsychiatric disorders later in life. Given the immunomodulatory role of GC and the crucial roles of microglia shaping the brain circuits, we hypothesized a contribution of microglia for the etiology of these disorders.

Indeed, previous data regarding the female offspring of rats treated with DEX during pregnancy, showed morphological alterations in microglia, which were maintained until adulthood, and correlated with an anxious behaviour (Henriques, 2015). Importantly, irregardless of the gender, there were morphological alterations in microglia at PND1 and PND7, crucial stages of neurodevelopment (Caetano, 2014). Considering the ability of $A_{2A}R$ to control and modulate microglia function and morphology, the blockade of $A_{2A}R$ as a therapeutic strategy was addressed, and it was reported that the treatment has contrasting gender-specific effects. Whereas in the female offspring it was not able to recover, either the morphological alterations in microglia or behavioural features (Henriques, 2015), the behaviour in the male offspring was reverted (unpublished data).

Considering the previous data, the main aims of this thesis were:

- To determine if prenatal exposure to DEX leads to permanent morphological alterations in microglia from males;
- To assess if the recovery of behavioural alterations in males upon $A_{2A}R$ treatment correlates with morphological alterations in microglia.

Once the main aims of the thesis were fulfilled, we initiated a project using an *in vitro* model with the purpose to shed light on the influence of GC treatment in the interaction between microglia and neurons during neuronal development.

The main aims of this project were:

- To establish a low density primary culture of hippocampal neurons, which allows the assessment of neuronal morphology during development;
- To analyse the morphological alterations induced by DEX treatment directly on neurons;
- To assess the potential of $A_{2A}R$ blockade as a preventive tool;
- To establish a co-culture of low-density primary hippocampal neurons and primary microglia;
- To analyse the influence of microglia upon neuronal morphogenesis, under the influence of DEX;

- To assess the potential of $A_{2A}R$ blockade as a preventive tool, in the presence of microglia;
- To evaluate the influence of DEX and $A_{2A}R$ antagonist exposure in synaptic formation.

This is an on-going work and, at the present time, we were able to complete the first three main aims referred above, whose results are featured in this thesis.

3. METHODS

3.1. REAGENTS

Table 1| Reagents

Reagent	Supplier
2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES)	Sigma Aldrich
4',6-diamidino-2-phenylindole (DAPI)	Invitrogen
B-27 Supplement (B27)	Alfagene
Bovine Serum Albumine (BSA)	NZYtech
Ethylene Glycol	Sigma Aldrich
Fetal Bovine Serum (FBS)	Alfagene
Gentamycin	Alfagene
Glucose	Sigma Aldrich
Glutamate	Sigma Aldrich
Glycergel mounting medium	DAKO
Isofluorane	Sigma Aldrich
L-Glutamine	Sigma Aldrich
Neurobasal Medium	Thermo-Fischer
Optimal Cutting Temperature Compound (OCT)	Thermo Scientific
Paraformaldehyde	Sigma Aldrich
Poly-D-Lysine	Sigma Aldrich
Potassium Chloride (KCl)	Merck
Potassium Dihydrogen Phosphate (KH_2PO_4)	Sigma Aldrich
Potassium Phosphate Dibasic (K_2HPO_4)	Sigma Aldrich
Sucrose ($C_{12}H_{22}O_{11}$)	Sigma Aldrich
Sodium Chloride (NaCl)	Sigma Aldrich
Sodium Dihidrogen Phosphate Monohydrate ($NaH_2PO_4.H_2O$)	Merck
Sodium Hydrogen Carbonate ($NaHCO_3$)	VWR International
Sodium Hydrogen Phosphate (Na_2HPO_4)	Sigma Aldrich
Sodium Pyruvate	Alfagene
Sucrose	Sigma Aldrich
Triton X-100	Sigma Aldrich
Trypsin	Sigma Aldrich

3.2. DRUGS

Table 2| Drugs used in *in vivo* and *in vitro* pharmacological treatments

Drug	Supplier	Concentration (*in vitro* ; *in vivo*)	Function
(Dexamethasone; $C_{22}H_{29}FO_5$)	Acros Organics	1 μM ; 1 mg/kg s.c.	Synthetic GC
7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4 triazolol[1,5c]pyrimidine (SCH58261; $C_{18}H_{15}N_7O$)	Tocris	50 nM ; 0.1 mg/kg/day for 21 days	$A_{2A}R$ selective antagonist
11β-[p-(Dimethylamino)phenyl]-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one (Mifepristone; RU486; $C_{29}H_{35}NO_2$)	Tocris	1 μM ; --	GR antagonist

3.3. ANTIBODIES

Table 3| Primary and secondary antibodies used in immunohistochemistry and immunocytochemistry

Antibody	Supplier	Host	Type	Dilution	Function
Anti Iba1	Wako Cat.#019-19741	Rabbit	Polyclonal	1:1000	Microglia marker
Anti TUJ1	Covance PRB-435P-100	Rabbit	Polyclonal	1:1000	Neuronal marker
Anti-rabbit Alexa Fluor 488	Thermo-Fisher A-11008	Goat	Polyclonal	1:1000	Detection of primary antibodies

3.4. EXPERIMENTS WITH SAMPLES FROM AN ANIMAL MODEL

3.4.1. Animal model of anxiety

Wistar rats were kept at 22°C and 55% relative humidity in a dark/light cycle of 12 h each (lights on at 8:00 am). Food and sterile tap water were available *ad libitum*. Pregnant females were injected subcutaneously with dexamethasone (DEX, 1 mg/kg) or saline (NT), at days 18 and 19 of the gestation period (Oliveira et al., 2006). Animals from the offspring were treated during the three last weeks before PND90 with the selective $A_{2A}R$ antagonist, SCH58261 (0.1 mg/kg/day, SCH) or saline by intraperitoneal injection (Figure 8). This protocol of chronic administration has been described as anxiolytic in adult rodents subjected to stress protocols (Kaster et al., 2015). Animals were anesthetized, perfused with saline followed by 4 % paraformaldehyde solution, and sacrificed by decapitation. Brain regions were dissected on ice using a stereomicroscope and stored at - 80°C. Animal manipulation was done according to the local regulations (European Union Directive 2010/63/EU) and National Institute of Health (NIH) guidelines for animal care and experimentation.

The pharmacological treatments and maintenance of the animals, which gave origin to the brain samples used in this part of the work, were conducted by others in the facilities and with the assistance of researchers from the Life and Health Sciences Research Institute (University of Minho).

Figure 8| Schematic view of the animal model pharmacological treatment

3.4.2. Immunohistochemistry and 3D morphometric analysis of microglia

Adult rat brains (PND90) were sectioned using a cryostat (Leica CM3050S, Germany) set at - 21°C (chamber temperature) and - 19°C (object temperature). Involved in OCT, brains were aligned in the cutting platform. 50 µm slices were transferred to 24 well plates filled with cryoprotection solution (50 mM $NaH_2PO_4.H_2O$, 50 mM K_2HPO_4, 30% sucrose, 30% ethylene glycol, diluted in MilliQ H_2O, pH 7.2) and stored at - 20°C.

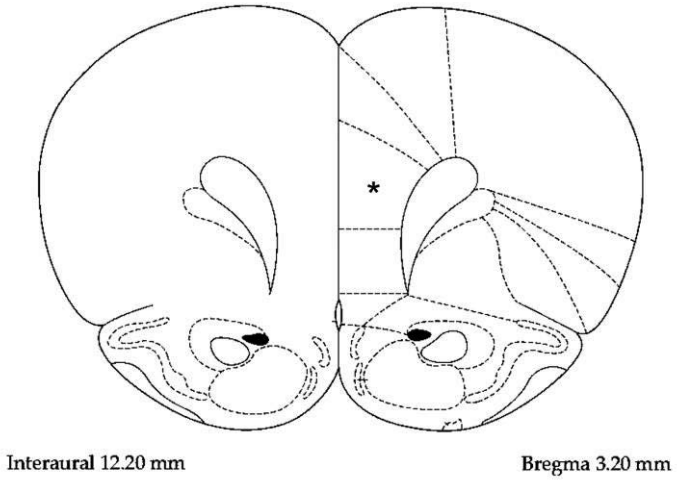


Figure 9| Representative image of the rat brain in the analysed section. (*) indicates the region acquired by confocal microscopy after immunohistochemistry, from *(Paxinos and Watson, 1998)*

For immunodetection of microglia, free-floating prefrontal sections located at the stereotaxic coordinates of interaural 12.20 mm and bregma 3.20 mm (Figure 9) (Paxinos and Watson, 1998) were washed three times with phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM $NaH_2PO_4.2H_2O$, 1.8 mM KH_2PO_4 in miliQ water, pH = 7.4), 10 min each time, in mild agitation. To allow the access of the antibodies to the respective antigen and increase specific binding, the sections were incubated in a permeabilization and blocking solution of 5% BSA and 0.1% Triton X-100 in PBS, for 2 hours at room temperature (RT) in mild agitation. The same solution was used to dilute the antibodies and DAPI. Incubation of slices with the primary antibody (1:1000, rabbit anti-Iba1) took place for 48 hours at 4°C, in mild agitation. After washing the excess of primary antibody with PBS at RT, three times, 10 min each time, the sections were incubated with the secondary antibody (1:1000, goat anti-rabbit) for 2 hours at RT in mild agitation and washed again with PBS as described. The sections were incubated with DAPI (1:5000) for 10 min, at RT, to stain cell nucleus (DAPI is a fluorescent dye which binds strongly to DNA). Negative controls (incubation with secondary antibody only) were included. After washing the excess of DAPI, sections were mounted on gelatinized microscope slides using glycergel mounting medium, covered with coverslips and allowed to air dry overnight. After sealing the coverslips with transparent nail polish, the slides were kept in the dark at 4°C.

Both primary and secondary antibodies solutions were centrifuged for 20 min at 16000 g, 4°C, in order to precipitate antibody aggregates.

Images of 15 random microglial cells from each animal were acquired in the PFC (region indicated by an asterisk in *Figure 9*), with a laser scanning confocal microscope LSM 710 META connected to ZEN Black software (Zeiss Microscopy, Germany), using a 63x objective lens (oil immersed, Plan-Apochromat 63x/1.40 Oil DIC M27). Settings were chosen to optimize the labelling of microglial processes, the structures of main interest in this study, and kept throughout all sets of acquisitions.

After importing Z-stacks to the Neurolucida software (MBF Bioscience, USA), an optimization allowed microglia to be manually drawn along the acquired planes, granting a 3D image of each cell. For each individual, 15 cells were reconstructed. The criterion established for the reconstruction of the cell processes was that all ramifications, regardless their length, should be taken into consideration, so that the final result was as representative of the original cell as possible. Morphometric data (branched structure analysis) was extracted by Neurolucida Explorer, an extension of Neurolucida software, and the number and length of processes *per* branch order was analysed. Processes emerging directly from the cell body were categorized as belonging to branch order 1, the ones proceeding from ramifications of these processes were considered order 2, and so forth, as schematized in Figure 10.

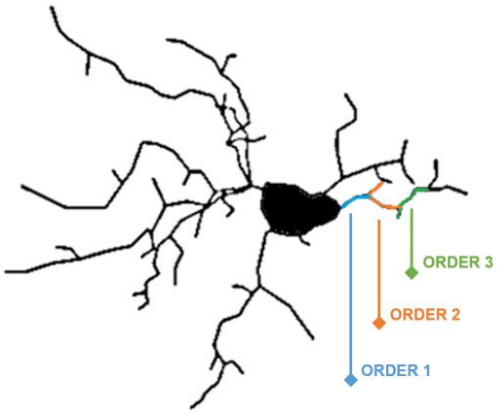


Figure 10| Representative scheme showing the discrimination of microglial processes by branch order.

3.5. *IN VITRO* EXPERIMENTS

3.5.1. Culture of rat hippocampal neurons

Primary cultures from hippocampal neurons were obtained from Wistar rats, as previously described (Baptista et al., 2013). Pregnant females (gestational day 18) were anesthetised with isoflurane, and sacrificed by cervical dislocation. Pups were delivered by caesarean operation and sacrificed by decapitation using surgical scissors.

All pups' brains were collected and maintained in cold Hank's Balanced Salt Solution (HBSS; 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 4.16 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 5 mM glucose, supplemented with 1 mM sodium pyruvate and 10 mM HEPES) throughout the macrodissection of the hippocampus from each hemisphere. Thereafter, the hippocampi were maintained for 15 min (manually agitated every 5 min) in a sterile trypsin solution (HBSS 0.15% trypsin), in a water bath at 37°C. The trypsin solution was discarded and a solution containing FBS (HBSS 10% FBS) was added, to stop trypsinization. The supernatant was removed and the hippocampi were washed with HBSS once, to remove the remaining FBS, which stimulates glial differentiation. The hippocampi were mechanically dissociated in Neurobasal medium 0.025 mM glutamate (supplemented with 0.5 mM L-glutamine, 2% B27, 0.1% gentamycin) and plated at a low density (3000 cells/coverslip) in 16 mm coverslips previously coated with poly-D-lysine (0.1 mg/ml), in 1 mL of medium.

Hippocampal neurons were maintained in an incubator at 37ºC, 5% CO_2, until the end of the experiments. Four days after plating, at day *in vitro* (DIV) 4, half of the total medium volume was replaced by supplemented Neurobasal medium without glutamate, to avoid excitotoxicity.

3.5.2. Hippocampal neurons pharmacological treatment

At DIV1, hippocampal neurons were treated with DEX (1 μM), once this concentration was able to induce an increase in the density of $A_{2A}R$ in a microglia cell line, as seen in previous studies by our group (Caetano, 2014), and/or the selective $A_{2A}R$ antagonist SCH58261 (50 nM) [this concentration is selective for $A_{2A}R$ (Zocchi et al., 1996)], and/or the GR antagonist mifepristone (MIF; 1 μM); DEX binds preferentially to GR (Kornel et al., 1982) and this concentration of MIF is able to abolish DEX effects *in vitro* (Kamradt et al., 2000, Kimura et al., 2011) (Figure 11).

When the effects of DEX were tested in the presence of the $A_{2A}R$ antagonist, SCH was added 15 min before DEX, whereas in the case of the GR antagonist, MIF was added immediately before DEX.

Neuron morphology was analysed at DIV2 and DIV5 (Figure 11). DIV2 was chosen to analyse the influence of the pharmacological treatment upon the initial development of the axon, once the neurons are already asymmetric, characterized by a "major" long process and several small processes. The major process continues to grow in culture, giving origin to the axon (Dotti et al., 1988). For naming purposes, in this study, the major processes at DIV2 were solely considered as axons. By DIV5, the axon is already branched and dendrites just started to elongate (Dotti et al., 1988). Considering this, DIV5 was chosen as an endpoint suitable to study the effects on dendritic elongation.

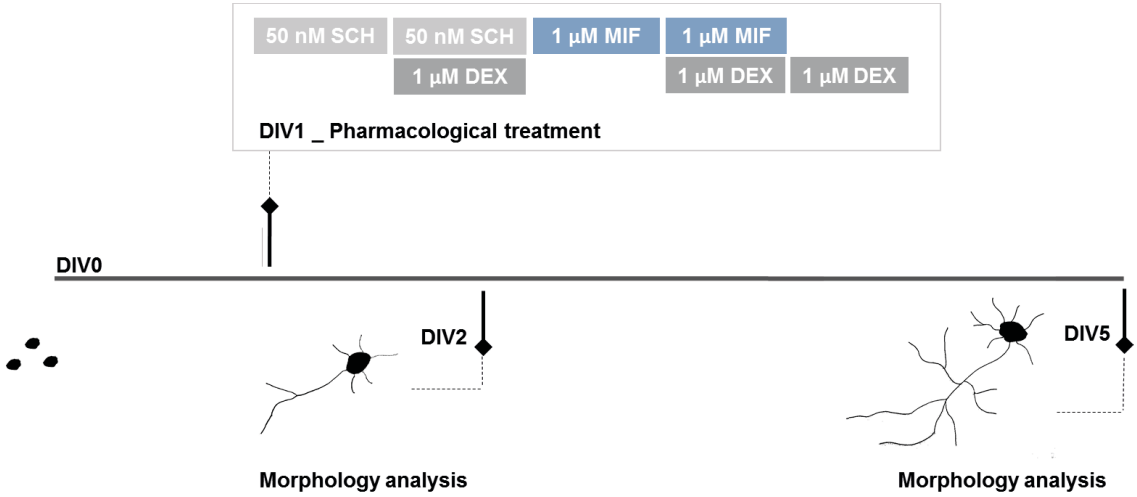


Figure 11| Schematic overview of the experiments with hippocampal neurons and pharmacological treatments. Cells were drawn according to the description of the development of hippocampal neurons in culture by (Dotti et al., 1988).

3.5.3. Immunocytochemistry

At DIV2 and DIV5, neurons were washed carefully with PBS at 37°C and fixed with a solution of 4 % paraformaldehyde and 4% sucrose in PBS. After washing three times with PBS in mild agitation, the coverslips were incubated for 2 hours at RT with the permeabilization and blocking solution (PBS 5% BSA 0.1% Triton X-100). The same solution was used to prepare the antibodies dilution and DAPI. Coverslips were incubated overnight with the primary antibody (1:1000, rabbit anti-TUJ1), at 4°C. After washing the excess of primary antibodies with PBS solution (three times, for 10 min in mild agitation),

the coverslips were incubated at RT for 2 h with the secondary antibody (1:1000, goat anti-rabbit). The primary and secondary antibodies solutions were centrifuged for 20 min at 16000 g, 4°C, in order to precipitate antibody aggregates.

Coverslips were washed again with PBS to remove the excess of the secondary antibodies, incubated for 10 min with DAPI (1:5000) and washed once more. Then, coverslips were mounted on microscope slides with glycergel mounting medium, and allowed to air dry overnight before being sealed with nail polish. Microscope slides were maintained at 4°C in the dark.

3.5.4. Morphometric analysis of hippocampal neurons

Images of neurons were acquired in a fluorescence microscope Zeiss Axio Imager 2 linked to Zeiss AxioCam, using a 20x objective lens (Plan Apochromat 20x/0.8), and processed by Zen Blue software (Zeiss Microscopy). The settings of the acquisition were maintained throughout all experiments. Two main criteria were taken into consideration at this phase: the acquisition of neurons whose neurites were clearly distinguishable and not overlaid with others, and the proximity to other neurons, in a radius of 1000 μm.

Images were imported to the Neurolucida software and manually reconstructed distinguishing axons and dendrites. The major branch in each cell was regarded as the axon. If the cell presented two major neurites of similar length, both were classified as axons. All ramifications were considered, regardless of their length.

At DIV2, 180 cells were reconstructed for each condition, in a total of six different experiments. At DIV5, 120 cells were reconstructed for each condition, in a total of five different experiments.

Morphometric data (branched structure analysis) was obtained in the Neurolucida Explorer, and the number of axons/dendrites, mean length of axons/dendrites and total number of ramifications of each were taken in consideration.

3.6. DATA ANALYSIS

The statistical analysis was carried out in GraphPad Prism version 5 (GraphPad Software Inc., USA). All graphic values are expressed as mean ± standard error of the mean (SEM).

Comparison between two independent means was done by a Student's t test. To assess differences between three groups, a one-way analysis of variance (ANOVA) was used, followed by a Tukey's Multiple Comparison Test, to compare all groups. Differences were considered significant at $p<0.05$.

For the morphometric analysis of microglia, 15 cells of each animal were reconstructed and the mean was considered for further analysis. 4 to 5 animals were used *per* treatment. In the case of neuronal morphometric analysis, the values of each cell were directly considered for statistical analysis, given the high variability of neurons' morphology in culture.

4. RESULTS

4.1. ANALYSIS OF MICROGLIA MORPHOLOGY IN A MODEL OF PRENATAL EXPOSURE TO GLUCOCORTICOIDS

In this study, we analysed microglia morphology of 3 months old male rats prenatally exposed to DEX, in the PFC, a main region involved in anxiety-related disorders. During three weeks before the sacrifice, the animals were treated daily with an $A_{2A}R$ antagonist (SCH). Given the modulatory role of $A_{2A}R$ upon microglia morphology (Gyoneva et al., 2009, Orr et al., 2009), and its anxiolytic effects (Kaster et al., 2015), we analysed the blockade of $A_{2A}R$ as a therapeutic tool.

The prenatal exposure to DEX was shown to induce morphological alterations in microglia during development (Caetano, 2014), which were maintained in females until adulthood. DEX induced an overall decrease in the number of ramifications in microglia in the PFC (Henriques, 2015). The chronic treatment with $A_{2A}R$ antagonist did not revert the behaviour phenotype (Caetano, 2014) and increased the atrophy of microglia morphology (Henriques, 2015). Conversely, in males prenatally exposed to DEX, which also displayed an anxious behaviour in adulthood, the treatment with $A_{2A}R$ antagonist was anxiolytic (unpublished data).

Thus, considering the gender-specific colonization of the brain by microglia (Schwarz et al., 2012), it was considered of importance to analyse possible gender bias in microglia morphology.

Pregnant Wistar rats were injected with DEX (1 mg/kg) at the ED18 and ED19 of the gestation period, and the morphology of PFC microglia was analysed in the male progeny, at PND90. The analysis of microglia morphology was assessed by manual tridimensional reconstruction in the Neurolucida Software, and the number and length of processes were quantified.

4.1.1. Prenatal exposure to dexamethasone induced microglia hyper-ramification in the PFC of adult males

The prenatal exposure to DEX induced a significant increase in the number of processes in male PFC microglia, from the first branch order, corresponding to processes directly coming from the cell body, to the tenth branch order (Figure 12, c). Regarding the length of the processes, there was a general increase in all orders, and a tendency to increase in orders 1 and 6 (Figure 12, d).

All values regarding morphometric analysis of microglia are summarized in the Supplementary Tables I (number of processes) and II (length of processes) in Section 8.

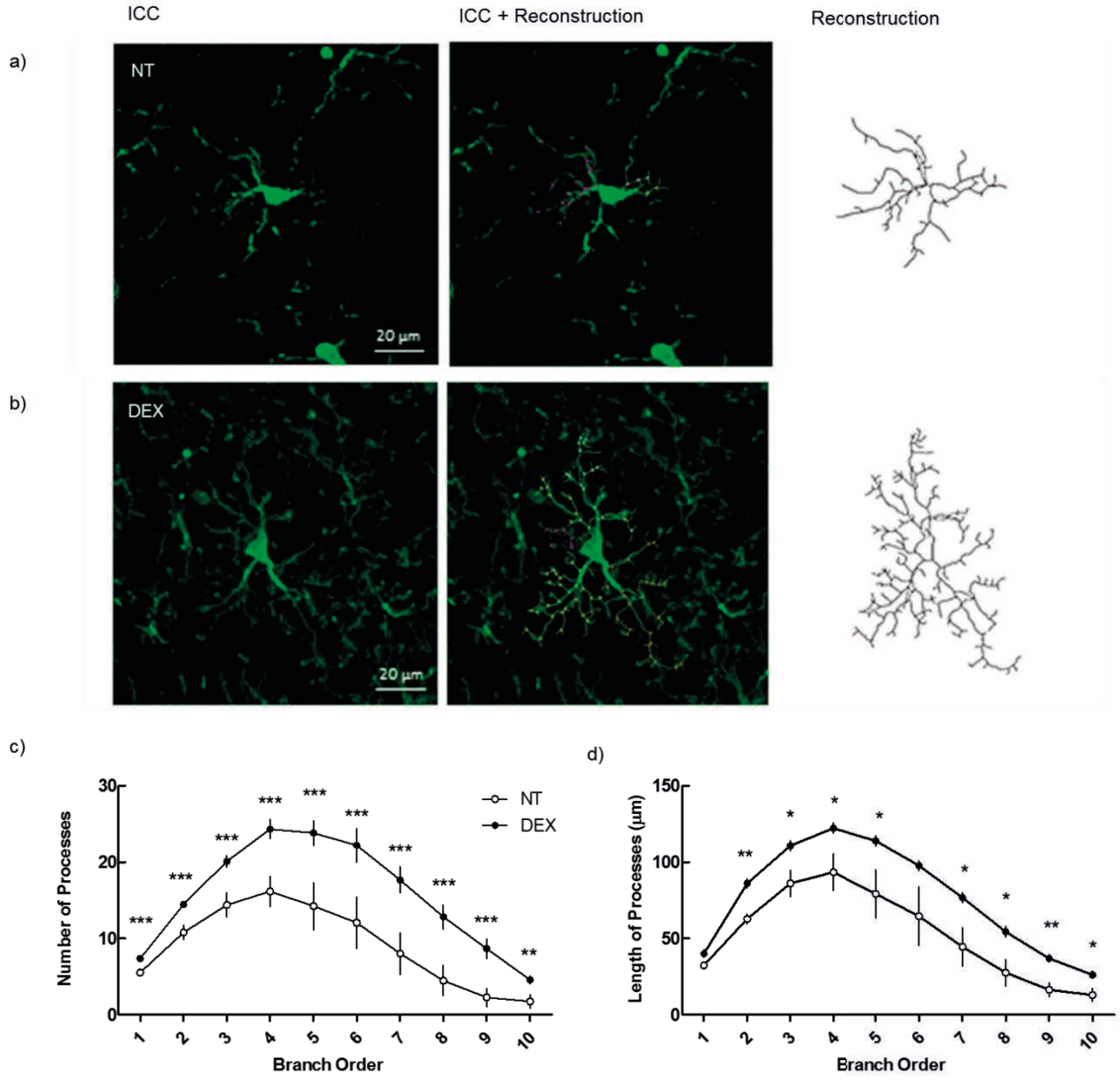


Figure 12| Effect of prenatal exposure to DEX in the number and length of PFC microglia processes. Pregnant females were injected with saline (a, NT) or DEX (b, DEX; 1 mg/kg s.c.) at the ED18 and ED19. Adult male progeny was sacrificed at PND90. Microglia morphology was assessed by tridimensional manual reconstruction in Neurolucida software (a, b) and morphometric data was acquired in Neurolucida Explorer regarding the number (c) and length (d) of processes. Results are expressed as mean ± SEM of 4-5 animals (statistical significance was assessed by t-student: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, comparing DEX treatment with NT).

4.1.2. Chronic treatment with adenosine A_{2A} receptor antagonist *per se* had a mild effect on microglia morphology in the PFC of adult males

Given the implications of $A_{2A}R$ in the control of microglia morphology (Gyoneva et al., 2009, Orr et al., 2009) and previous data showing an overall atrophy of microglia in the PFC of female adult rats subjected to $A_{2A}R$ antagonist chronic treatment (Henriques, 2015), we analysed the effect of the chronic treatment with $A_{2A}R$ antagonist *per se* in the morphology of microglia in the PFC of male adult rats.

We report that the chronic treatment with the $A_{2A}R$ antagonist had a mild effect on microglia morphology in the PFC of male adult rats, slightly increasing the number of processes in the branch orders closer to the cell body (branch orders 1 and 2), and at branch order 9, more distal to the cell body (Figure 13, c). The treatment had no effect on the length of microglia processes (Figure 13, d).

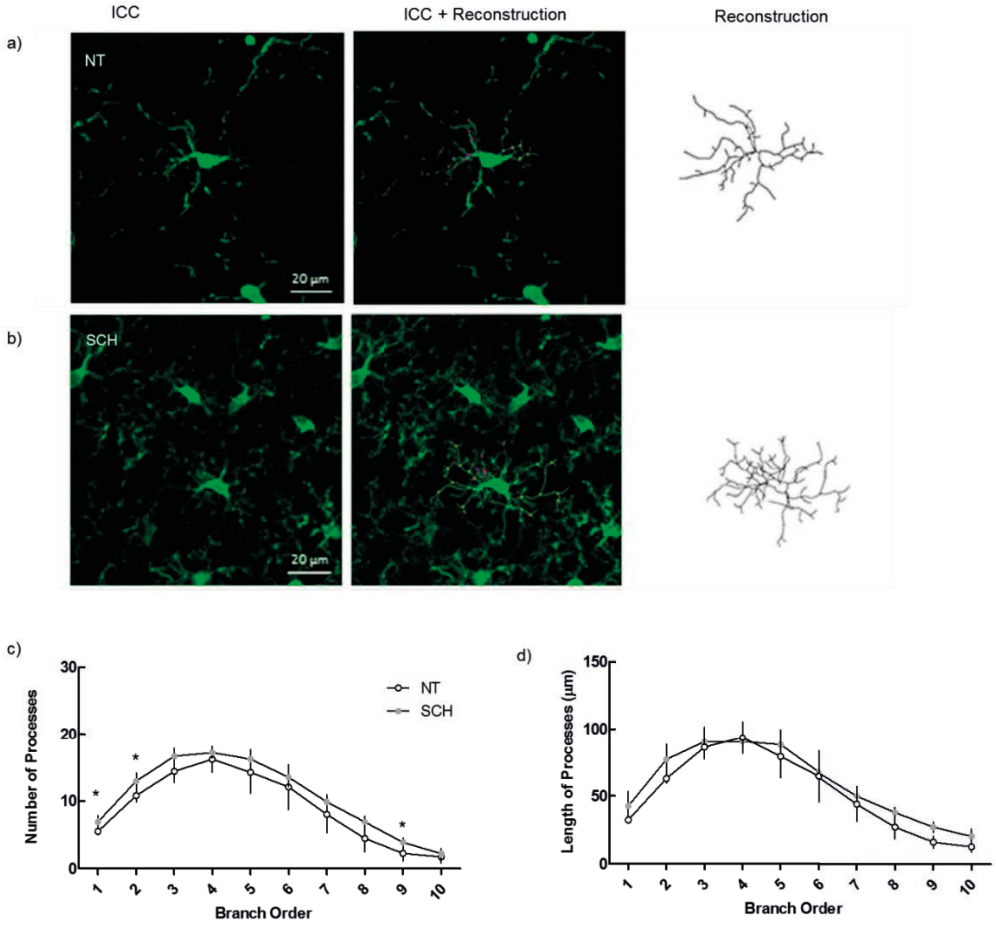


Figure 13| Effect of $A_{2A}R$ chronic blockade on the number and length of PFC microglia processes. Adult male rats were treated for 21 consecutive days with saline (a, NT) or the $A_{2A}R$ antagonist SCH58261 (b, SCH; 0.1 mg/kg/day i.p.) and sacrificed at PND90. Microglia morphology was assessed by tridimensional manual reconstruction in Neurolucida software (a, b) and morphometric data was acquired in Neurolucida Explorer regarding the number (c) and length (d) of processes. Results are expressed as mean ± SEM of 4 animals (statistical significance was assessed by t-student: * $p<0.05$, comparing chronic treatment with $A_{2A}R$ antagonist with NT).

4.1.3. Adenosine A_{2A} receptor antagonist chronic treatment ameliorated microglia hyper-ramification induced by prenatal exposure to DEX in the PFC of adult males

Given the anxiolytic effect of $A_{2A}R$ chronic blockade in adult male rats prenatally treated with DEX (unpublished data), we analysed the morphology of microglia in the PFC, to assess if the improvement in behavioural phenotype was temporarily coincident with an improvement in the alterations induced in microglia morphology.

Microglia from the PFC of animals prenatally exposed to DEX and treated with $A_{2A}R$ antagonist in adulthood exhibited a general hyper-ramification in all branch orders, as assessed by the increase in the number of processes (Figure 14, e). Regarding the length of the processes, there was an increase only in the processes of branch order 9 (Figure 14, f). Comparing these with the results from animals solely exposed to DEX (Figure 14, b), there was an overall tendency to decrease in the number of processes, statistical significant in the branch orders 8 and 9 (Figure 14, e), and in length of processes (Figure 14, f), approximating microglia morphology to that of NT animals.

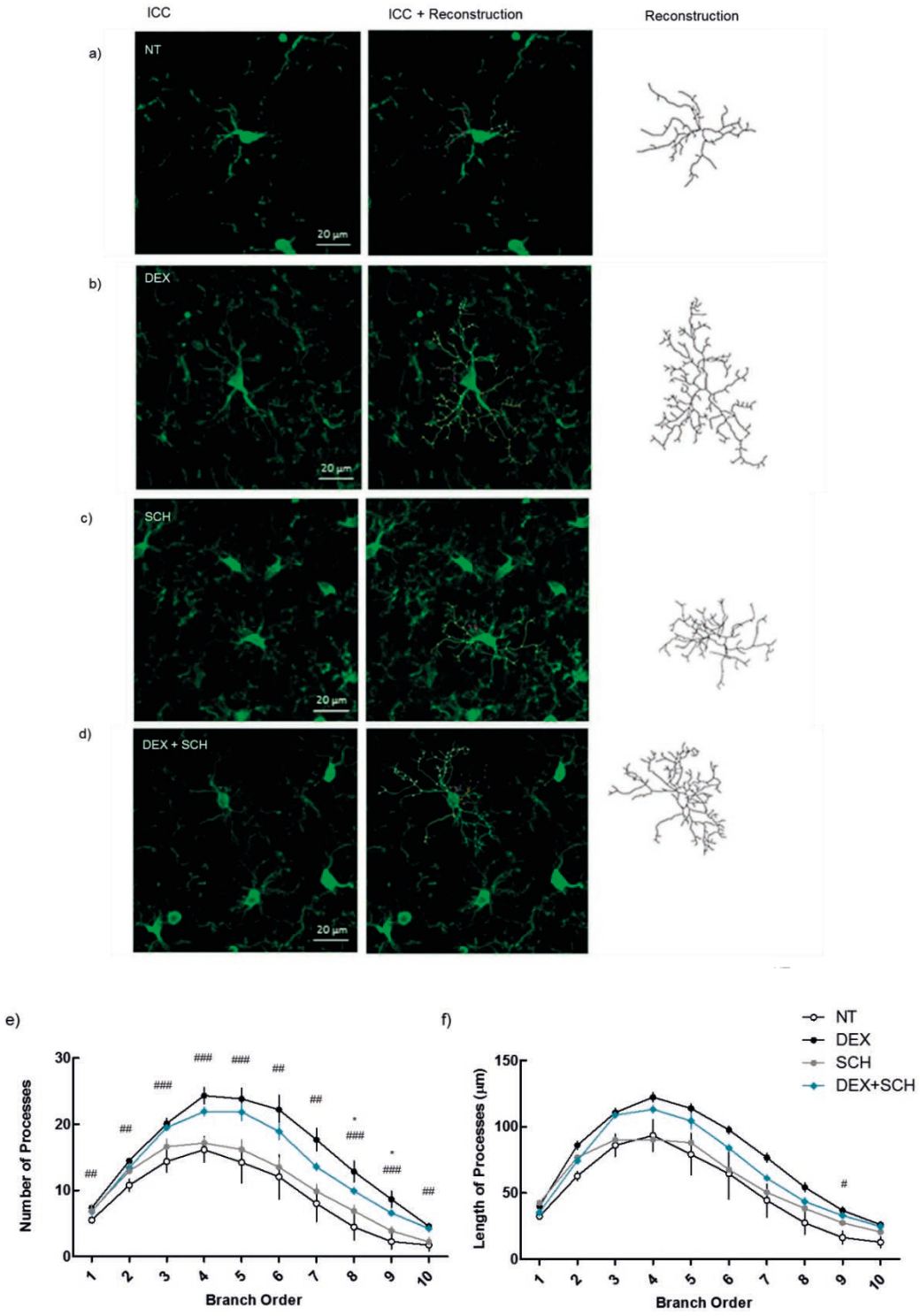


Figure 14| Effect of $A_{2A}R$ chronic blockade in the morphologic alterations induced by prenatal exposure to DEX on the number and length of PFC microglia processes. Pregnant females were injected with saline (a, NT) or DEX (b, DEX; 1 mg/kg s.c.) at the ED18 and ED19 and/or treated for 21 consecutive days with $A_{2A}R$ antagonist SCH58261 (c and d, SCH; 0.1 mg/kg/day i.p.) and sacrificed at PND90. Microglia morphology was assessed by tridimensional manual reconstruction in Neurolucida software (a, b, c, d) and morphometric data was acquired in Neurolucida Explorer regarding the number (e) and length (f) of processes. Results are expressed as mean ± SEM of 4-5 animals (statistical significance was assessed by one-way ANOVA, followed by Tukey's Multiple Comparison Test: * $p<0.05$, comparing prenatal treatment with chronic treatment with $A_{2A}R$ antagonist after DEX prenatal exposure (DEX+SCH); # $p<0.05$, ## $p<0.01$, ### $p<0.001$, comparing NT with prenatal treatment with DEX and chronic treatment with $A_{2A}R$ antagonist (DEX+SCH)).

4.2. GLUCOCORTICOID EFFECTS ON NEURONAL MORPHOGENESIS

Having in mind the importance of the maintenance of GC levels during pregnancy, and already published data showing alterations in neuronal morphology upon HPA deregulation, we analysed the effects of the exposure to DEX during development in neuronal morphology, using a simpler *in vitro* model. This initiated project aims to dissect mechanistic components of the effect of GC on neurons.

The results presented above regarding microglia morphology, show an amelioration induced by the chronic blockade of $A_{2A}R$. However, there was only a partial recover of microglia cells morphology, whereas the treatment completely reverted the anxious phenotype (unpublished data). We strongly believe the complete recover is the result of the blockade of $A_{2A}R$ in multiple cell types, namely neurons. Thus, and supported by the observations of $A_{2A}R$ implication in neuronal morphogenesis, we also proposed to study the impact of its modulation.

4.2.1. Hippocampal neurons development *in vitro*

After two days *in vitro* (DIV2), the neurons had already established neuronal polarity, displaying a major process (1.1 ± 0.1), corresponding to the axon, and several dendrites (6.9 ± 0.3; Figure 15, a). This was not the case of all cells, once some presented more than one long process with the morphological characteristics of axons. However, this was highly unusual. The mean length of the dendrites was 77.9 ± 3.1 µm (Figure 15, d) and the medium length of the axon was 275.6 ± 13.5 µm (Figure 15, g). At DIV2 both axon and dendrites were ramified. The number of ramifications in the dendrites was 3.9 ± 0.4 (Figure 15, e) and in the axon was 3.4 ± 0.3 (Figure 15, h). Note that the number of ramifications of the dendrites corresponds to the total number distributed by several dendrites, and not to the mean of ramifications *per* dendrite.

At DIV5 the complexity of the dendrites and axon increased (Figure 15, b). The number of dendrites was maintained, but there was a one-fold increase in their mean length (150.7 ± 8.1 µm; Figure 15, d) and a 2-fold increase in the overall number of ramifications (13.1 ± 1.0; Figure 15, e). The increase in the complexity of the axon was more pronounced. There was a 2-fold increase in the length (811.5 ± 57.6 µm) and a 3-fold increase in the number of ramifications (12.5 ± 1.2; Figure 15, h).

Note that in the reconstruction panel in the representative images, the axon is coloured in green, to facilitate its identification. Also, considering the small size of neurons at DIV2, the image was amplified to allow a proper visualization of its neurites.

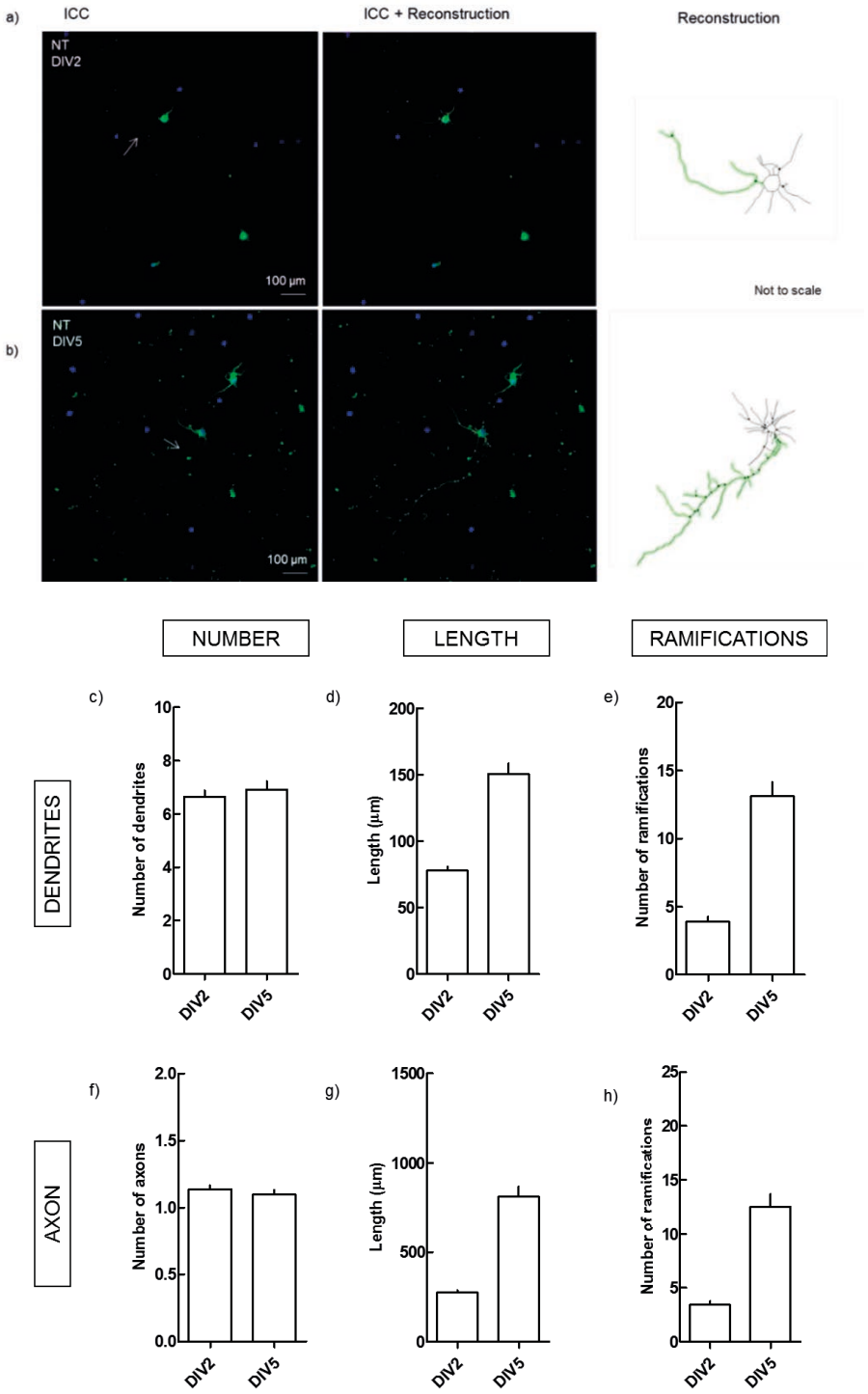


Figure 15| Developmental state of hippocampal neurons at DIV2 and DIV5. Hippocampal neurons from ED18 rats were cultured *in vitro* for 2 (DIV2) and 5 (DIV5) days, without any treatment (NT). Neuron morphology was assessed by manual reconstruction in Neurolucida software (a, b) and morphometric data was acquired in Neurolucida Explorer regarding the number (c), length (d) and number of ramifications (e) of dendrites and the number (f), length (g) and number of ramifications (h) of axons. Results are expressed as mean ± SEM of 120-180 cells, from 5 to 6 independent experiments. No statistical analysis was performed.

4.2.2. Dexamethasone induced a differential effect on dendrites and axon length

To assess the effects of DEX upon neuronal morphogenesis, hippocampal primary neurons were treated with DEX (1 μM) after 24h in culture and maintained throughout the duration of the experiment. At DIV2 and DIV5, the neurons were manually reconstructed using Neurolucida software. Morphometric data was analysed considering the number and length of dendrites and axons, and the ramification of both.

At DIV2, DEX did not induce any alterations in the morphometric parameters analysed (Figure 16). However, the exposure to DEX led to alterations in the morphology of both axon and dendrites at DIV5 (Figure 17).

DEX induced a pronounced decrease in the mean length of the dendrites (107.6 ± 6.6 μm, $p < 0.001$, as compared with NT [150.7 ± 8.1 μm]; Figure 17, d), but had no statistical effect upon their number and number of ramifications. In the axon, DEX induced the opposite effect, increasing its length (1139.2 ± 86.1 μm, $p < 0.005$, as compared with NT [811.5 ± 57.6 μm]; Figure 17, g). As reported for the dendrites, there was no statistical effect of DEX on the number of the axon or the number of ramifications.

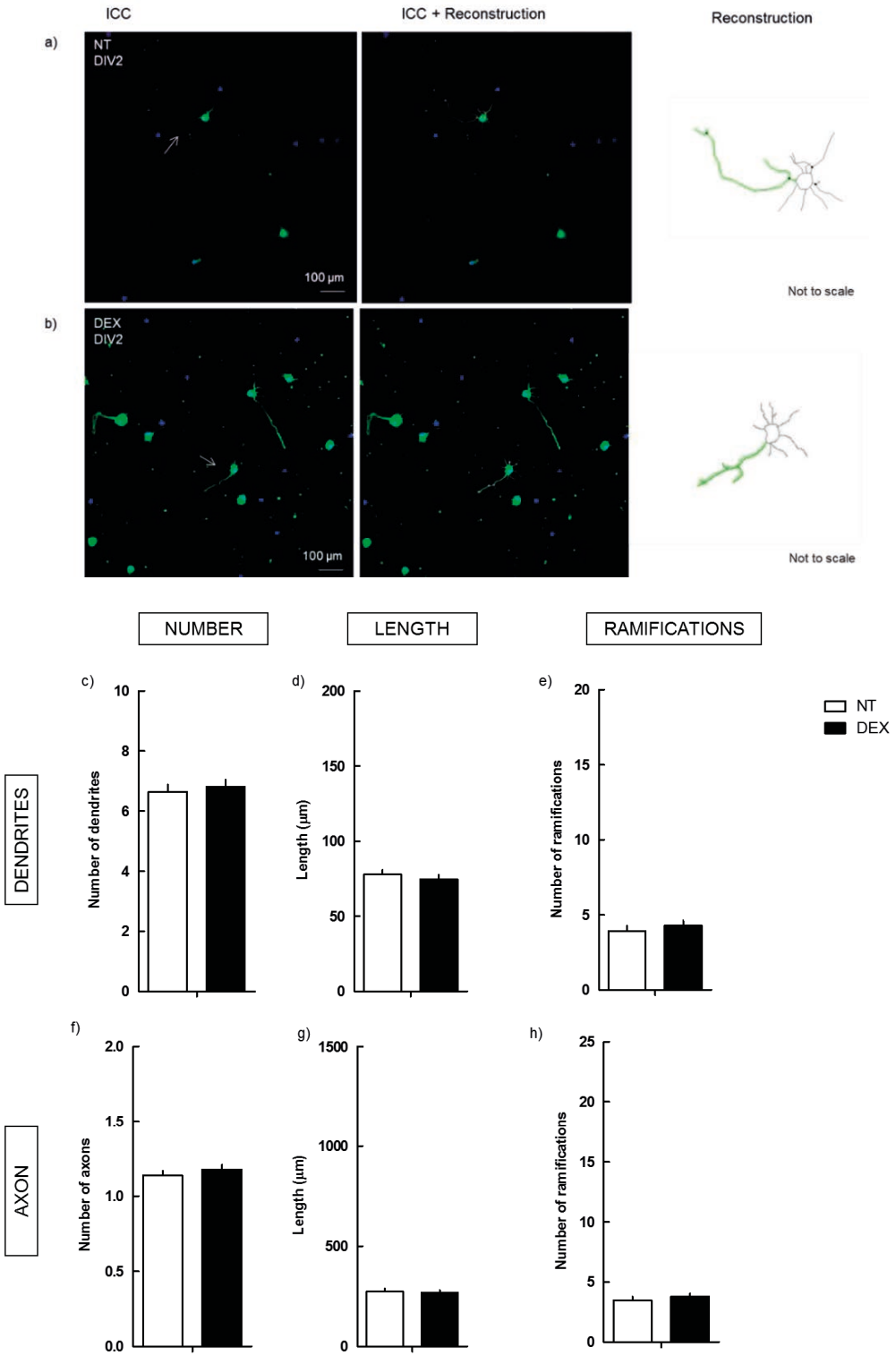


Figure 16| Effect of DEX treatment on DIV2 hippocampal neurons (24 hours treatment). Hippocampal neurons from ED18 rats were cultured *in vitro* for 2 days (DIV2) and treated with DEX (1 µM) at 24 hours in culture. Neuron morphology was assessed by manual reconstruction in Neurolucida software (a, b) and morphometric data was acquired in Neurolucida Explorer, regarding the number (c), length (d) and number of ramifications (e) of dendrites and the number (f), length (g) and number of ramifications (h) of axons. Results are expressed as mean ± SEM of 180 cells, from 6 independent experiments (no statistical significance comparing NT with DEX treatment, assessed by t-student).

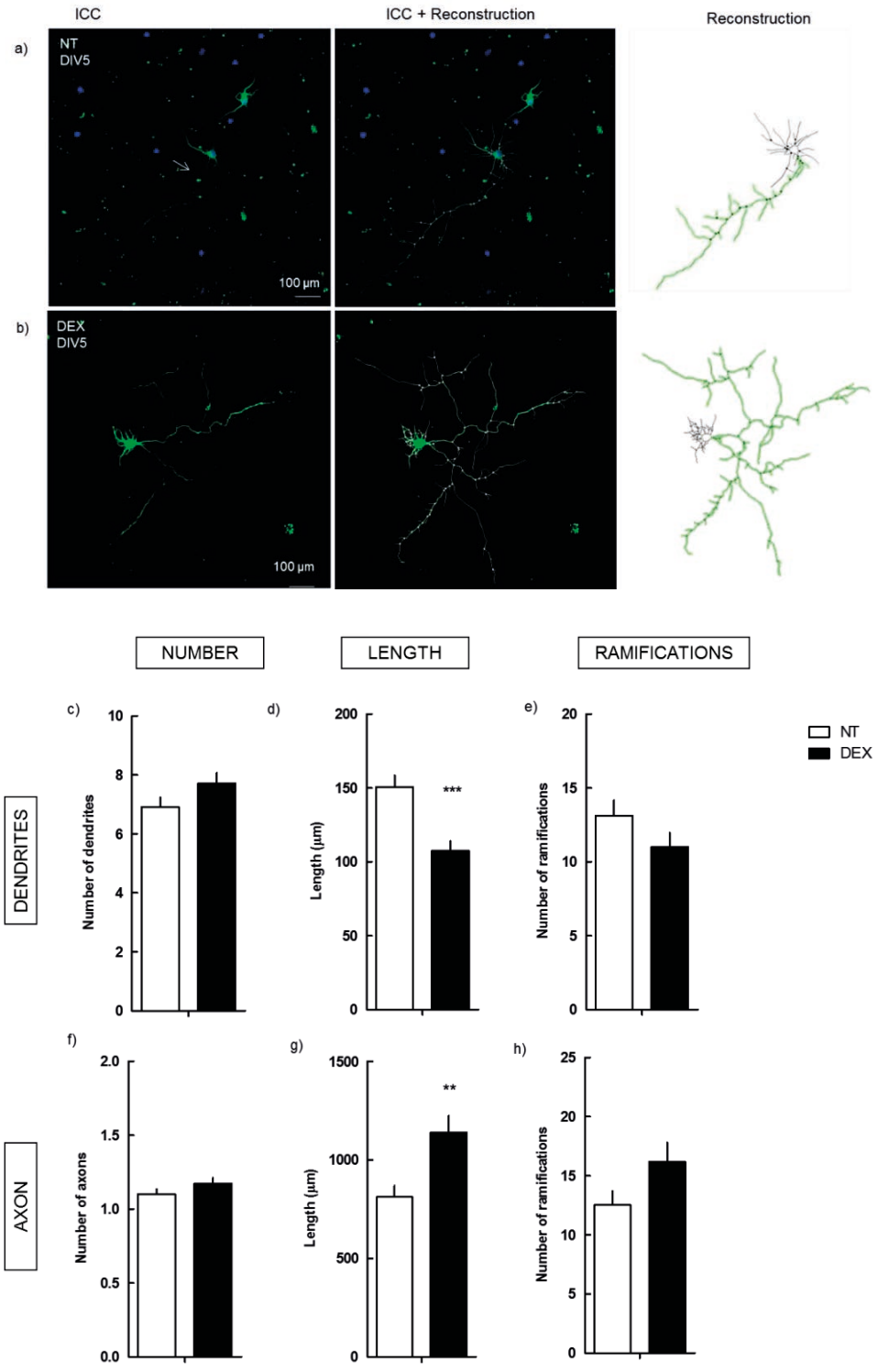


Figure 17| Effect of DEX treatment on DIV5 hippocampal neurons (4 days treatment). Hippocampal neurons from ED18 rats were cultured *in vitro* for 5 days (DIV5), and treated with DEX (1 µM) at 24 hours in culture. Neuron morphology was assessed by manual reconstruction in Neurolucida software (a, b) and morphometric data was acquired in Neurolucida Explorer, regarding the number (c), length (d) and number of ramifications (e) of dendrites and the number (f), length (g) and number of ramifications (h) of axons. Results are expressed as mean ± SEM of 120 cells, from 5 independent experiments (statistical significance was assessed by t-student test: ** $p < 0.005$, *** $p < 0.001$ comparing DEX treatment with NT).

4.2.3. Dexamethasone-induced decrease in dendrite length was dependent on the activation of glucocorticoids receptors

Considering that synthetic glucocorticoids, as DEX, have high affinity to GR (Kornel et al., 1982), we sought to clarify if the effects of DEX on the morphology of neurons were dependent on the activation of GR.

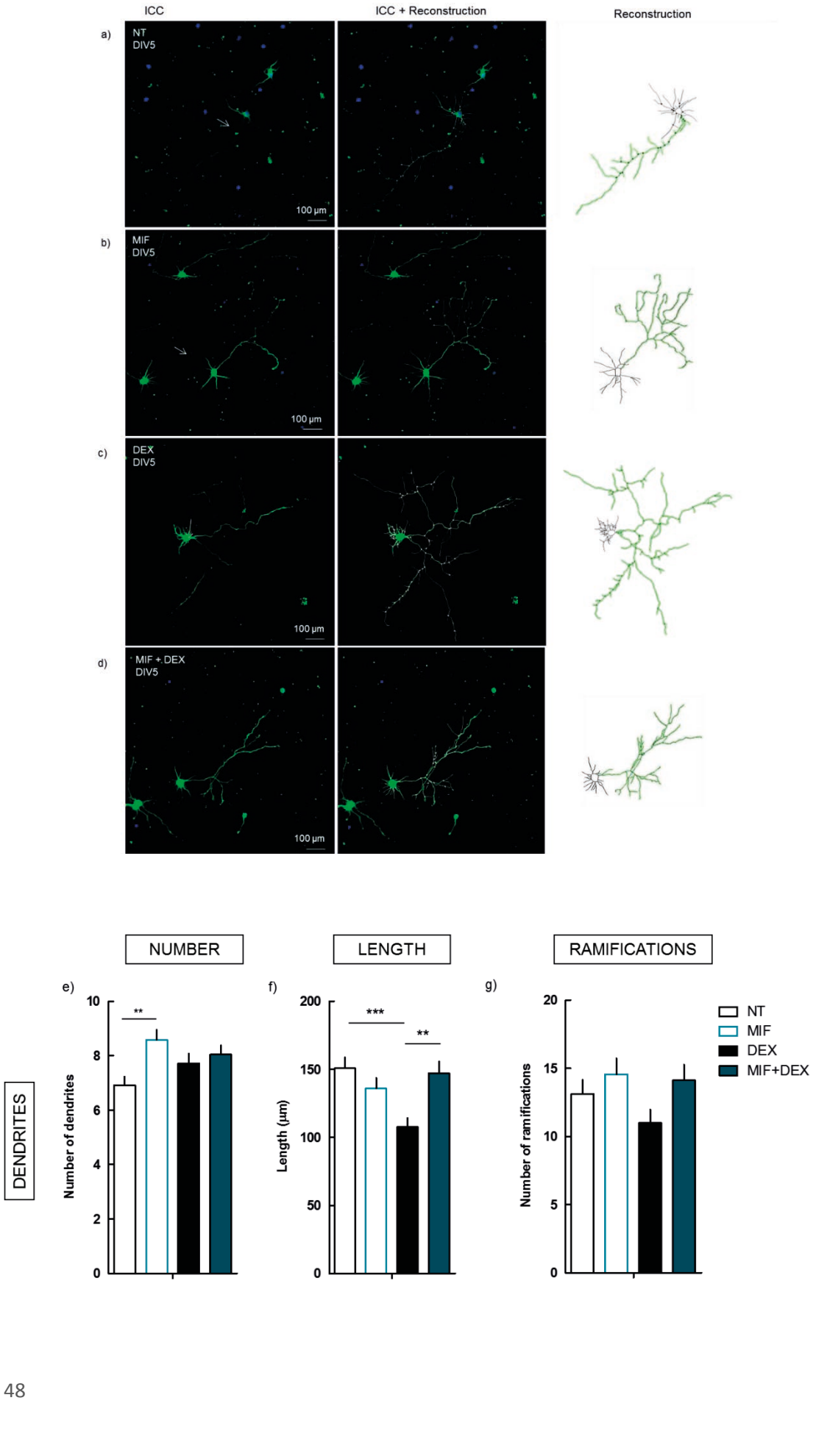
To test this hypothesis, primary hippocampal neurons were treated with the antagonist of GR, MIF (1 μM) and/or DEX (1 μM). After 4 days of treatment, at DIV5, neurons were manually reconstructed, using Neurolucida software. Morphometric data was analysed considering the number and length of dendrites and axons, and the ramification of both.

The blockade of GR prevented the alteration in the length of the dendrites induced by DEX (147.1 ± 8.6 μm, n.s. as compared with NT [150.7 ± 8.1 μm], $p < 0.005$ as compared with DEX [107.6 ± 6.6 μm]; Figure 18, f). However, the effect of DEX in the increase in the length of the axon was not prevented by the blockade of GR (1069.1 ± 88.7 μm, n.s. as compared with NT [811.5 ± 57.6 μm], n.s. as compared with DEX [1139.2 ± 86.1 μm]; Figure 18, i).

4.2.4. Hypertrophic effect of endogenous glucocorticoids blockade

The blockade of the action of endogenous levels of GC, as seen by the treatment with GR antagonist *per se*, had an overall hypertrophic effect on dendrites and axon. Four days after treatment, there was a small, but significant increase in the number of dendrites (8.6 ± 0.4, $p < 0.005$ as compared with NT [6.9 ± 0.3]; Figure 18, e), and in the complexity of the axon. The number of ramifications of the axon had a significant increase (18.7 ± 2.0, $p < 0.01$, as compared with NT [12.5 ± 1.2]; Figure 18, j). The length of the axon also showed a tendency to increase, although not statistical significant (1056.9 ± 74.1 μm, comparing with NT [811.5 ± 57.6 μm]; Figure 18, i).

Although at DIV2 DEX had no effect upon the morphogenesis, the blockade of endogenous GC slightly decreased the number of dendrites (5.7 ± 0.2, $p < 0.05$ as compared with NT [6.6 ± 0.2] assessed by t-student; Supplementary Figure 1). This effect was transient, once at DIV5 there was an increase in the number of dendrites.


ICC
ICC + Reconstruction
Reconstruction
a)
NT
DIV5
100 µm
b)
MIF
DIV5
100 µm
c)
DEX
DIV5
100 µm
d)
MIF + DEX
DIV5
100 µm
NUMBER
LENGTH
RAMIFICATIONS
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NT
MIF
DEX
MIF+DEX

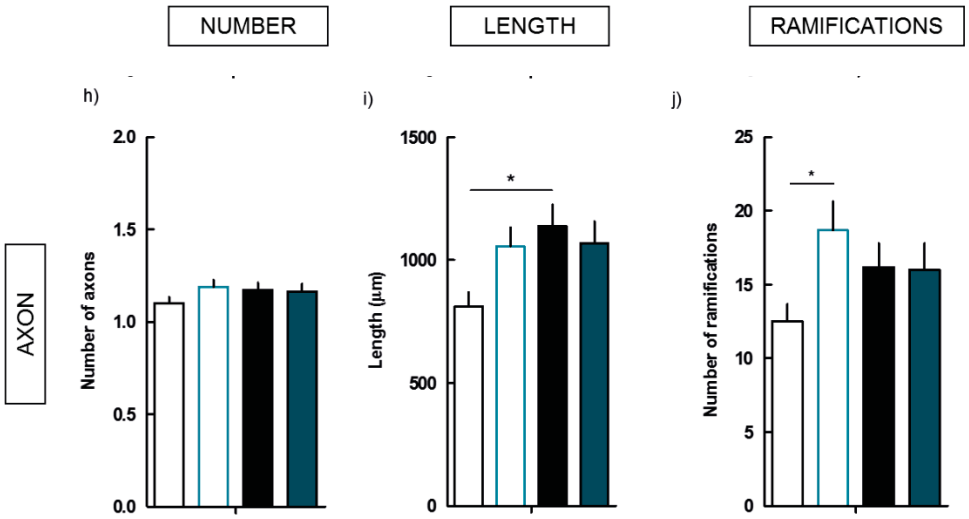


Figure 18| Effect of the blockade of GR and/or DEX on DIV5 hippocampal neurons (4 days treatment). Hippocampal neurons from ED18 rats were cultured *in vitro* for 5 days (DIV5), and treated with the GR antagonist, MIF (1 µM) and/or DEX (1 µM) at 24 hours in culture. Neuron morphology was assessed by manual reconstruction in Neurolucida software (a-d) and morphometric data was acquired in Neurolucida Explorer, regarding the number (e), length (f) and number of ramifications (g) of dendrites and the number (h), length (i) and number of ramifications (j) of axons. Results are expressed as mean ± SEM of 120 cells, from 5 independent experiments (statistical significance was assessed by one-way ANOVA followed by Tukey's Multiple Comparison Test: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ as indicated by the horizontal lines above the columns).

4.2.5. Dexamethasone-induced increase in axon length was dependent on the activation of adenosine A_{2A} receptors

It was recently described by Ribeiro and colleagues that the activation of $A_{2A}R$ induces axonal elongation (Ribeiro et al., 2016). Once the trophic effect of DEX in the length of the axon was not mediated by the activation of GR (Figure 18, i), we analysed if it was due to $A_{2A}R$ activation.

Hence, primary hippocampal neurons were treated with DEX (1 µM) in the presence or absence of an $A_{2A}R$ antagonist (SCH; 50 nM). Four days after treatment, at DIV5, neurons were manually reconstructed, using Neurolucida software. Morphometric data was analysed considering the number and length of dendrites and axons, and the ramification of both.

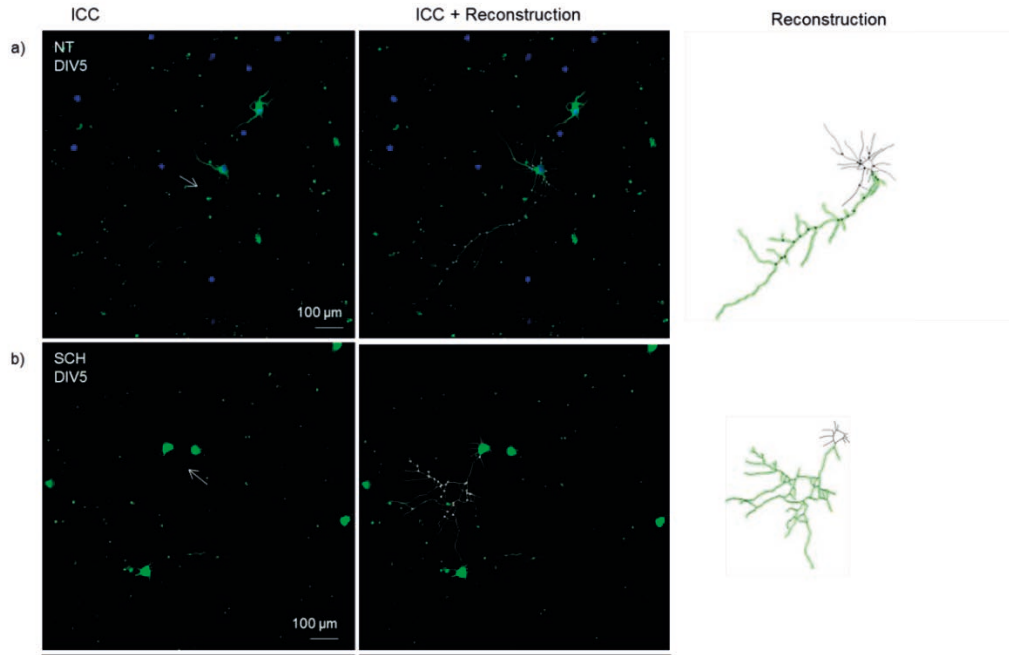
The blockade of $A_{2A}R$ was not able to prevent the atrophy in the length of the dendrites induced by DEX (110.0 ± 5.4 µm, n.s. comparing with DEX [107.6 ± 6.6 µm], $p < 0.001$, comparing with NT [150.7 ± 8.1 µm]; Figure 19, f). However, the blockade of $A_{2A}R$ *per se* induced a small increase in the number of dendrites (8.4 ± 0.3, $p < 0.05$ comparing with NT [6.9± 0.3]), which was promoted in the presence of DEX (9.0 ± 0.4, $p < 0.001$, comparing with NT [6.9 ± 0.3]; Figure 19, e).

In what comes to the axon, $A_{2A}R$ blockade was able to prevent the increase in the length induced by DEX (752.4 ± 60.8 μm, n.s. comparing with NT [811.5 ± 57.6 μm]; $p < 0.001$ comparing with DEX [1139.2 ± 86.1 μm]; Figure 19, i).

Thus, the differential effect of DEX upon dendrites and axon is likely mediated by GR and $A_{2A}R$, respectively.

4.2.6. Adenosine A_{2A} receptors blockade affected dendritic development

The exposure to the $A_{2A}R$ antagonist *per se* led to alterations in dendritic development, suggestive of an impact of endogenous adenosine. Four days after treatment, there was a small increase in the number of dendrites (8.3 ± 0.3, $p < 0.05$ comparing to NT [6.9 ± 0.3]; Figure 19, e) and a decrease in their mean length (105.5 ± 5.0 μm, $p < 0.001$, as compared with NT [150.7 ± 8.1 μm]; Figure 19, f). The alterations were restricted to the dendrites, whereas the axon morphology was similar to NT (Figure 19, h-j).



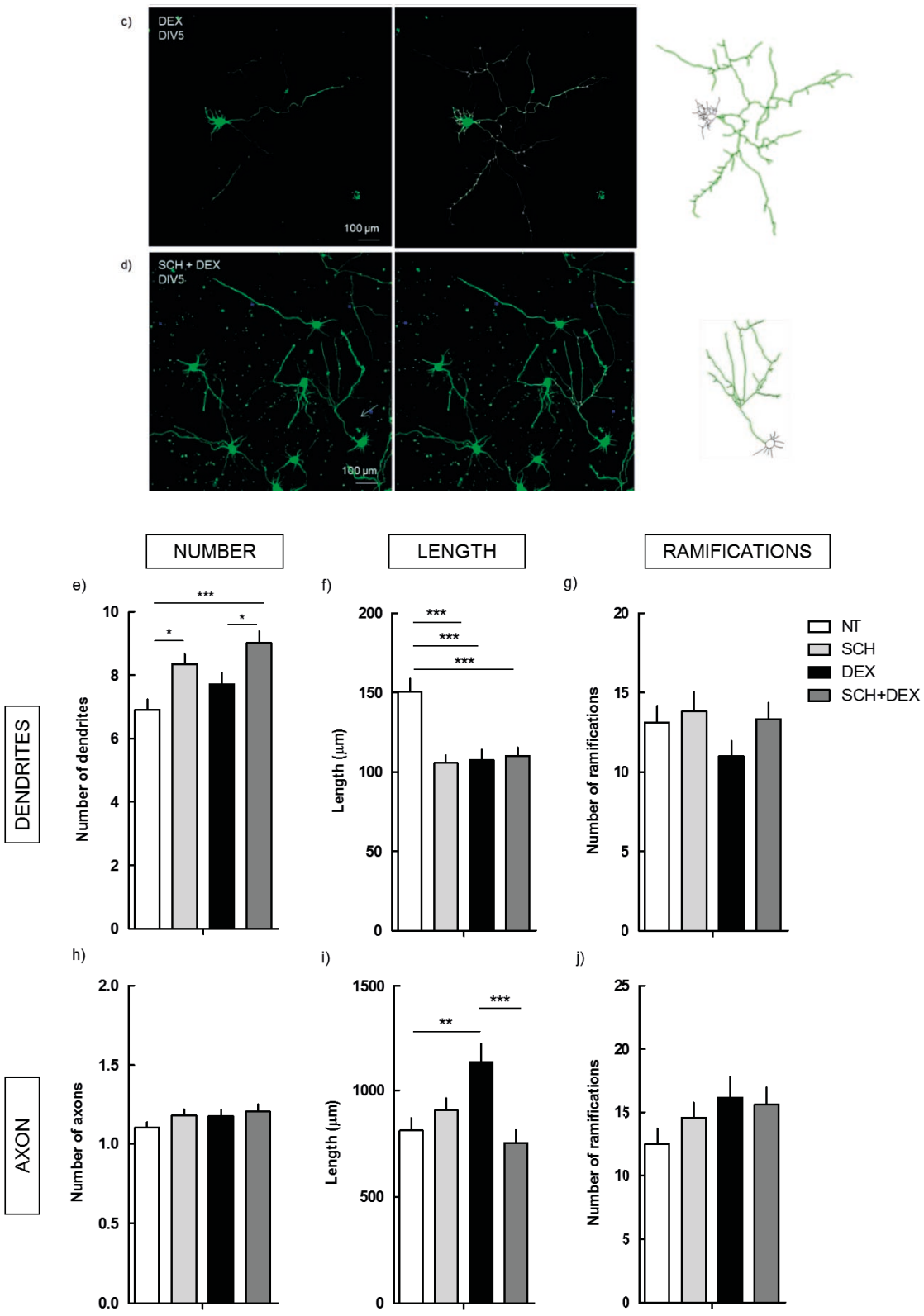


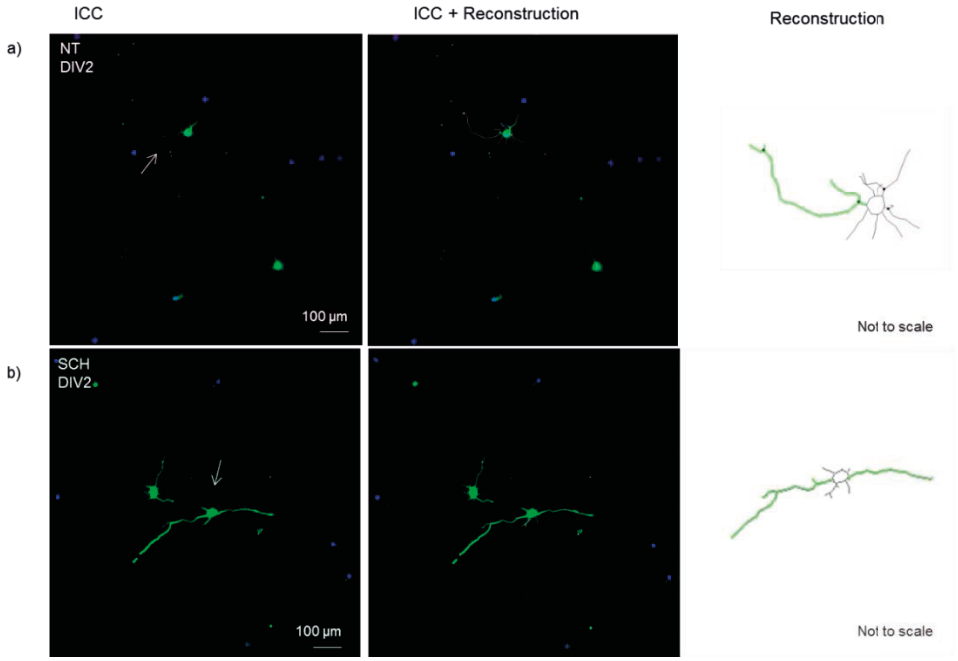
Figure 19| Effect of the blockade of $A_{2A}R$ and/or DEX on DIV5 hippocampal neurons (4 days treatment). Hippocampal neurons from ED18 rats were cultured *in vitro* for 5 days (DIV5), and treated with the $A_{2A}R$ antagonist, SCH (50 nM), and/or DEX (1 µM) at 24 hours in culture. SCH was added 15 min before DEX. Neuron morphology was assessed by manual reconstruction in Neurolucida software (a-d) and morphometric data was acquired in Neurolucida Explorer, regarding the number (e), length (f) and number of ramifications (g) of dendrites and the number (h), length (i) and number of ramifications (j) of axons. Results are expressed as mean ± SEM of 120 cells, from 5 independent experiments (statistical significance was assessed by one-way ANOVA followed by Tukey's Multiple Comparison Test: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ as indicated by the horizontal lines above the columns).

4.2.7. Adenosine A_{2A} receptors blockade transiently affected axonal development

We further analysed the effects of $A_{2A}R$ blockade, focusing on the influence upon the initial development of neurons. Hippocampal neurons were incubated with SCH (50 nM) at 24 hours in culture, and maintained until DIV2. Then, morphometric data was obtained as established.

The blockade of $A_{2A}R$ activation had short-term effects on neuronal development; it slightly decreased the length of the dendrites (69.0 ± 2.8 μm, $p < 0.05$ comparing with NT [77.9 ± 3.1 μm]; Figure 20, c), although it did not affect their number (Figure 20, c). In respect to the axon, there was an increase in the number of cells with more than one axon-like structure (1.4 ± 0.1, $p < 0.001$ comparing with NT [1.1 ± 0.1]; Figure 20, f), whereas in normal conditions most neurons had only one axon at this phase of development, as referred in section 4.2.1. From 180 cells analysed from 6 independent experiments, 113 cells had only one longer structure with the morphological features of the axon, while the remaining had two or, in more unusual cases, three (data not shown). The increase in the number of axon-like structures was accompanied by an overall decrease in their mean length (229.60 ± 9.71 μm, $p < 0.005$ comparing with NT [275.58 ± 13.53 μm]; Figure 20, g).

This effect upon the axon was transient, once at DIV5 there were no alterations comparing with NT (Figure 19, h-j). Contrastingly, the effect of $A_{2A}R$ blockade on dendrites, was maintained and promoted by DIV5 (discussed in Section 4.2.6).



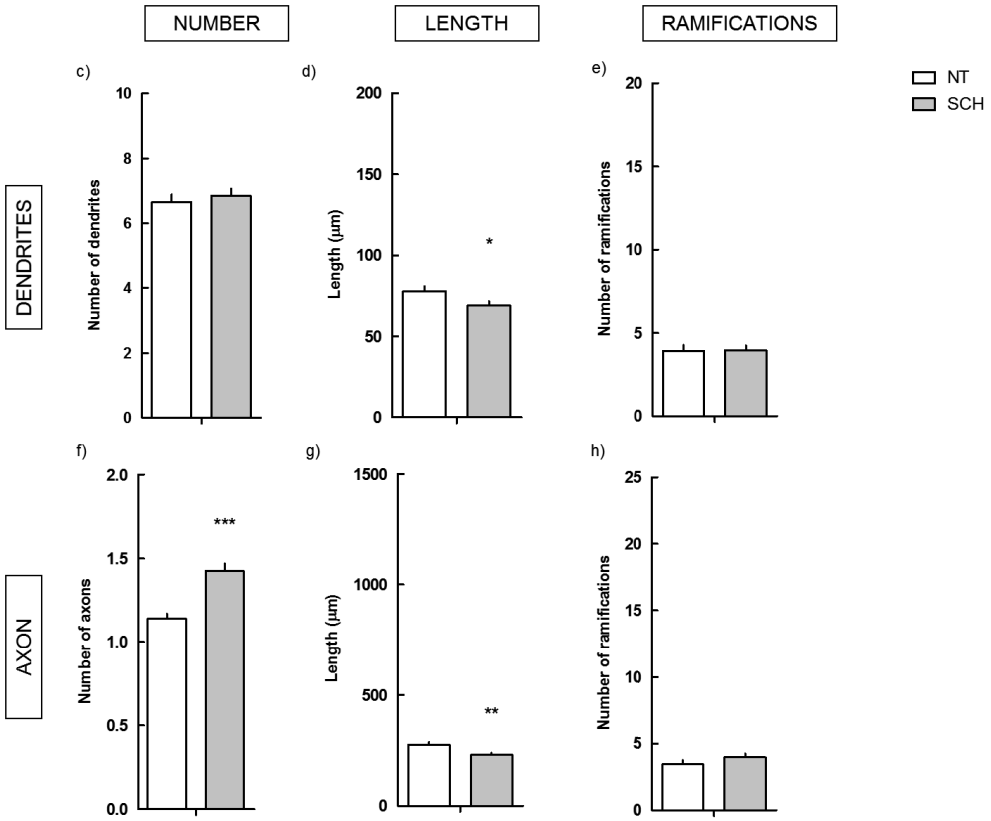


Figure 20| Effect of the blockade of $A_{2A}R$ on DIV2 hippocampal neurons (24 hours treatment). Hippocampal neurons from ED18 rats were cultured *in vitro* for 2 days (DIV2), and treated with the $A_{2A}R$ antagonist, SCH (50 nM) at 24 hours in culture. Neuron morphology was assessed by manual reconstruction in Neurolucida software (a-b) and morphometric data was acquired in Neurolucida Explorer, regarding the number (c), length (d) and number of ramifications (e) of dendrites and the number (f), length (g) and number of ramifications (h) of axons. Results are expressed as mean ± SEM of 180 cells, from 6 independent experiments (statistical significance was assessed by t-student test: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ comparing with NT).

5. DISCUSSION

The development of the brain is tightly regulated by environmental factors. In the present work, we used an established model of neuropsychiatric disorder with neurodevelopmental origin, in order to analyse the effects of GC on microglia (Oliveira et al., 2006).

The prenatal exposure to DEX has a longterm impact in behaviour, as our group (Caetano, 2014) and others observed, increasing the susceptibility to anxiety disorder and depression (Oliveira et al., 2006) and disturbing male sexual behaviour (Oliveira et al., 2011).

In this model, the behavioural features seen in adulthood were shown to be correlated with alterations in the peripheral immune system (Roque et al., 2011). However, immune alterations in the CNS were not studied. Considering the importance of microglia in the immune response in the CNS, their responsiveness to GC, and to the normal development of the neuronal circuits, we hypothesized these cells were likely to be altered after exposure to DEX, contributing to the neuropsychiatric alterations.

In fact, previous studies by our research team showed that the morphology of microglia from the PFC of rats is altered at PND1 and PND7 (decreased number and length of processes), after prenatal exposure to DEX (Caetano, 2014). Surprisingly, given the high plasticity of microglia, the atrophy of microglia processes was maintained in adulthood, in female rats (Henriques, 2015).

Having in mind the gender differences in microglia brain colonization, which are maintained at least until PND60 (Schwarz et al., 2012), our main aim was to characterize microglia morphology in the PFC of adult male rats exposed to DEX during the prenatal period, to verify if the response to DEX is gender-specific.

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Prenatal DEX induced contrasting morphological microglia remodelling in the PFC of males and females. The chronic treatment with $A_{2A}R$ antagonist ameliorated morphologic alterations in males, but exacerbated the phenotype in females.

Microglia in the PFC of adult male rats presented an overall hyper-ramification (whereas in the case of the females it induced an atrophy (Henriques, 2015)). The alterations in male microglia were pronounced, displaying a general significant increase in the number and length of microglia processes, from the processes directly coming from the cell body to order

10 (in the length, the increase was not statistically significant only in processes from orders 1 and 6).

$A_{2A}R$ blockade was successful in males, both by rescuing the anxiety-like behaviour (unpublished data), and improving DEX-induced alterations in microglia morphology. There was a tendency to decrease the number and length of microglia processes comparing to DEX, mainly from the orders 4 and higher, which reached statistical significance in the number of processes from orders 8 and 9.

The correlation of microglia morphology improvement in response to $A_{2A}R$ blockade with the complete rescue in behaviour, further reinforces the putative role of microglia in the pathophysiology of this disorder. This hypothesis is strengthened by the observation that in females, $A_{2A}R$ blockade did not revert the behavioural alterations, while exacerbating microglia atrophy (Henriques, 2015).

$A_{2A}R$ have been implicated in the dynamics of microglia morphology (Gyoneva et al., 2009, Orr et al., 2009, Gyoneva et al., 2014a, Gyoneva et al., 2014b). In fact, the treatment with $A_{2A}R$ antagonist *per se* in females induced an atrophy in microglia processes (decrease in number and length), more pronounced than the effect of DEX (Henriques, 2015). Strikingly, the treatment with $A_{2A}R$ antagonist had little effect in microglia morphology in males (there was a significant decrease in the number of processes only in orders 1, 2 and 9). This differential response reinforces the impact of $A_{2A}R$ in microglia morphology, and shows its gender specificity.

What leads to this gender-specific response is still unexplored. It could be due to gender differences in the density of $A_{2A}R$ in the PFC, or in the availability of adenosine. Indeed, a *post mortem* analysis of human brains revealed gender variations in the levels of adenosine in several brain areas, namely lower levels of adenosine in women total cortex (Kovacs et al., 2010).

It is important to understand the basis of gender-specific microglia responses (which were seen both in the response to DEX and to $A_{2A}R$ antagonist treatments), once they possibly contribute to the higher susceptibility of women to stress related neuropsychiatric disorders (Van de Velde et al., 2010).

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The chronic treatment with $A_{2A}R$ antagonist was able to completely revert the anxious-like behaviour induced by prenatal DEX in males, while only partially recovering microglia hyper-ramification.

The observation that in rats prenatally exposed to DEX and treated with $A_{2A}R$ antagonist in adulthood, there was a partial recovery in microglia hyper-ramification, coincident with a complete recovery in behaviour (unpublished data), suggests the contribution of other cell types to the pathophysiology of anxiety.

Considering the alterations induced by stress or GC exposure in neurons, in several brain regions, such as the PFC (Brown et al., 2005, Cerqueira et al., 2005), hippocampus (Sousa et al., 1999, Sousa et al., 2000, Haynes et al., 2001, Donohue et al., 2006) and striatum (Haynes et al., 2001, Copeland et al., 2005), we believe there is a major contribution of these cells.

Following this hypothesis, two major possibilities arise: (1) GC and $A_{2A}R$ directly mediate neuronal alterations and (2) microglia influence neuronal cytoarchitecture and function. Rather than one or the other, the behavioural phenotype is likely due to the sum of these effects.

Although the treatment with the $A_{2A}R$ antagonist reverted the behavioural alterations in adulthood, microglia was shown to be already altered by DEX exposure in early stages of life (PND1 and PND7; Caetano, 2014). Thus, considering the importance of microglia in the development of neuronal circuits, we decided to pursue the alterations at this stage.

In order to do so, we started by analysing the direct effects of DEX and $A_{2A}R$ antagonist exposure in primary hippocampal neurons morphogenesis.

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Exposure to DEX induced delayed contrasting neuronal morphologic alterations in dendrites and axon.

We reported that, while we did not observe any alteration in neuronal morphology after a short-term exposure to DEX, four days after treatment, it induced an atrophy of the dendrites and hypertrophy of the axon, seen by the decrease/increase in their respective lengths. This can be due to a delayed action of DEX on neuronal morphology.

Several reports showed dendritic atrophy resulting from either stress or glucocorticoid exposure in brain regions, such as as the PFC (Brown et al., 2005, Anderson et al., 2016) and the hippocampus (Sousa et al., 1999, Sousa et al., 2000, Silva-Gomez et al., 2013). However, alterations in axonal morphology were not yet reported.

Once synthetic GC, such as DEX, have a higher affinity to GR, it was expected that the effects of DEX on neuronal morphology were mediated by these receptors. In order to verify this hypothesis, we used an antagonist of GR. We observed that, whereas the effect on dendrites was prevented by the blockade of GR, the effect on the axon was maintained, suggesting a different mechanism of DEX-induced alterations.

The recent report that $A_{2A}R$ activation induces axonal elongation (Ribeiro et al., 2016), pointed $A_{2A}R$ as hypothetical modulators of these alterations. Accordingly, the increase in axon length induced by DEX exposure was prevented in the presence of an $A_{2A}R$ antagonist.

We previously reported that the exposure to DEX leads to an increase in the levels of $A_{2A}R$ in male PFC, and a decrease in females (Henriques, 2015). This observation is also indicative of a putative GR-$A_{2A}R$ crosstalk.

It would be interesting to understand how the exposure to DEX influences $A_{2A}R$ signalling. It is possible that DEX can raise the levels of endogenous adenosine, leading to the overactivation of $A_{2A}R$, increase the levels of $A_{2A}R$ (e.g. by modulation of *ADORA2A* gene expression), and/or lead to the translocation of receptors to the axon.

In late stages of development and early postnatal life, the expression of $A_{2A}R$ is already known to increase in the rat brain (Aden et al., 2000), but the subcellular location of these receptors was not addressed.

The blockade of GR *per se*, had an overall hypertrophic effect on dendrites and axon, reported at DIV5, inducing an increase in the number of dendrites and an increase in the complexity of the axon. These observations indicate that GR tonic activation by endogenous GC is important to control neuronal development. These morphological alterations could be due to the overactivation of MR, once the blockade of GR can increase the binding of GC to MR.

The fact that the blockade of GR had an opposite effect of DEX treatment in dendrites, but not in the axon, reinforces the observation that the dendritic effects are mediated by GR, whereas the effects on the axon have a different mechanism.

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$A_{2A}R$ tonic activation is important for normal neuronal development.

Considering the results presented in this work, the activation of $A_{2A}R$ is essential for the normal development of neuronal morphology in culture. The first observations of adenosine implications in neuronal morphogenesis, by Heilbronn and Zimmermann, showed that the increase in adenosine levels in PC12 cells led to neurite outgrowth, whereas the decrease in adenosine drove the cells to round up and cluster (Heilbronn and Zimmermann, 1995). Accordingly, it was reported that $A_{2A}R$ agonist induced an increase in the number and length of neurites in the same cell line (Cheng et al., 2002).

In a more complex model, primary cortical neurons, the activation of $A_{2A}R$ by an agonist led to the increase in dendritic branching and axonal elongation (Ribeiro et al., 2016).

Thus, $A_{2A}R$ activation over the tonic activation by endogenous adenosine, exerts a trophic effect upon dendrites and axon.

Indeed, it was previously reported in an animal model of caffeine (A_1R and $A_{2A}R$ non-selective antagonist) administration in the early postnatal period, an increase in dendritic length and ramification in PFC pyramidal neurons, maintained until PND70 (Juarez-Mendez et al., 2006). This indicates that, either the effects are mainly modulated by A_1R antagonism, or the blockade of $A_{2A}R$ also exerts a trophic effect on neuronal development, as in the case of its activation above physiological levels.

In line with this hypothesis, Batalha and colleagues observed that the animal treatment with an $A_{2A}R$ selective antagonist, increases dendritic branching in hippocampal pyramidal neurons (Batalha et al., 2013), reverting an atrophy induced by maternal separation. Even considering that *in vivo* there are different variables to take into account, and the alterations could be due to the interplay between several cell types, this observation is consistent with the hypothesis that $A_{2A}R$ have a modulator role upon neuronal morphology.

Our results, showed that the exposure to the $A_{2A}R$ antagonist led to a decrease in the length of the dendrites, which is in accordance with published data showing that $A_{2A}R$ activation leads to dendrite outgrowth (Ribeiro et al., 2016). However, we detected an increase in the number of dendrites, showing that $A_{2A}R$ antagonism does not induce dendritic atrophy. It is possible that the decrease in the length is not only due to an effect upon elongation, but is also a consequence of the increase in the number. The observation that the short-term exposure to the $A_{2A}R$ antagonist already led to a decrease in the length of the dendrites, while not altering their number, reinforces the first hypothesis.

At this time in neuronal development (five days in culture), the alterations induced by the $A_{2A}R$ antagonist were restricted to dendrites. Yet, the short-term exposure to the antagonist led to significant, although transient, alterations in the axon.

At DIV2, after 24 hours of treatment with $A_{2A}R$ antagonist, there was an increase in the number of axon-like structures, and a decrease in their average length. Two possibilities arise: either the activation of $A_{2A}R$ is essential to regulate the number of axons in the cell, or it has an impact upon axonal polarization.

The potential impact of $A_{2A}R$ signalling in neuronal polarization is not yet published; however, a recent work by Rodrigues *et al.*, presented at a conference of the *International Society for Neurochemistry* (PS02-15; 7th ISN Special Neurochemistry Conference, June 1-4, 2016, Coimbra, Portugal), described that the treatment of neurons in culture with an $A_{2A}R$ agonist induces the formation of axons, analysed at DIV3.

Considering that we observed that by DIV5 the number of axons had recovered to the regular number of one axon *per* cell, we speculate that the blockade of $A_{2A}R$ negatively influences neuronal polarization, leading to the presence of two competing neurites for a longer time period.

Taking into account the observation by Shelly and colleagues that BDNF and cAMP can induce axonal initiation and differentiation, this effect could be dependent on the impairment of cAMP and/or BDNF signalling (Shelly et al., 2007).

Once $A_{2A}R$ are positively coupled to adenylate cyclase (converts ATP into cAMP) (Fredholm, 1995), the blockade of these receptors will decrease the levels of cAMP. Also, it will promote the binding of endogenous adenosine to other receptors, such as A_1R and A_3R, which inhibit adenylate cyclase.

On the other hand, several functions of BDNF are dependent on the activation of $A_{2A}R$ (Jeronimo-Santos et al., 2014, Colino-Oliveira et al., 2016). In the absence of neurotrophins, the activation of $A_{2A}R$ can directly lead to transactivation of tropomyosin receptor kinase B (TrkB) (Lee and Chao, 2001). Thus, the blockade of $A_{2A}R$ can potentially inhibit BDNF-signalling, and therefore, negatively impact upon axon differentiation.

6. CONCLUSIONS

Although the use of synthetic GC in clinics is essential due to their beneficial effects in several pathologies, it is important to have a full understanding of their potential deleterious effects, namely in the CNS.

In this work, we found that the prenatal treatment with DEX can induce long-lasting morphological alterations in PFC microglia in males, a region involved in the response to stress. These alterations were correlated to an anxious-like phenotype (unpublished data). Previous work by our group had shown that DEX also had a long-lasting effect in the morphology of PFC microglia in females (Henriques, 2015). Surprisingly, DEX induces contrasting microglia remodelling in males and females (hyper-ramification and atrophy, respectively).

Considering previous observations of the anxiolytic effect of $A_{2A}R$ blockade (El Yacoubi et al., 2001, Kaster et al., 2015) and $A_{2A}R$ involvement in microglia morphology (Gyoneva et al., 2009, Orr et al., 2009, Gyoneva et al., 2014a, Gyoneva et al., 2014b), we tested if its chronic blockade could revert the anxious-like phenotype. $A_{2A}R$ antagonist chronic treatment successfully reverted behavioural alterations in males (unpublished data), but not in females (Caetano, 2014). This was correlated with an amelioration in males' microglia morphologic alterations, and an aggravation in females'. The different response of males and females to the pharmacological treatment is a matter of urgent notice, once it can bias the development and establishment of new therapies.

Having in mind the roles of microglia during brain development (Frost and Schafer, 2016) and the importance of its morphology, we infer that DEX-induced alterations contribute to alterations in the correct formation of neuronal circuits.

Once DEX can directly impact on neurons, we studied its effect upon the development of neuronal morphology. Once more, the normal development and maturation of axonal and dendritic trees is essential for their integration in the circuitry.

We report that, while the effect upon dendrites is atrophying, axon development is favoured. This differential effect depends upon the activation of different receptors, GR and $A_{2A}R$, respectively.

Thus, it is important to consider the influence of DEX upon the various cell types in the CNS, which are certainly involved in the higher susceptibility to neuropsychiatric disorders.

7. FUTURE PERSPECTIVES

Do microglia have gender?

Considering the contrast between males and females in the response of microglia to DEX and to the chronic treatment with $A_{2A}R$ antagonist, it is interesting to further analyse the differences between male and female microglia underlying such discrepant responses.

These differences can be due to the levels of expression of GR and $A_{2A}R$; so, it would be interesting to analyse if there are differences in the expression of these receptors in microglia, in the PFC, between males and females, in a physiological context.

Also, it is important to analyse the influence of sex specific hormones in the response to DEX and $A_{2A}R$ antagonist treatment, having in mind females' higher susceptibility and resilience to treatment. Thus, we could analyse, *in vitro*, the response of microglia to DEX and $A_{2A}R$ blockade in the presence or absence of male and female hormones.

The fact that microglia in the brain are distributed in several populations with a different signature (Grabert et al., 2016), the analysis of the morphology of microglia in other regions related to stress, such as the amygdala and ventral hippocampus, in the same model, could give us an overall view of the impact of microglia in the anxious phenotype, once we cannot infer that the alterations will be the same.

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In the big picture of anxiety, do microglia play an important role?

One of the limitations of this work is the impossibility to assign the features we observe to a specific cell type. *In vivo,* we always see the influence of treatments in the system, and not in a specific cell type. So, it is difficult to ascertain the contribution of microglia alterations. To try to overcome this limitation and ascertain if microglia do play an important role in the pathophysiology and recovery of the anxious-phenotype, we could specifically delete $A_{2A}R$ in microglia in rats prenatally exposed to DEX, by electroporation, and assess anxious behaviour.

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What is the influence of microglia upon neuronal cytoarchitecture and circuitry?

Given the tight association of these cells to synaptic pruning, we believe that the alterations in their morphology, induced by the exposure to DEX, can alter the normal rate of synaptic pruning, thus affecting the formation of neuronal circuits. Once complement factors are already established as a marker for synaptic pruning, we could analyse the levels of these proteins in a model treated with DEX.

However, even if there is no alteration in the levels of synaptic “tags” to phagocytosis, the ability of microglia to phagocytose can be altered by DEX exposure. Given the tight correlation between microglia function and morphology, this would not be surprising.

To try to overcome the problem of cell specificity in *in vivo* models, we can analyse microglia ability to phagocytose when expose to DEX *in vitro*, using co-cultures of neurons and microglia (primed and non-primed with DEX), and quantifying the number of synapses. Considering that some neuropsychiatric disorders have alterations in the balance of excitatory and inhibitory synapses, it would be interesting to analyse if microglia increase or decrease in phagocytosis was directed to specific kinds of synapses. Also, electrophysiology studies could give us important information regarding the wiring and the maturation of synapses.

Furthermore, one of the major future perspectives is to state the influence of microglia in neuronal morphogenesis, which could be done by the repetition of the experiments presented in this thesis, regarding the analysis of neuronal morphology, using co-culture of neurons and microglia. Additionally, conditioned medium of microglia primed with DEX could be used. The first experiment would shed light on the direct effect of microglia upon neuronal morphogenesis, whereas the second would only focus on the effects mediated by soluble factors.

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How does endogenous adenosine impact neuronal polarization?

One striking observation in this work was the alterations induced by the $A_{2A}R$ antagonist in axonal differentiation. Regarding this point, it is important to repeat the experiment using a specific neuronal marker, to verify the hypothesis that, rather than inducing the formation of more than one axon, $A_{2A}R$ antagonist delays axon specification, leading to the observation of several competing neurites at a stage in development when most neurons are already polarized.

Then, to address the hypothesis that this effect can be mediated by TrkB or cAMP levels, we could examine if this effect was reverted in the presence of exogenous BDNF or cAMP, or mimicked in the presence of respective inhibitors.

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Which are the mediators of the direct impact of DEX upon neurons?

Once we were only able to verify that DEX effect on dendrites and axon are dependent on the activation of different receptors, we could further pursue this path, and try to understand the mechanism behind these observations. Specially in what concerns the elongation of the axon, it could be interesting to check if it depends on microtubule destabilization, as was observed for the effect of $A_{2A}R$ agonist (Ribeiro et al., 2016), an hypothesis particularly promising, since the effects of DEX were dependent on these receptors.

Considering that this observation in the elongation of the axon was only seen *in vitro*, it is important to use a more complex model, to understand if the effect is maintained. Also, since these experiments were done in early stages of development, the effects can be transient. So, it would be interesting to observe the same effect at later stages.

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9. SUPPLEMENTARY DATA