Miguel Maria Restolho Mateus Pinheiro

Glucocorticoid Effects in the Developing Hippocampus: A Morphometric Assessment

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GLUCOCORTICOID EFFECTS IN THE DEVELOPING HIPPOCAMPUS:

A MORPHOMETRIC APPROACH

MIGUEL MARIA RESTOLHO MATEUS PINHEIRO

Dissertation presented to the Faculty of Science and Technology of the University of Coimbra to fulfil the necessary requirements to obtain the Master degree in Cellular and Molecular Biology. The work was performed in the Retinal Dysfunction and Neuroinflammation Lab from the Institute of Biomedical Imaging and Life Sciences (IBILI) in collaboration with the Center for Neuroscience and Cell Biology (CNC) and the Life and Health Sciences Research Institute (ICVS), under the supervision of Doctor Catarina A. Reis Vale Gomes and co-supervision of Doctor Ana Luísa Monteiro de Carvalho.

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We have to remember that what we observe is not nature herself,

but nature exposed to our method of questioning.

Werner Heisenberg

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ABBREVIATION LIST

A_1R	Adenosine A_1 receptor
$A_{2A}R$	Adenosine A_{2A} receptor
$A_{2A}R$ KO	Adenosine A_{2A} receptor
$A_{2B}R$	Adenosine A_{2B} receptor
A_3R	Adenosine A_3 receptor
ACTH	Adenocorticotropic hormone
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
AVP	Vasopressin
BDNF	Brain derived growth factor
cAMP	Cyclic adenosine monophosphate
CB1R	Cannabinoid receptor 1
CBG	Corticosteroid binding globlulin
CNS	Central nervous system
COX-2	Ciclooxigenase 2
CRH	Corticotropin releasing hormone
CTRL	Control
CX_3CL_1	Fractalkine
CX_3CR_1	Fractalkine receptor
D_1R	Dopaminergic receptor 1
D_2R	Dopaminegic receptor 2
DAPI	4’, 6-diamino-2-phenylindole
DEX	Dexamethasone
DG	Dentate gyrus
DIV	Days *in vitro*
ED	Embryonic day
GC	Glucocorticoid
GR	Glucocorticoid receptor
GW	Gestational week
HIP	Hippomcapus
HPA	Hipothalamus-pituitary-adrenal
HPT	Hipothalamus
Iba1	Ionized calcium-binding adapter molecule 1
IPC	Intermediate progenitor cell
LPS	Lipopolysaccharide
MAGUK	Membrane-associated guanylate kinase homologs
MR	Mineralocorticoid receptor

MS	Maternal stress
NMDA	N-methyl-D-aspartate
OCT	Optimal cutting temperature compound
OPC	Oligodendrocyte precursor cell
PBS	Phosphate buffer solution
PFC	Prefrontal cortex
PGE2	Prostaglandin E2
PND	Postnatal day
PS	Prenatal stress
PSD95	Post-synaptic protein 95
RT	Room temperature
SAP102	Synapse-associated protein 102
SCH	SCH58261; selective $A_{2A}R$ antagonist
TTX	Tetradotoxin

ABSTRACT

The neurobiological mechanisms underlying brain development rely on the constant harmony between the endogenous and exogenous factors that coordinate them. In case of compromised fetal environment, the neurodevelopmental programming can deviate from its normal course, leading to dysfunctional brains with altered functionality, as is the case of glucocorticoid exposure.

Indeed, it is widely reported that the exposure to high levels of glucocorticoids during development (due to pharmacological treatment or stress) can have deleterious effects in the brain. Recently, we demonstrated that rats prenatally exposed to dexamethasone, a synthetic glucocorticoid, present anxious like behaviour which positively correlates with morphological alterations in prefrontal cortex microglial cells. Interestingly, the cytoarchitectural remodelling had a strong gender-biased effect, since dexamethasone elicited different structural alterations according to sex. To overcome the behavioural deficits, a pharmacological chronic blockade of adenosine A_{2A} receptors, important modulators of microglia morphology, proved to be efficient, but only in males. The improvement in behaviour was correlated with an amelioration regarding microglia structure, while further compromising microglia in females.

Regarding the hippocampus, which has a central role in behaviour, we observed that antenatal dexamethasone also induces long-term structural alterations in microglia in females. These alterations were accompanied by connectivity deficits between the prefrontal cortex and the hippocampus, further suggesting that the structural integrity of the hippocampal region is compromised.

In this work, to explore gender specificity regarding the hippocampus and the extent of its compromise upon dexamethasone exposure, we assessed the cellular structure in the hippocampal formation (CA1 and dentate gyrus) in adult male rats exposed to dexamethasone *in utero*. Manual reconstructed pyramidal neurons from the CA1 presented heightened dendritic length in both basal and apical arborization with a mild increase in dendritic ramification, showing an overall structural hypertrophy. This structure remodelling was also noticeable at the synaptic level. In organotypical hippocampal slices, acute dexamethasone stimulus showed minor tendencies in promoting spine maturation in the same neuronal population.

Concerning microglia morphology, prenatal dexamethasone promoted a slight increase in the length and number of processes in the dentate gyrus. Upon a chronic blockade of adenosine A_{2A} receptors in adulthood, dexamethasone exposed animals revealed a

marked structural hypertrophy, with increased length and number of processes. These results contrasted with females, since adenosine A_{2A} receptor blockade induced a partial recovery in microglia morphology in the hippocampus. The implication of the adenosine A_{2A} receptors was furthered validate in knockout mice for this receptor, where adult male microglia exhibited some minor structural alterations.

This study further portrays the cellular structural remodelling in the developing brain exposed to elevated glucocorticoid levels, clearly emphasizing the importance of both sex and brain region in the modulation of these effects. Thus, when accounting structural alterations and their impact in brain function and behaviour, it is essential to have in mind the differential responses of each gender not only towards the insult but also to the pharmacological treatments. Finally, this awareness is imperative in the development of new pharmacological treatments, particularly regarding disorders with gender-specific susceptibilities such as psychiatric disorders.

KEYWORDS: brain development, glucocorticoids, hippocampus, CA1 pyramidal neurons, microglia, adenosine A_{2A} receptors

RESUMO

Os mecanismos neurobiológicos subjacentes ao desenvolvimento cerebral dependem da constante harmonia entre os fatores endógenos e exógenos que os coordenam. Em circunstâncias em que o ambiente fetal se encontre alterado, como no caso da exposição a glucocorticoides, o desenvolvimento neurológico poder-se-á desviar do seu curso normal, culminando na formação de cérebros anómalos e disfuncionais.

De facto, está extensivamente descrito que a exposição a elevadas concentrações de glucocorticoides (devido a tratamentos farmacológicos ou stress) pode ter efeitos deletérios no cérebro. Recentemente, demonstrámos que ratos expostos a dexametasona (glucocorticoide sintético) durante o período pré-natal, apresentam comportamento tipo ansioso. Por sua vez, estas modificações comportamentais encontram-se positivamente correlacionadas com alterações estruturais nas células da microglia do córtex pré-frontal. Curiosamente, a remodelação citoarquitectural das células da microglia apresentou efeitos dependentes do sexo, uma vez que a dexametasona promoveu modificações morfológicas diferentes entre machos e fêmeas. De modo a superar os défices comportamentais observados, o bloqueio farmacológico crónico dos recetores de adenosina A_{2A}, importantes reguladores fisiológicos da microglia, provou ser eficaz,apenas em machos. A melhoria a nível comportamental também se refletiu numa melhoria a nível estrutural nas células da microglia, comprometendo ainda mais a morfologia destas células em fêmeas.

Relativamente ao hipocampo, uma região cerebral intimamente ligada ao comportamento, observámos que a dexametasona no período pré-natal induz alterações estruturais a longo prazo na microglia de fêmeas. Estas modificações foram acompanhadas por défices de conectividade entre o córtex pré-frontal e o hipocampo, sugerindo possíveis alterações na integridade estrutural do hipocampo.

No presente trabalho, de forma a explorar a possível especificidade de género na região do hipocampo e a extensão das alterações induzidas pela exposição a dexametasona, analisou-se a estrutura celular na formação hipocampal (CA1 e giro denteado) em ratos machos adultos submetidos a dexametasona *in utero*. Modelos tridimensionais de neurónios piramidais da região CA1 reconstruídos manualmente apresentaram um aumento nas ramificações dendríticas basais e apicais, com um ligeiro aumento no número de ramificações. Esta restruturação também foi notável a nível sinático. Em culturas organotípicas de hipocampo, um breve estímulo com dexametasona mostrou tendência a promover a maturação de espículas dendríticas na mesma população neuronal.

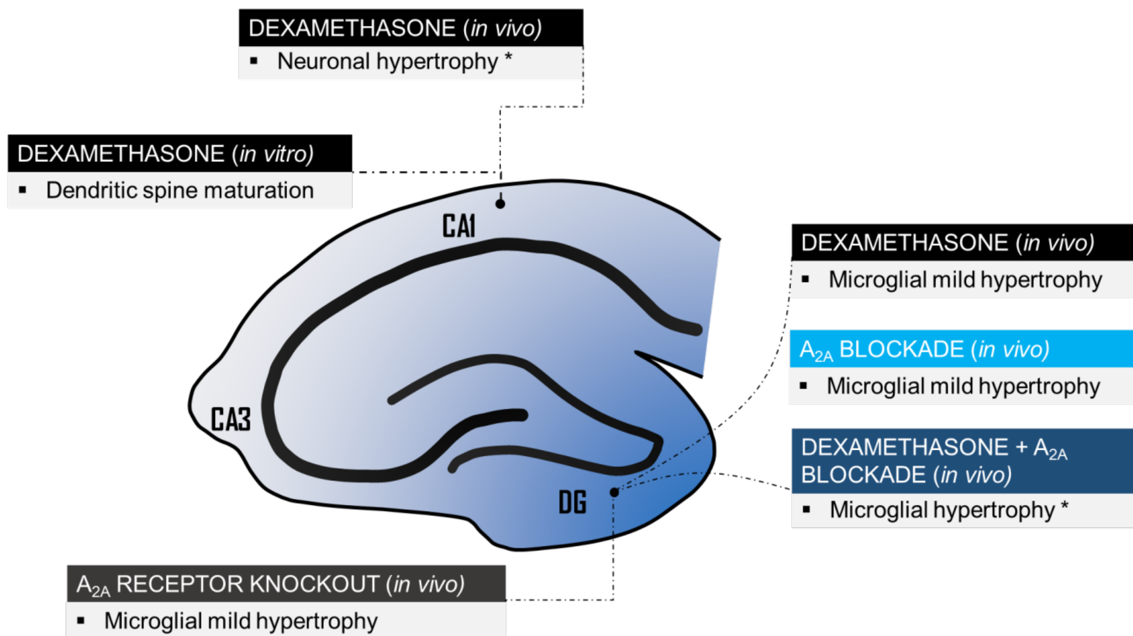
Quanto à morfologia da microglia, a dexametasona promoveu um ligeiro aumento no comprimento e número de processos no giro denteado. Após o bloqueio crónico dos recetores adenosinérgicos A_{2A} na idade adulta, animais expostos a dexametasona durante o período gestacional apresentaram células da microglia hipertróficas, com um aumento acentuado no comprimento e número de processos. Estes resultados contrastaram com os observados em ratos fêmeas, nos quais o bloqueio crónico dos recetores A_{2A} induziu uma recuperação parcial na morfologia da microglia no hipocampo. A implicação dos recetores adenosinérgicos A_{2A} como moduladores morfológicos da microglia foi igualmente validada em murganhos *knockout* para este recetor, onde machos adultos exibiram microglia com ligeiras alterações estruturais.

Este estudo detalhou a remodelação estrutural a nível celular no cérebro em desenvolvimento exposto a concentrações elevadas de glucocorticoides, enfatizando o sexo e as regiões cerebrais enquanto moduladores diferenciais destes efeitos. Demonstrámos queé essencial discriminar as repostas de cada sexo não apenas perante distúrbios mas também perante tratamentos farmacológicos aquando da avaliação de alterações estruturais e das suas implicações no funcionamento cerebral e no comportamento. Esta consciencialização é imperativa para o desenvolvimento de novas ferramentas farmacológicas, principalmente no tratamento de patologias com diferentes suscetibilidades entre sexos, como os distúrbios psiquiátricos.

PALAVRAS-CHAVE: desenvolvimento neurológico, glucocorticoides, hipocampo, neurónios piramidais CA1, microglia, recetores adenosinérgicos A_{2A}

GRAPHICAL ABSTRACT

Graphical abstract depicting the main results presented in this thesis and the corresponding hippocampal regions where cell morphometry was assessed. All experiments were done using male rats except for the adenosine A_{2A} receptor knockouts, which were done in male mice.



** Statistical significant alterations*

INTRODUCTION

1.1. NEURODEVELOPMENT – AN OVERVIEW

Human brain development is an intricate process which involves a plethora of neuronal and non-neuronal events that culminates in the maturation of the healthy brain. It begins around the third gestational week (GW), with the formation of the first neural structure, the neural tube (Stiles and Jernigan, 2010), along with the differentiation of neural progenitor cells into neurons and glial cells, which progresses until late adolescent, debatably throughout the lifespan (Yamaguchi et al., 2016). This overlapping occurrence of neurodevelopmental mechanisms is tightly regulated at an endogenous molecular level, coupled to a spatial and temporal switch of gene expression (Kang et al., 2011), sensible to environmental inputs (Bock et al., 2015). Hence, an overall harmony between these processes is needed in order to assure the correct development of the brain.

By the end of the embryonic period (eighth week post conception), the basic structures of the human brain and of the central nervous system (CNS) are formed. Until the end of the gestational period, cortical and subcortical structures develop at a rapid pace, with the formation of primitive major fiber pathways (Kostović and Jovanov-Milosević, 2006). At a cellular level, neuron production begins at embryonic day (ED) 42 concomitantly with gliogenesis, giving rise to glial cells (Bystron et al., 2008). Produced neurons will then migrate to different brain areas where they will establish the first interneural connections, in a process called synaptogenesis, allowing the formation of the first rudimentary neural networks (Stiles and Jernigan, 2010; Knuesel et al., 2014).

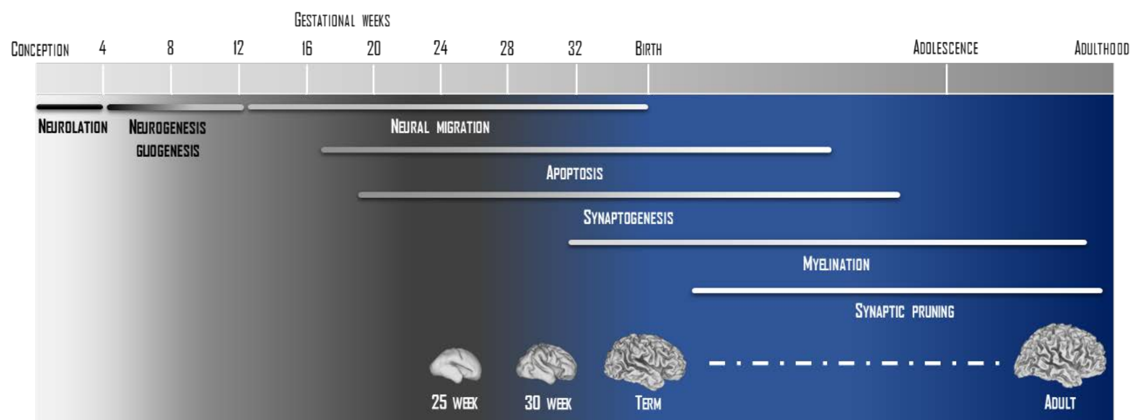


Figure 1| Timescale of brain development main events from conception until adulthood (adapted from Knuesel et al., 2014**).**

1.1.1. NEURAL DEVELOPMENT

The human brain is composed by billions of neurons and glial cells, most of which are produced by mid-gestation (von Bartheld et al., 2016; Bayer et al., 1993). The first event to take place in neurogenesis consists in the augmentation of the neural progenitor cell population through symmetrical division. Then, these cells will undergo a shift from symmetrical division to asymmetrical, originating both neural progenitors and neurons (one of each per division) (Weissman et al., 2003). Once neurons are formed, they will migrate and populate the neocortex, forming distinct cortical layers with different neuronal populations (Cooper, 2008). Meanwhile, neuroepithelial cells (which coat the cerebral ventricles and spine canal) begin to transform into radial glial cells as soon as neurogenesis begins. These cells can then become astrocytes or give rise to intermediate progenitor cells and oligodendrocyte precursor cells (OPCs), which in turn produce both neurons and oligodendrocytes (Figure 2) (Rowitch and Kriegstein, 2010). However, one type of glial cell has a distinct ontogeny. Microglia cells derive from progenitors of the yolk-sac, colonizing the brain during development (Alliot et al., 1999; Cuadros and Navascués, 1998; Ginhoux et al., 2010). These cells will be thoroughly described in a following section.

1.1.1.1. NEURONAL ARBORIZATION

Neurons possess a well-defined arborization that is responsible for the synaptic transmission. Soon after neurogenesis, the nascent neuron emanates several processes from the soma. One of the neurites will commence a rapid growth and thus becomes the axon, the output structure. The remaining immature neurites will later become dendrites, where electrical signals are encoded (Figure 2) (Dotti et al., 1988). These two different subcellular compartments are highly specialized and are distinguishable in terms of electrical excitability and morphology (Cáceres et al., 2012).

1.1.1.2. DENDRITIC SPINE DEVELOPMENT

Dendrites present specialized structures along their shafts, the dendritic spines, which are small cytoplasmic protrusions that integrate synaptic signaling, increasing the area for signaling input. These small structures receive the majority of excitatory connections in the brain, being key structures in the regulation of neural activity (García-López et al., 2010). They appear early in development and are more abundant in higher brain regions, such as the hippocampus (HIP) (Bhatt et al., 2009).

These synaptic structures also undergo various stages of development, from filopodia (headless immature spines) to fully matured mushroom spines, named after their bulbous heads. Dendritic spines also present other intermediate forms, each one representing different maturation rates. Thin spines differ from the mushroom types due to their smaller head. On the other hand, stubby spines have equal neck width to spine length ratios while bifurcated have more or two heads (Figure 2) (Dumitriu et al., 2011)

Spine development, which directly correlates with synaptogenesis, begins with an adhesive contact between motile filopodia and the presynaptic axon. Some of them receive synaptic inputs, which consequently converts them in dendritic spines while others form shaft synapses, being able to later reemerge as spines (Bourne and Harris, 2008).

Immature spines are identified by the presence of NMDA receptors while AMPA receptors are absent (Hanse et al., 2009). The emergence of the latter will promote a dynamic reorganization of the cytoskeleton, thus having an important role in spine maturation (Washbourne et al., 2004). The stabilization of these structures also requires the function of different proteins such as membrane-associated guanylate kinase homologs (MAGUKs) (Oliva et al., 2012), SAP102 and PSD95 (Elias et al., 2008).

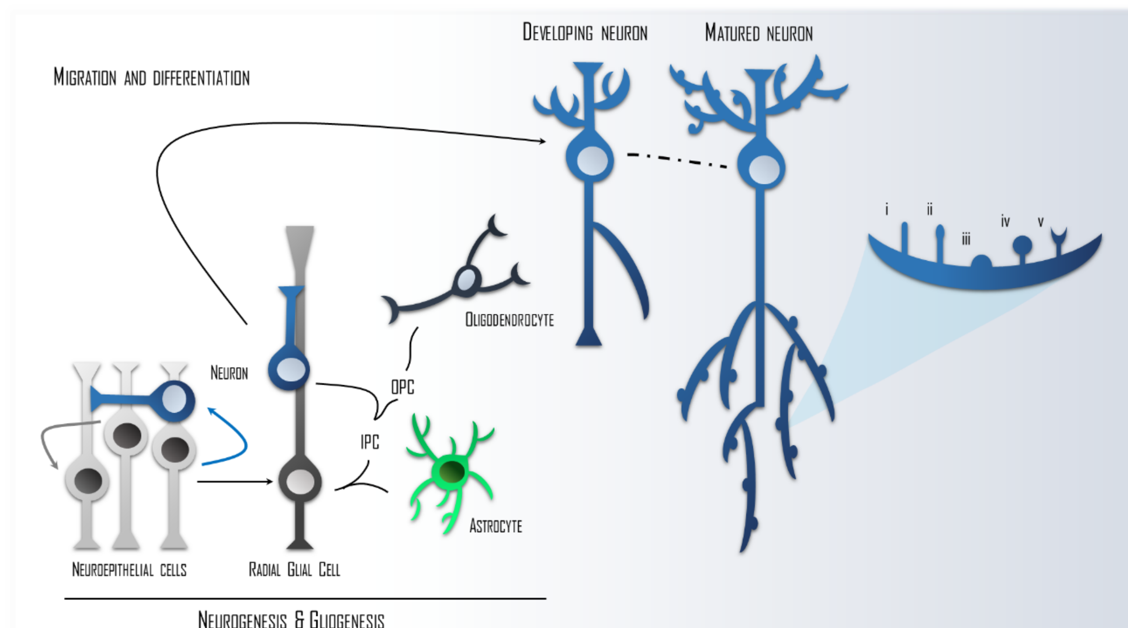


Figure 2|Main steps in neurogenesis/gliogenesis and neuronal development of a pyramidal neuron. Neuroepithelial cells replenish the neural progenitor population through symmetrical divisions (grey arrow) or give rise to new neurons by asymmetrical divisions (blue arrow). These cells can then transform in radial glial cells which can further turn into intermediate precursor cells (IPCs). IPCs cells can the transform to neurons or oligodendrocytes through the generation of oligodendrocytes precursor cells (OPCs). During migration, immature pyramidal neurons start to develop their dendritic arborization, culminating in the formation of matured neurons with dendritic spines (i – filopodia; ii – thin; iii – stubby; iv – mushroom; v – bifurcated) along their dendritic shafts (basal and apical).

Nonetheless, is important to state that several models for spinogenesis have been proposed (García-López et al., 2010), suggesting that different mechanisms may underlie the formation of dendritic spines and their structural plasticity.

1.1.1.3. SYNAPTOGENESIS

After the maturing neuron reaches its final destination in the brain, it then needs to establish contact with their surrounding partners, some located at a significant distance. Synaptogenesis occurs concurrently with dendritic and axonal growth, along with the myelination of the subcortical white matter (Huttenlocher and Dabholkar, 1997). Extending dendrites and axons reach their targets through a specialized structure, the growth cone, present at the tip of the developing neurite (Tessier-Lavigne and Goodman, 1996). This growth is not randomly conducted, being guided by a variety of diffusible chemotactic factors (McAllister, 2002) that establish either repulsive or attractive gradients (Dent et al., 2011), allowing the correct neuronal approach. The assembly of the pre- and postsynaptic compartment will follow through a vast combination of vesicle trafficking and local recruitment of synaptic proteins. Ultimately, synaptic activity will promote synapse strengthening and stabilization, or weakening and elimination (Waites et al., 2005).

1.1.2. REGRESSIVE EVENTS IN BRAIN DEVELOPMENT

Neurodevelopment consists not only on the sole proliferation, but also in the controlled elimination of neural elements. Regressive events during brain development happen naturally, including cell death and pruning supranumerary synapses (Stiles and Jernigan, 2010). Both processes play an essential role in modeling our brain circuitry.

1.1.2.1. NEURONAL DEATH

Neuronal death can occur by two distinctive well described mechanisms. Necrotic cell death is a mechanism for removing damaged tissue from biological systems, consequence of pathological events that resulted in the injury of a population of cells. On the other hand, apoptosis is a cell-intrinsic process that culminates in cell death through a cascade of regulated processes which occur in physiological conditions (Majno and Joris, 1995). This process, however, does not always implies a previous commitment to cell death by neurons since recent data indicates that phagocytosis can promote the death of viable neurons. This process is termed as primary phagocytosis or simply phagoptosis (Brown and Neher, 2012).

During brain development, it is widely thought that neurons compete for neurotrophic factors, which are crucial for their survival over time. This runs accordingly with an already established theory, the neurotrophic hypothesis, stating that neurons which manage to establish stable connections are able to compete and obtain surrounding neurotrophic factors more effectively (Oppenheim, 1989). Therefore, cell death has a preponderant effect in neural development, since it supports the establishment of brain circuitry, influencing both neuronal production and migration (Buss and Oppenheim, 2004).

1.1.2.2. SYNAPTIC PRUNING

The density of synaptic connections undergoes significant changes throughout neurodevelopment. It was long believed that neural pruning, the elimination of synapses, was a brief event that ended shortly after birth. However, this view came to term in 1979, when the late neurologist Peter Huttenlocher demonstrated that synaptic pruning actually continues long after birth. He observed synaptic density peaks in mid- to late childhood, decreasing afterwards during adolescence (Huttenlocher, 1979) in a activity-dependent manner (Huttenlocher, 1990).

The complex refinement of our brain circuitry has very likely important consequences for normal and abnormal brain functions. Additionally, abnormal synaptic pruning is intrinsically related to neuropsychiatric disorders, possibly explaining why many mental illnesses emerge in adolescence (Wittchen et al., 1998). Sekar and colleagues recently showed that the onset of schizophrenia is connected to the dysregulation of the complement cascade (Sekar et al., 2016), an important component of the immune system that promotes synaptic elimination (Stevens et al., 2007). This molecular system coordinates synaptic pruning by marking neurons to be eliminated, a process which is accomplished by microglia, the CNS macrophages (Paolicelli and Gross, 2011). Briefly, in the developing brain, astrocytes trigger the production of the complement protein C1q in neurons via an unknown molecular signal. C1q originated from neurons and microglia tag weak or superfluous synapses for removal through the classical complement pathway, culminating in C3 cleavage and synaptic C3b deposition. Complement-tagged synapses are ultimately removed through phagocytosis by microglia (Figure 3) (Stephan et al., 2012).

The implication of microglia in neural restructuring process is crucial for brain development and being validated as the homeostatic guardians of the brain, microglia impairment can have severe implications in brain health and disease (Hong et al., 2016).

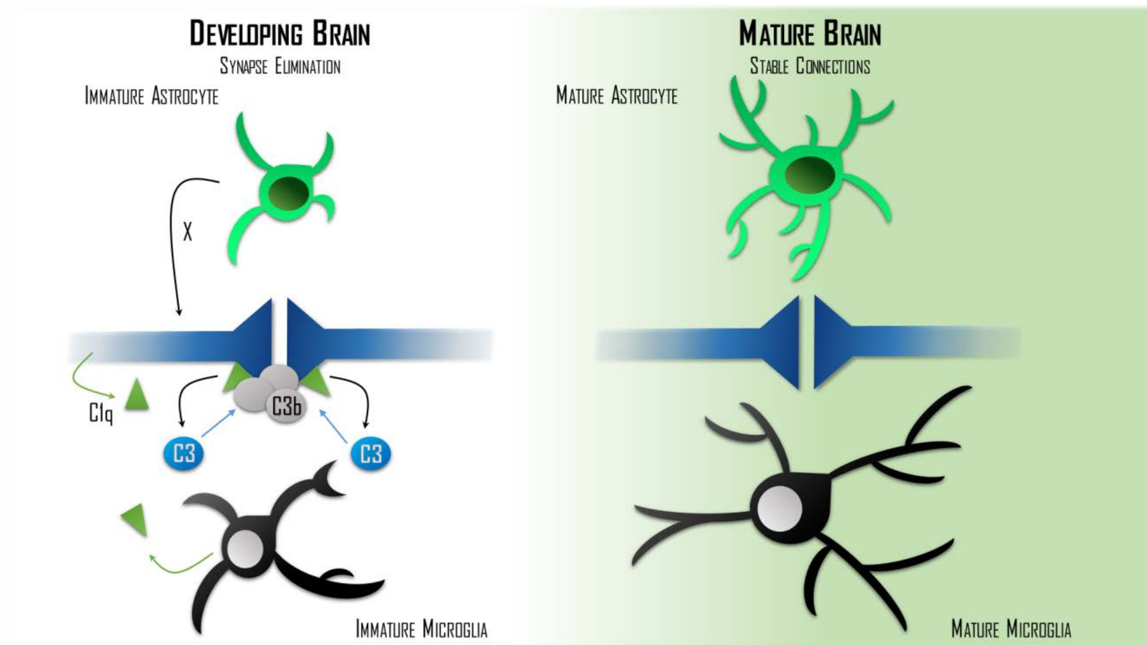


Figure 2| The classical complement cascade mediates synapse elimination in the developing brain. During brain development, astrocytes induce the production of C1q by an undetermined signal ("X"). C1q from both neuronal and microglial populations mark synapses for elimination, inducing C3 cleavage and the deposition of synaptic C3b. Ultimately, tagged synapses are phagocytized by microglia. In the absence of the complement molecular signaling, synapses remain stable (adapted from Stephan et al., 2012).

1.2. MICROGLIA – ORIGIN AND IMPLICATION IN BRAIN HEALTH

1.2.1. HISTORICAL OVERVIEW

Hitherto, gentlemen, in considering the nervous system, I have only spoken of the really nervous parts of it. But if we would study the nervous system in its real relations in the body, it is extremely important to have a knowledge of that substance also which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or less degree.

Rudolf Virchow, *Die Cellularpathologie* (Virchow, 1858)

Historical evidence regarding microglia takes us back to the nineteen century, when Rudolph Virchow introduced the term neuroglia , translated from the word “Nervenkitt”, meaning nerve-glue (Virchow, 1856). A few years later, Camillo Golgi made a breakthrough with his remarkable staining technique, the “Reazione Negra” (Black Reaction), which allowed him to depict glial cells as a distinct population from neurons (Golgi, 1885).

Ramon y Cajal was the first to identify microglia, characterizing them as a unique cell population. The name “corpuscles without processes” was the first name given to microglia, since the gold chloride-sublimate staining didn’t allow him the observation of the cellular processes (Rezaie and Male, 2002). It was not until 1919 that Pio del Río-Hortega finally depicted an accurate representation of microglia, thus calling them the “third element” of the CNS (Río-Hortega, 1919).

1.2.2. MICROGLIA ONTOGENY

The ontogeny of microglial cells was a subject of intense controversy until recently. Evidence has now emerged demonstrating that microglia are mesodermally-derived mononuclear cells that arise during primitive hematopoiesis in the yolk sac (Alliot et al., 1999; Cuadros et al., 1993; Ginhoux et al., 2010). In humans, microglia can be identified in the extracerebral mesenchyme as early as 4.5 GW, invading the parenchyma around the 5 GW (Monier et al., 2006, 2007), while microglia progenitors appear near embryonic day 9 in rodents (Figure 4) (Alliot et al., 1999; Ginhoux et al., 2010; Kierdorf et al., 2013). Brain colonization by microglia is tightly coupled with embryonic vascularization (Ajami et al., 2007), relying on the formation of blood vessels and blood pressure (Ginhoux et al., 2010). Nonetheless, microglia has also been observed in the CNS in regions devoid of vascularization (Hurley and Streit, 1995), indicating alternative entering pathways such as

the brain ventricles and the para-meningeal routes (Cuadros and Navascués, 1998). Other areas have been implicated in humans, such as the meninges, the ventricular zone and the choroid plexus (Monier et al., 2006, 2007).

1.2.3. MICROGLIA SPATIAL DYNAMIC

The spatial distribution of microglia in the embryonic brain occurs in a gender-specific manner. Male rodents have higher microglial density in the early postnatal period, while females present an increased microglial density in adulthood (Figure 4) (Schwarz et al., 2012). Additionally, the colonization of the brain is accompanied by microglia differentiation throughout neurodevelopment. In early developmental stages, a reduced amount of amoeboid microglia are present (Andjelkovic et al., 1998) near highly vascularized regions (Payam Rezaie et al., 2005). As neurons start to migrate and populate the brain, neuronal clearance is substantially reduced. This promotes a shift regarding microglia morphology, from an amoeboid structure to a more complex and ramified one, bearing long, thin and branched processes (Monier et al., 2006). By the end of the 35 GW, microglia exhibits a fully matured, ramified cytoarchitecture (Esiri et al., 1991). This shift in microglia morphology is accompanied by a change in microglia physiology. During the early stages of neurodevelopment, amoeboid microglia are able to phagocytize both neuronal precursor cells, controlling neurogenesis rate (Peri and Nüsslein-Volhard, 2008; Cunningham et al., 2013) and apoptotic neurons, efficiently containing toxic degradation elements (Peri and Nüsslein-Volhard, 2008). In adulthood, amoeboid "reactive" microglia are implicated in inflammatory responses such as cytokine production or pathogens phagocytosis, while ramified "resting" microglia constantly and actively scan their environment for endogenous or exogenous signals that my indicate a

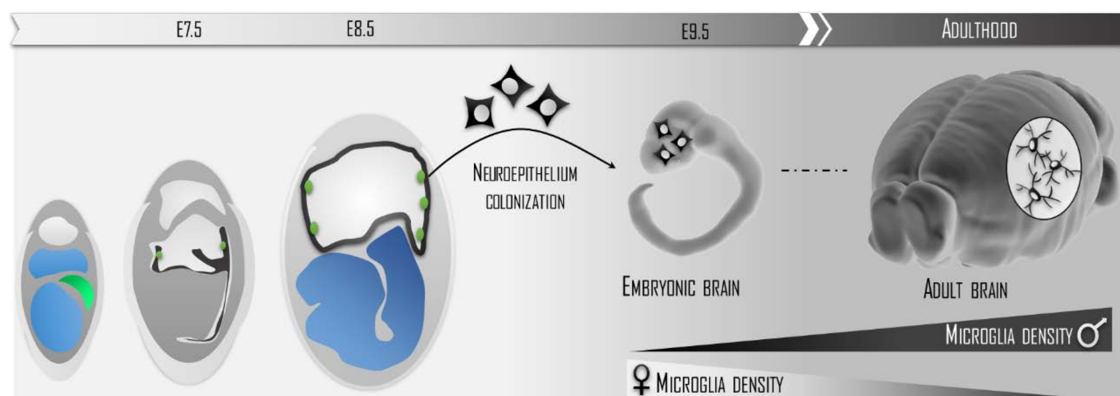


Figure 3| Microglia ontogeny and colonization in the rodent brain. At the onset of circulation, primitive hematopoiesis takes place in the yolk sac of the embryo, forming primitive macrophages. These cells later colonize the brain between E8.5-E9.5, giving rise to microglia. The density of microglial cells in the brain is gender-biased, since females present more microglia in the immature brain than males. Nonetheless, these differences are reversed over development, with males displaying more microglia than females (adapted from Ginhoux et al., 2013).

threat to brain homeostasis (Kettenmann et al., 2011).

In the adult, it was first suggested that microglial population is maintained by self-renewal (Lawson et al., 1992). Accordingly, a recent study demonstrated that in the adult mouse and human brain, microglia display a high proliferation rate, allowing the renewal of the whole population several times during their lifespan (Askew et al., 2017).

1.2.4. MICROGLIA IN BRAIN HEALTH - A RESTLESS VIGILANT

For many years, microglia was portrayed as an inert/resting component in the brain parenchyma, becoming reactive only in response to external stimuli (Ransohoff and Perry, 2009). In the late nineties, there was already a plethora of evidence implicating microglia in brain pathology, further validating microglia role in brain insult (Nakajima and Kohsaka, 1993). However, with the development of more advanced microscopic equipment, this view was rapidly refuted. Through the coupling of recent tools at that time, such as the $CX_3CR_1^{GFP}$ (fractalkine receptor) transgenic mouse model and two-photon *in vivo* fluorescence microscopy, two separate studies uncovered the true dynamics of microglia, which display a highly dynamic cell motility and plasticity while in their “resting”-ramified state (Davalos et al., 2005; Nimmerjahn et al., 2005). The rapid motility of microglia processes allow these cells to interact with nearby neurons to assess neuronal synaptic integrity (Wake et al., 2009) in an activity-dependent mechanism (Tremblay et al., 2010). This neuron-microglia crosstalk is further exemplified with the expression of all neuronal receptors (Kettenmann et al., 2011), along with the expression of the CX_3CR1 receptor which interacts with CX_3CL_1 (fractalkine), released by neurons (Sheridan and Murphy, 2013). The CX_3CR_1 signaling is responsible for mediating numerous cellular processes during postnatal development. These receptors can modulate microglial recruitment to neuronal circuits by increasing their process dynamics and cellular migration, as well as modulating neuronal survival via the release of trophic factors (Paolicelli et al., 2014), reflecting a tight physiological link between both neurons and microglia.

This change of paradigm regarding microglia physiology raised awareness concerning microglia role in the healthy brain, leading to the discovery of unique microglia properties within the CNS.

1.2.5. MICROGLIA AS A BRAIN ARCHITECT

The physiological polyvalence of microglia put this cell population in a highlighted position concerning the patterning of the developing CNS. Being depicted as professional macrophages, microglia also participate in a vast array of homeostatic processes. In the

early stages of brain development, microglia control the number of neuronal precursor cells through phagocytose (Cunningham et al., 2013). In later stages, microglia continues eliminating excess neurons by two distinct mechanisms. The first occurs as a natural response towards programmed cell death, where microglia phagocytizes dead or dying neurons and associated debris (Fricker et al., 2012). The second implicates an active role in inducing cell death through soluble or contact-mediated cues, a process termed phagoptosis (Neniskyte et al., 2014).

The most prominent role of microglia in brain development is implicated in circuitry refinement through the elimination of exceeding synapses. This mechanism involves a neuron-microglia interaction, since the disruption of CX_3CR1 signaling culminated in an increase in spine density and immature synapses (Paolicelli et al., 2011a). This intercellular communication can also be mediated by the complement system. Complement proteins are widely expressed in both neurons and glia in the postnatal brain. In mice lacking the C1q, the initiating protein in this signaling cascade, or the complement protein C3, there are impairments in synapse elimination and synaptic connectivity (Stevens et al., 2007).

Conversely, microglia can also promote synaptogenesis through the release of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) (Parkhurst et al., 2013) and anti-inflammatory cytokines, such as interleukin-10 (Lim et al., 2013). Our group further validated the importance of the immune modulation by demonstrating that microglia cells promote synapse formation after being primed with an inflammatory stimuli (Cristovão et al., 2014).

Having a dynamic profile in our brain, microglia impairment can severely compromise brain development, being fundamentally implicated in the onset of neuropsychiatric disorders (Prinz and Priller, 2014).

1.3. GLUCOCORTICOID SIGNALING – A STRESS RESPONSE

At the initial stages of brain maturation, our neural circuitry has a malleable nature that enables its continuous restructuration in accordance with internal and external stimuli. Although being a lifelong process, the earliest phases of maturation are important due to its higher plasticity (Toga et al., 2006). This enhanced plasticity is more noticeable in brain areas where maturation takes a slower pace, such as the HIP and the prefrontal cortex (PFC) (Taverna et al., 2014), making these regions more vulnerable to deleterious factors, such as stress.

In 1989, Barker and colleagues postulated the fetal programming hypothesis, stating that the disruption of the uterine environment during critical periods of organogenesis may permanently alter organ structure and function (Barker et al., 1989). This theory has been corroborated by studies showing structural abnormalities promoted by stress in brain regions linked to higher cognitive functions, such as the HIP, the PFC and the amygdala (Shirazi et al., 2015) (Figure 5). Moreover, prenatal stress (PS) has been implicated in the onset of neuropsychiatric illness, such as depression (Watson et al., 1999) and schizophrenia (Holloway et al., 2013). These findings place stress in the core of emotional related disorders.

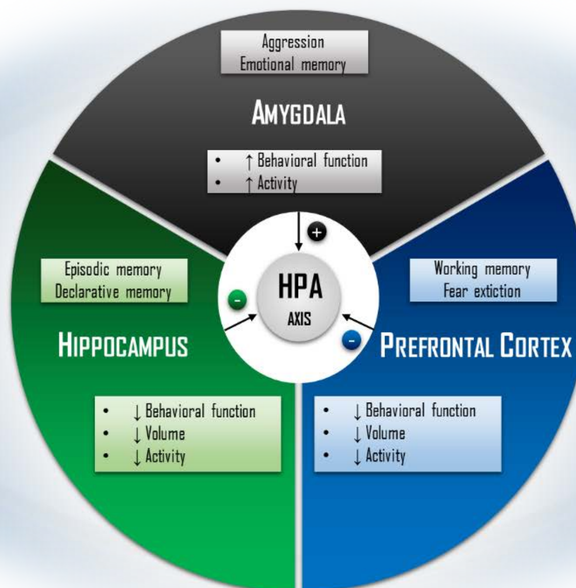


Figure 4| Brain regions implicated in stress responses. The amygdala, hippocampus and prefrontal cortex undertake structural adaptations, as well as functionality alterations in stress-related disorders. In turn, each region regulates differentially the HPA axis (adapted from Charttaji et al., 2015).

1.3.1. THE HYPOTHALAMIC-PITUITARY-ADRENOCORTICAL AXIS

Glucocorticoids (GCs) are stress-responding hormones that mediate a plethora of physiological changes in order to restore homeostasis upon stress stimuli (Smith and Vale, 2006).

The levels of these steroids within our organism are tightly regulated by the hypothalamic-pituitary-adrenocortical (HPA) axis, characterized by a hormonal cascade between those regions which culminates in the synthesis and release of GCs (cortisol in humans and corticosterone in some animals, as rodents and chicks) to the bloodstream. Briefly, upon a stressful situation, the hypothalamus will potentiate the release of corticotropin-releasing hormone (CRH) and vasopressin (AVP), which stimulate the production and secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary. ACTH then stimulates the release and synthesis of GC from the adrenal cortex. This cascade is negatively regulated by a feedback mechanism which inhibits signal transduction in each previously mentioned structures, plus the HIP (Figure 6) (Sandi, 2004).

In the brain, GCs will bind to both glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), preferentially binding to MR due to the higher affinity for these receptors (Sloviter et al., 1993). However, upon a stressful event, elevated levels of GCs will promote their binding to GR, hence promoting a stress response (Myers et al., 2014). It has been postulated that a "yin-yang" relationship between the activation of both receptors is essential to maintain homeostasis and health. Since stress disrupts this balance, early life stress is thought to potentiate the onset of mental illness by precisely impairing the

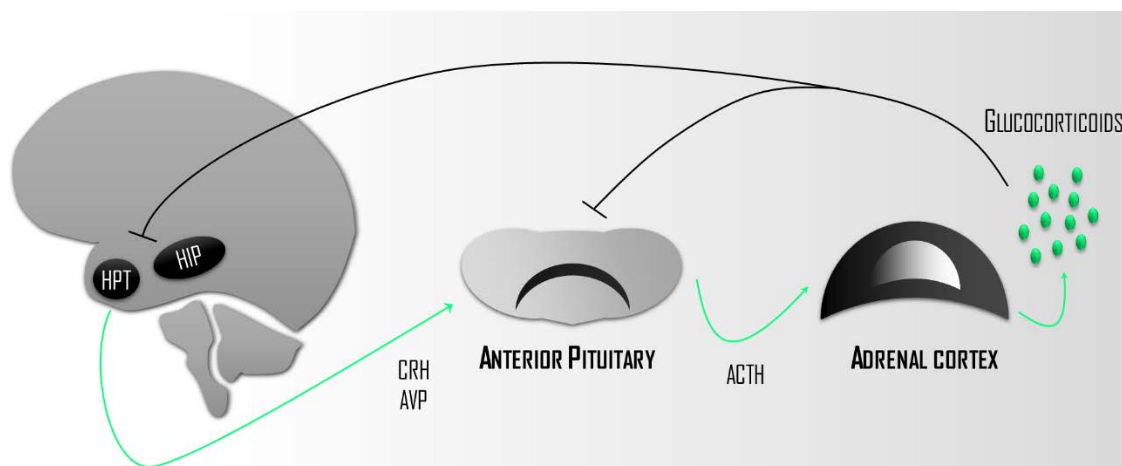


Figure 5| The hypothalamic-pituitary-adrenal hormonal cascade. Upon a stress input, the hypothalamus (HPT) promotes the release of corticotropin-releasing hormone (CRH) and vasopressin (AVP). These hormones will act on the anterior pituitary, eliciting the secretion of adrenocorticotropic hormones (ACTH) that will stimulate the production of glucocorticoids in the adrenal cortex. A negative-feedback mechanism then takes place ceasing the hormonal production in all sites implied in the axis (adapted from Lupien et al., 2009).

GCs negative feedback mechanism, having reverberating effects throughout an organism's lifespan (Khalife et al., 2013).

1.3.2. DEXAMETHASONE – A CLINICAL CASE

One crucial step during gestation involves the maturation of the pulmonary system, in which a peak of endogenous GCs is needed for this process to occur (Sullivan et al., 2003). When dealing with premature births, the use of dexamethasone (DEX), a synthetic GC, is a common practice nowadays since it mimics the natural GC surge, allowing pulmonary maturation (Brownfoot et al., 2013).

While fetal production of GCs occurs relatively late in gestation, maternal corticosteroids can cross the placental barrier, as well as synthetic corticosteroids such as DEX (Gitau et al., 2001). Their actions are buffered by two placental enzymes, the corticosteroid binding globulin (CBG) and the 11β-hydroxylase 2, which control the levels of GC between the placenta and the fetus (Mesquita et al., 2009). However, DEX does not bind to CBG and it's not metabolized by 11β-hydroxylase 2. As such, neither the mother nor the fetus can buffer its action (Oliveira et al., 2006). Furthermore, it has been shown that DEX displays a 25-fold greater selectivity for GR when compared to their endogenous counterparts (Nagano et al., 2008). Taking in account the role of GR in promoting systemic maturation and in modulating a stress response, GR overactivation can result in the diversion of the natural developmental programing of the fetus, permanently changing brain structure and function (Mesquita et al., 2009).

1.3.3. PRENATAL GLUCOCORTICOID EXPOSURE IN THE DEVELOPING BRAIN

Oliveira and colleagues observed that a brief antenatal exposure to DEX induces long-term behavioral alterations, such as decreased locomotor activity and exploratory activity, as well as an increase vulnerability to develop depressive-like behavior in adulthood (Oliveira et al., 2006, 2012). Structural abnormalities in dendritic arborization were also detected in both amygdala and bed nucleus of the stria terminalis. While there was a neuronal atrophy in the first, an increased dendritic length was found in the later, reflecting a DEX-induced regional-dependent effect in the brain (Oliveira et al., 2012). Using a similar model in primates, DEX intramuscular injection to pregnant dams demonstrated a dose-dependent degenerative effect and reduction of hippocampal neurons in fetuses close to term (Uno et al., 1990).

The antenatal exposure to GCs also expands to other cellular populations besides neurons. Recent work from our group revealed that antenatal DEX modulates microglia

morphology in a long-term gender-specific manner. While male microglia in the PFC present a hyper-ramification and increased length of microglia processes in adulthood, females exhibited a decrease in the number and in their length, while both exhibit anxiety traits (Caetano et al., 2016).

Since microglia morphology is intrinsically related to microglia physiology and inflammatory profile (Eggen et al., 2013), changes in the cytoarchitecture of these cells suggest alterations in microglia function. In fact, PS has been demonstrated to alter microglia structure and also augment microglia proinflammatory response to an immune challenge in adulthood (Diz-Chaves et al., 2012, 2013). This indicates that, like neurons, alterations in microglia can be maintained throughout adulthood.

Although these results highlight the harmful effects of PS and antenatal exposure to GCs concerning neuronal development, it seems that this is not always the case. Fujioka and colleagues demonstrated that PS can have both enhancing and suppressing effects on the development of hippocampal neurons, being its effect intensity-dependent. Whereas short-lasting (30 min) mild PS enhanced neonatal neurogenesis and differentiation of processes of hippocampal neurons, long-lasting (240 min) severe PS greatly compromised neuronal morphology. This contrasting effect mirrors the harmony between GR and MR activation, since the same study also showed that MR in the HIP promoted neurogenesis and neurite differentiation in culture hippocampal neurons, whereas GR activation compromised neuronal maturation (Fujioka et al., 2006).

1.4. THE ADENOSINERGIC SYSTEM

Adenosine is a by-product of purine nucleotide metabolism that is ubiquitously distributed throughout our entire organism (Cunha, 2001). In the CNS, adenosine participates in a vast array of homeostatic processes, having a neuromodulatory role in neuronal excitability and synaptic plasticity (Cunha, 2016). The synthetic pathways of adenosine involves both intra and extracellular nucleotides (e.g. ATP), which are converted into adenosine by a series of ectonucleotidases such as CD73 and CD39, culminating in the production of adenosine (Delaney and Geiger, 1998; Dunwiddie and Masino, 2001).

Adenosine receptors are included in the P1 purinergic receptor family. They are G coupled receptors which are divided in four subtypes (A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) based in their response towards agonist and antagonist which was assessed by their ability to inhibit (A_1R and A_3R) or stimulate ($A_{2A}R$ and $A_{2B}R$) adenylate cyclase (AC), responsible for the synthesis of cyclic AMP (cAMP), an important signaling molecule (Figure 7) (Londos et al., 1980).

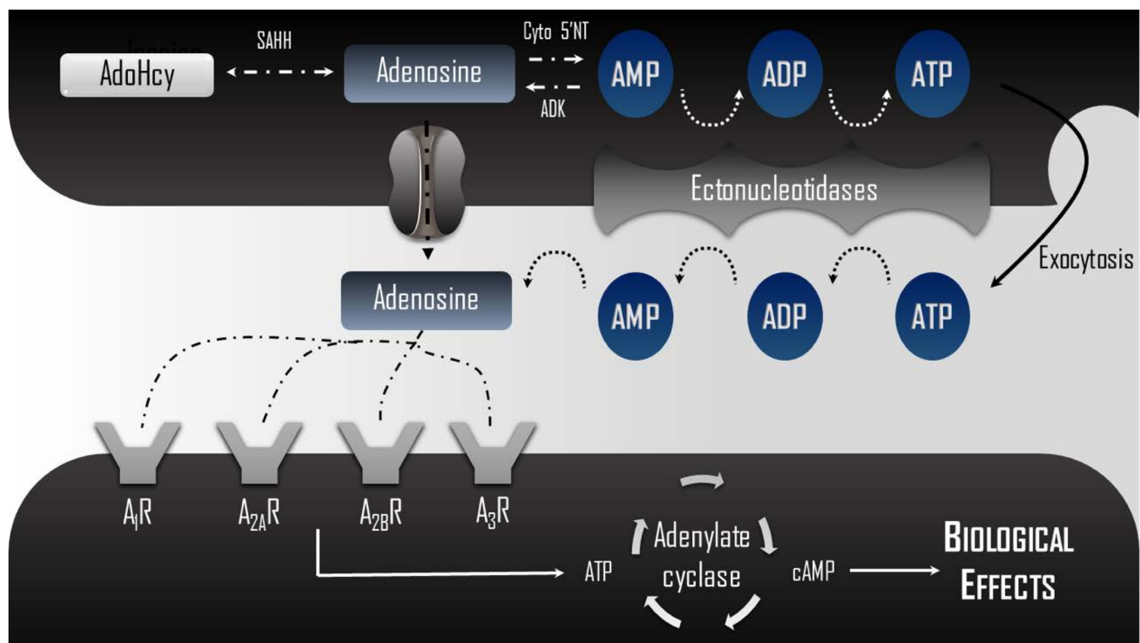


Figure 6|Overview of adenosine metabolism in P1 receptors. Adenosine is generated intracellularly and extracellularly through the metabolism of adenine nucleotides. Inside the cell, the conversion of S-adenosyl-homocysteine (AdoHcy) to adenosine is catalyzed by the S-adenosyl-homocysteine hydrolase (SAHH). Adenosine monophosphate (AMP) can be desphophoralized by intracellular cytosolic enzyme 5'-nucleotidase (Cyto 5'NT), also contributing for the intracellular adenosine pool. Adenosine is then transported by bidirectional nucleoside transporters. Intracellular adenosine can also be phosphorylated by the adenosine kinase (ADK) turning into AMP, which can be further phosphorylated, originating adenosine diphosphate (ADP) and ultimately adenosine trisphosphate (ATP). ATP can be released by neurons by exocytosis and suffer consecutive desphosphorilations by ectonucleotidases, turning into adenosine, which will then bind to A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. The activation of these receptors will modulate the activity of adenylate cyclase, eliciting a biological response.

1.4.1. ADENOSINE A_{2A} RECEPTOR

From all the P1 receptors, the A_{2A} receptors ($A_{2A}R$) received special consideration for being implicated in several brain pathologies due to their therapeutic potential (Preti et al., 2015). The ontogeny of these receptors portrays a dynamic spatial distribution across different brain areas during neurodevelopment. $A_{2A}R$ are first detected at GW 14 in the striatum (Weaver, 1993), suggesting potential roles as neurodevelopmental regulators. Global knockouts of $A_{2A}R$ ($A_{2A}R$ KO) by the deletion of the ADORA2 gene are viable, without gross anatomic abnormalities and fertile, indicating that these putative actions by $A_{2A}R$ can be somehow circumvented without compromising development (Wei et al., 2011). However, CD1 $A_{2A}R$ KO mice present anomalous symptoms later in development (Ledent et al., 1997), as well as an increase in striatal dopaminergic receptor 1 (DR_1) and 2 (DR_2) expression, emphasizing the importance of considering the influence of genetic background when comparing studies where different rodent strains are used.

The $A_{2A}R$ is widely expressed in different tissues at varying levels, being predominant in the brain, spleen and thymus (Peterfreund et al., 1996). Within the brain, $A_{2A}R$ are mainly expressed on striatopallidal medium spiny neurons from both dorsal and ventral striatum and in the olfactory tubercle (Rosin et al., 1998), being also found at lower levels in other brain regions, such as the HIP and cortex (Dixon et al., 1996; Svenningsson et al., 1997). $A_{2A}R$ localization is almost exclusive to the post-synaptic area, although it has been found in pre-synaptic sites in cortico-striatal terminals and in HIP (Rebola et al., 2005)

These receptors act mainly through the AC-cAMP-PKA (phosphokinase A) signaling pathway, though a PKC (phosphokinase C) dependent pathway has been detected in hippocampal synaptosomes (Cunha and Ribeiro, 2000). $A_{2A}R$ activation can also trigger alternative signaling cascades by interacting with other receptors, such as A_1Rs, D_2Rs, CB_1Rs and NMDARs (Ciruela et al., 2006; Hillion et al., 2002; Ribeiro, 1999; Tebano et al., 2009) . In the HIP, it has been reported that $A_{2A}Rs$ modulate synaptic activity through brain-derived neurotrophic factor BDNF (Potenza et al., 2007).

1.4.2. $A_{2A}R$ IN NEUROPROTECTION

The $A_{2A}R$ have been target of intensive research since the late 90's, when selective pharmacological tools to manipulate this receptor activity were created (Ongini et al., 1999). Since then, a burgeoning amount of evidence regarding $A_{2A}R$ relevance in brain pathology has emerged, all indicating neuroprotective roles of $A_{2A}R$ in a plethora of brain diseases (Chen et al., 2013).

$A_{2A}R$ inactivation has been shown to decrease the infarcted area upon ischemic insults, as well as better outcomes in animal models of traumatic brain injury (Melani et al., 2006; Ning et al., 2013). Likewise, $A_{2A}R$ blockade prevents neurodegeneration in amyotrophic lateral sclerosis (Ng et al., 2015) Machado-Joseph disease (Gonçalves et al., 2013) and experimental autoimmune encephalomyelitis (Mills et al., 2012). It is relevant to stress that in the last case, $A_{2A}R$ activation provided neuroprotective effects in the early stages of the disease, while having detrimental effects in later stages (Ingwersen et al., 2016), emphasizing the contrasting effects of $A_{2A}R$ activity in the progression of diseases. Additionally, $A_{2A}R$ antagonist has proved to be efficient in ameliorating depressive-like symptoms, such as social withdrawal, anhedonia and helpless behavior (Kaster et al., 2015).

1.4.3. $A_{2A}R$ AS MICROGLIA MODULATOR

Microglial cells are equipped with purinergic receptors, including the $A_{2A}R$ (Färber and Kettenmann, 2006). This receptor has been described to modulate microglia inflammatory profile since $A_{2A}R$ blockade decreased microglia activation in the HIP when primed with lipopolysaccharide (LPS) (Rebola et al., 2011). Nonetheless, $A_{2A}R$ activation decreased pro-inflammatory cytokines in LPS activated microglia *in vitro* (Newell et al., 2015). Ergo, microglial $A_{2A}R$ have a non-defined status concerning inflammatory responses, since $A_{2A}R$ can modulate both anti and pro-inflammatory responses.

$A_{2A}R$ also mediate the regulation of COX-2 mRNA and prostaglandin E2 (PGE2) synthesis, important mediators in the immunopathology in chronic infections and cancer (Fiebich et al., 1996), as well as the production of nerve growth factor (NGF) and BDNF release (Gomes et al., 2013; Heese et al., 1997). Moreover, microglial processes dynamics seem to be regulated by the $A_{2A}R$. Upon an inflammatory stimuli, $A_{2A}R$ activation promoted the retraction of microglial processes (Gyoneva et al., 2014a, 2014a; Orr et al., 2009), highlighting $A_{2A}R$ activity concerning microglia physiology.

In an already mentioned study performed by our group, a sex-specific $A_{2A}R$ activity was also described. In a PS model (intrauterine DEX administration), both adult males and females presented contrasting effects in microglia morphology, presenting also anxious-like behavior. $A_{2A}R$ antagonist treatment ameliorated males microglia phenotype and behavior, while aggravating female's microglia morphology without behavioral improvements, hence depicting a biased gender-effect concerning $A_{2A}R$ activity (Caetano et al., 2016)

This compilation of data clearly depicts that adenosine plays an important role in modulating microglial functions.

RATIONALE AND AIMS

During the gestational period, the maturation of the healthy brain encompasses a close sync between endogenous and exogenous factors. If the fetus environment is disrupted, the mechanisms underlying brain development can be affected, leading the neurodevelopmental programming to drift away from its normal course.

High levels of GCs have shown to exert deleterious effects in neurodevelopment, inducing long-term effects that perpetuate throughout an organism lifespan. Indeed, previous work done by our group has described both structural and behavioral alterations in the offspring of female rats treated with dexamethasone during pregnancy. Microglia cells from the progeny showed marked morphologic changes, being positively correlated with anxious behavior. Extraordinarily, the morphological alterations were different between genders (Caetano et al., 2016) and even among brain regions, namely the HIP and the PFC (Duarte et al, unpublished). The gender-bias concerning the modulation of microglia morphology was further strengthened by the pharmacological blockade of the $A_{2A}R$, already described as prominent regulators of microglia morphology. While $A_{2A}R$ chronic blockade was able to rescue behavioral alterations and partially normalize microglia morphology in adult males prenatally exposed to DEX, microglia structure in adult females was found to be further impaired without mood improvements (Caetano et al., 2016).

Regarding the dissimilarities between brain regions, we have showed that females subjected to DEX *in utero* present distinct microglia phenotypes between the HIP and PFC. Interestingly, electrophysiological data revealed that the neural connectivity between these two regions was decreased, which suggests that the integrity of the neural circuitry might have been compromised (Duarte et al, unpublished). Oliveira and colleagues have already described structural alterations in neuronal morphology using the same animal model (Oliveira et al., 2012), while other have found alterations in the density of dendritic spines using other stress models (Bessa et al., 2009; Cerqueira et al., 2005, 2007; Silvia-Gomez et al., 2013; Shors et al., 2001), which might explain the previously stated connectivity deficits.

Taking in account the above-mentioned data, the main aims for this thesis were:

- To determine if prenatal DEX exposure induced permanent structural changes in neurons from the hippocampal formation in adult males;
- To establish organotypical hippocampal cultures to address DEX influence in the number of dendritic spines in hippocampal neurons in early postnatal development;

- To validate putative alterations in microglia morphology in the HIP of adult males prenatally exposed to DEX and if $A_{2A}R$ treatment correlates with alterations in microglia;
- To assess microglia structure in $A_{2A}R$ knockout mice in order to understand the physiological relevance of $A_{2A}R$ in defining microglia morphology in the early stages of neurodevelopment.

METHODS

ANIMAL MODELS

3.1.1. 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 Wistar females were injected subcutaneously with dexamethasone (DEX, 1 mg/kg) or saline (CTRL), 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 (Caetano et al., 2016; Kaster et al., 2015). All animals were anesthetized and perfused with 4 % paraformaldehyde solution (for immunohistochemistry) or with 0.9 % saline (for Golgi-Cox staining) at PND90. After decapitation, the brains were involved in OCT (Optimal Cutting Temperature) and stored at - 80°C.

The pharmacological treatments and maintenance of these animals 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 7|Timeline of the animal model pharmacological treatment.

3.1.2. ADENOSINE $A_{2A}R$ KNOCKOUT MICE

Wild type and $A_{2A}R$ knockout ($A_{2A}R$ KO) C57BL/6 mice were housed under controlled conditions, same as the ones above mentioned. Both groups were genotyped using DNA extracted from tail biopsy and maintained until PND90. Animals were then anesthetized and perfused.

After dissection, the extracted brains were involved in OCT compound and stored at - 80°C.

Animal manipulation was done according to the European regulations (European Union Directive 2010/63/EU) and National Institute of Health (NIH) guidelines for animal care and experimentation.

Table 1|Drugs used in *in vitro* and *in vivo* experiments

DRUG	SUPPLIER	CONCENTRATION (*IN VITRO; IN VIVO*)	DESCRIPTION
(Dexamethasone; $C_{22}H_{29}FO_5$)	Acros Organics	0,25 μM;1 mg/kg	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	0.1 mg/kg/day for 21 days	$A_{2A}R$ selective antagonist

3.2. MORPHOMETRIC ASSESSMENT OF CELLULAR STRUCTURE IN BRAIN SLICES

3.2.1. HIPPOCAMPAL CA1 PYRAMIDAL NEURONS RECONSTRUCTION

3.2.1.1. GOLGI-COX STAINING OF BRAIN SLICES

This procedure was done in male rats exposed to DEX *in utero*. Brains from animals perfused with 0.9 % saline where processed for Golgi–Cox staining as previously described (Gibb and Kolb, 1998). Briefly, brains were placed in 20 ml Golgi-Cox solution and stored for 14 days, after which it was replaced with a 30 % sucrose solution. The brains were then allowed to sit in the dark for 2-5 days before sectioning in order to reduce the background staining. After being blotted dry, brains were mounted on sectioning stages with cyanocacrylic glue in a vibratome station. 200 μm coronal sections were saved and placed on 2 % gelatinized microscope slides. The sections were then subsequently alkalinized in 18,7 % ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated through a graded series of ethanol, cleared in xylene, mounted and coverslipped.

Samples preparation and mounting were performed by PhD student Carina Cunha from ICVS.

3.2.1.2. TRIDIMENSIONAL MORPHOMETRIC RECONSTRUCTION AND ANALYSIS

For the morphometric analysis of hippocampal neurons, only brain sections containing the dorsal HIP were considered. Images of 5 to 7 pyramidal neurons from each animal were acquired in the CA1 region from the dorsal HIP. For each selected neuron, all branches of the dendritic tree were reconstructed at x1000 magnification, using a motorized

microscope with oil objectives (Axioplan 2, Carl Zeiss, Thornwood, NY, USA) attached to a camera (DXC-390, Sony, Tokyo, Japan) and Neurolucida software (Microbrightfield, Williston, VT, USA). Sholl analysis and branched structure analysis was perfomed using NeuroExplorer software (Microbrightfield, Williston, VT, USA).

3.2.2. Hippocampal DG microglia reconstruction

3.2.2.1. Immunohistochemistry of brain slices

This procedure was done equally in both rats and mice. Brains 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 (Table 3) and stored at - 20°C.

Table 2| Antibodies used in immunohistochemistry in brain slices

Antibody	Supplier	Host	Type	Dilution	Description
Anti Iba1	Wako	Rabbit	Polyclonal	1:1000	Microglia marker
Anti-rabbit Alexa Fluor 488	Thermo-Fisher	Goat	Polyclonal	1:1000	Detection of primary antibodies

For immunodetection of microglia, free-floating brain sections containing the dorsal HIP were washed three times with phosphate buffer saline (PBS; Table 3), 10 min each time, in mild agitation. The sections were later incubated in a permeabilization and blocking solution (Table 3) for 2 hours at room temperature (RT) in mild agitation in order to increase specific binding. The same solution was used to dilute the antibodies and DAPI. Slices were incubated for 48 hours with the primary antibody (Table 2) at 4°C, in mild agitation. After removing the excess of primary antibody through successive washings with PBS (3 times, RT), the sections were incubated with the secondary antibody (Table 2) 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 cells nuclei. After washing the brain slices, sections were mounted on gelatinized microscope slides using glycergel mounting medium, covered with coverslips after properly dried. After sealing the coverslips with 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.

3.2.2.2. TRIDIMENSIONAL MORPHOMETRIC RECONSTRUCTION AND ANALYSIS

Images of 9 to 10 random microglial cells from each animal were acquired in the dentate gyrus (DG) of the dorsal HIP 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 better visualize microglial processes, to allow an accurate tracing of the processes of these cells, and maintained troughout the whole experiment.

After importing Z-stacks to the Neurolucida software (MBF Bioscience, USA), microglia cells were manually drawn along the image planes, granting a tridimensional image of each cell. Morphometric data (branched structure analysis) was extracted by Neurolucida Explorer and the number and length of processes per branch order was analyzed. 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 (Figure 9).

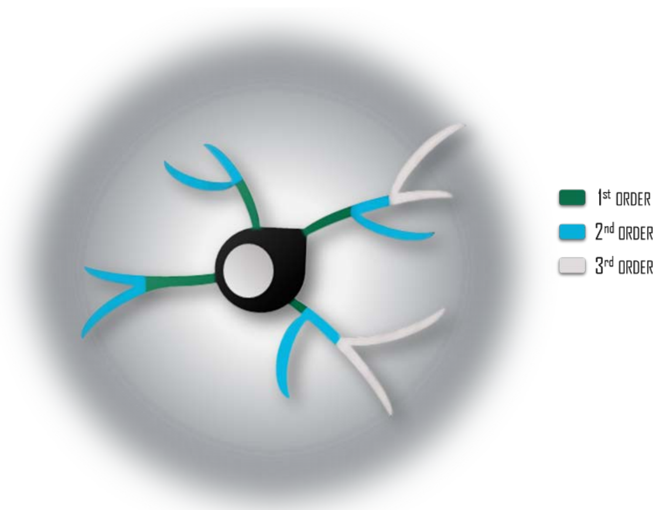


Figure 8| Representative scheme depicting the different branch orders in microglia processes.

3.3. SPINE ANALYSIS IN HIPPOCAMPAL ORGANOTYPICAL SLICE CULTURES

3.3.1. CULTURE PREPARATION

Hippocampi from P6 Wistar pups were isolated and dissected emerged in ice cold dissection solution (Table 3) gassed in 5% CO_2/95% air. Hippocampi were then cut in 250 µm thick slices using a McIlwain tissue chopper. After carefully separating the slices, 4 to 5 slices were placed onto a slice culture insert inside a well filled with 850 µL of culture medium (Table 3). Cultures were maintained in a humidified incubator with 5% CO_2/95% air at 35°C (Gähwiler et al., 1997). The culture medium was replaced once every two days. At DIV 5, hippocampal slices were treated with DEX (0.25 µM).

Table 3| List of solution used in all experiments

SOLUTION	COMPOSITION
Blocking/Permeabilization Solution	5% BSA and 0.1% Triton X-100 diluted in PBS
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
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
Dissection Solution	10 mM glucose, 4 mM KCl, 24 mM $NaHCO_3$, 234 mM sucrose, 0.5 mM $MgCl_2 \cdot 6H2O$, 0.7 $CaCl_2 \cdot 2H_2O$, 0.03 phenol red, pH = 7.4
Slice medium	Minimum Essential Media (MEM); supplemented with 20% (v/v) horse serum, 1 mM glutamine, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mg/l insulin, 0.0012% (w/v) ascorbic acid, 30 mM HEPES, 13 mM glucose, 5.2 mM $NaHCO_3$, pH = 7.25-7.26, 310-320 mOsm/l.
Artificial cerebral spinal fluid (ACSF)	127 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM Glucose

3.3.2. BIOLISTIC TRANSFECTION

3.3.2.1. PREPARATION OF MICROCARRIERS

Approximately 10 mg of 1 µm diameter gold microcarriers were weighted in a microtube, and 50 µL spermidine (0.05 M) was added to the gold, allowing the DNA vectors to couple to the gold microparticles. An equivalent amount of DNA coding for the mCherry Red fluorescent protein (5 µg of DNA/ 1 mg of gold) was later added. Afterwards, 100 µL $CaCl_2$ (1 M) was added dropwise to the previous mix while vortexing to facilitate DNA precipitation and to obtain an even DNA dispersion on the particles.

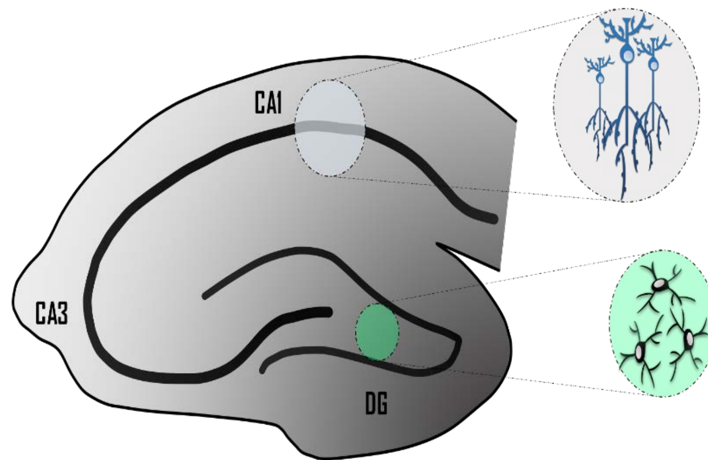


Figure 9| Regional map of the hippocampus region. For the neuronal morphometric analyses, pyramidal neurons from the CA1 regions (white circle) were randomly selected. Microglia cells were randomly selected from the dentate gyrus (DG; green circle) for tridimensional reconstruction.

The mix was allowed to stand for about 10 min at RT to enable DNA precipitation, after which it was centrifuged. The pellet (DNA microcarriers) was then washed three times in absolute ethanol, vortexed and centrifuged at 13000 g for 2 minutes. The supernatant was discarded after every centrifugation. After the final wash, the pellet was resuspended in absolute ethanol and 8 µL of 0.1 mg/mL polyvinylpyrrolidone (PVP) solution was added to the mix to serve as an adhesive. This suspension was transferred to a 5 ml polypropylene tube containing 2 mL of absolute ethanol and was vortexed before being aspired to a polypropilene tube (previously dried on a N_2 station). The suspension was left to settle for 15-20 min at RT. Once the microcarriers were attached to the tube, the supernatant was carefully aspired. The tube was rotated for 30-40 seconds to ensure an even distribution of the microcarriers through the tube. The tube containing the microcarriers was left to dry for 20 min on a N_2 gas station after which it was cut into cartridges of 1 cm length. The cartridges were separated and kept in a plastic flask with a desiccant at 4ºC.

3.3.2.2. GENEGUN TRANSFECTION

Helios gene gun (BioRad) contains a cartridge holder and a diffuser that enables the proper dispersion of the bullets and reduces the air pressure directly incident on the slice.

The diffuser was first sterilized in absolute ethanol during 5 min and then under UV Light until it was completely dried. The genegun was properly mounted with the cartridge holder filled with the cartridges and posteriorly attached to the helium cylinder. For the bombardment, the genegun was placed roughly 1,5 cm over each insert and the gas pressure was set for 180 psi. At DIV3, two shootings were done per insert in order to get a proper amount of transfected cells (adapted from Woods and Zito, 2008)

3.3.3. IMAGING

For the dendritic spine studies, hippocampal neurons from organotypical slices (DIV 6) were imaged as multiplane (z-series) image stacks on a LSM710 confocal microscope (Carl Zeiss, Gemany) using a 63× Plan-ApoChromat oil objective (N.A. 1.4), zoom 2. For the image acquisition, live neurons were kept in artificial cerebrospinal fluid (ACSF; Table 3) supplemented with 4 mM $CaCl_2$ and 4 mM $MgCl_2$, and 1 uM TTX (tetrodotoxin) at the time of the imaging (adapted from Oliveira and Yasuda, 2014). Secondary and tertiary dendrites of apical and basal dendrites from CA1 pyramidal neurons were chosen for imaging.

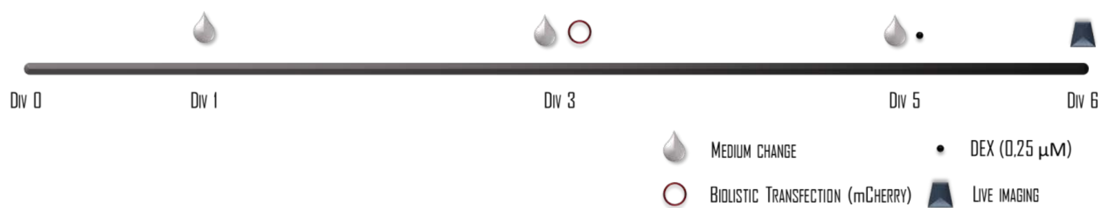


Figure 10| Schematic representation of the different procedures done in *in vitro* experiments using organotypic hippocampal slices

3.3.4. SPINE MORPHOLOGY CHARACTERIZATION

Spine characterization was done with the NeuronStudio software (Rodriguez et al., 2008). Dendritic spines were manually classified accordingly to the following criteria:

- Protrusions without head were identified as filopodia;
- Bulbous spines with no neck were identified as stubby spines;
- Big headed spines with a defined neck were identified as mushroom;
- Bifurcated spines were defined as branched;
- Spines with identifiable necks and small heads were identified as thin spines.

3.4. DATA ANALYSIS

The statistical analysis was carried out in GraphPad Prism version 6 (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 four groups, a one-way analysis of variance (ANOVA) was used, followed by a Tukey's Multiple Comparison Test, to compare all groups.

Data concerning dendritic spine number was analyzed by two-way ANOVA, followed by Bonferroni post-hoc test, with spine typology (filopodia, thin, stubby, mushroom and bifurcated) and treatment (CTRL and DEX) as independent factors. In all analysis, differences were considered significant at $p<0.05$.

Table 4| Table depicting the number of animals used in each set of experiments and the number of cells analysed.

<table>
<tr><th colspan="10">IN VIVO</th></tr>
<tr><th>STRAIN</th><th>CELL TYPE</th><th colspan="4">ANIMALS/CONDITION</th><th colspan="4">CELLS/ANIMAL</th></tr>
<tr><td rowspan="4">Wistar</td><td rowspan="2">Neurons</td><td colspan="2">CTRL</td><td colspan="2">DEX</td><td colspan="2">CTRL</td><td colspan="2">DEX</td></tr>
<tr><td colspan="2">3</td><td colspan="2">4</td><td colspan="2">6-7</td><td colspan="2">5-7</td></tr>
<tr><td rowspan="2">Microglia</td><td>CTRL</td><td>DEX</td><td>SCH</td><td>D+S</td><td>CTRL</td><td>DEX</td><td>SCH</td><td>D+S</td></tr>
<tr><td>3</td><td>4</td><td>2</td><td>3</td><td>10</td><td>10</td><td>10</td><td>10</td></tr>
<tr><td rowspan="2">C57BL/6</td><td rowspan="2">Microglia</td><td colspan="2">WT</td><td colspan="2">A_{2A}R KO</td><td colspan="2">WT</td><td colspan="2">A_{2A}R KO</td></tr>
<tr><td colspan="2">4</td><td colspan="2">4</td><td colspan="2">10</td><td colspan="2">10</td></tr>
<tr><th colspan="10">IN VITRO</th></tr>
<tr><th>STRAIN</th><th>CELL TYPE</th><th colspan="4">ANIMALS/CONDITION</th><th colspan="4">CELLS/ANIMAL</th></tr>
<tr><td rowspan="2">Wistar</td><td rowspan="2">Neurons</td><td colspan="2">WT</td><td colspan="2">DEX</td><td colspan="2">WT</td><td colspan="2">DEX</td></tr>
<tr><td colspan="2">4</td><td colspan="2">4</td><td colspan="2">4-5</td><td colspan="2">4-5</td></tr>
</table>

RESULTS

In this study, the effects of prenatal GC were addressed by analyzing both neurons and microglia morphology. Both cellular populations seem to have abnormal structures when primed with DEX during gestation, which were perpetuated until adulthood (Caetano et al., 2016; Oliveira et al., 2006; Silva-Gomez et al., 2013; Uno et al., 1990). Likewise, PS also promotes similar effects (Anderson et al., 2016; Diz-Chaves et al., 2013; Fujioka et al., 2006; Martínez-Téllez et al., 2009), once again reflecting the importance of the placental environment in influencing brain developing (Barker et al., 1989; Seckl and Holmes, 2007).

Our recent study approached these morphological changes in the PFC, a brain region involved in anxiety-related disorders, where microglia show a dimorphic cytoarchitecture among gender (Caetano et al., 2016) and between brain regions (Duarte et al, unpublished). In the last study, a decrease in the coherence between the HIP and the PFC in adult female rats was also detected.

This impairment in connectivity was also validated by deficits in memory recognition, suggesting that the HIP might also be affected in this model. In fact, it has been described stress can induce pronounced morphological rearrangements in hippocampal formation, since the overall structure of neurons from CA1, CA3 and DG seems to be compromised (Bessa et al., 2009; Lambert et al., 1998; Martínez-Téllez et al., 2009; Silva-Gomez et al., 2013; Sousa et al., 2000).

Hence, a morphometric analysis of neurons and microglia was performed in order to further explore the remodeling effects of antenatal GCs in brain development.

4.1. PRENATAL EXPOSURE TO DEXAMETHASONE INDUCED NEURONAL HYPERTROPHY IN CA1 PYRAMIDAL NEURONS IN THE HIP OF ADULT MALES

The exposure to DEX *in utero* induced significant alterations in the dendritic arborization of CA1 pyramidal neurons in the HIP. Basal dendrites suffered an increase in length [*Basal CTRL* = 411.3 ± 10.8 μm and *Basal DEX* = 515.0 ± 28.5 μm; $p < 0.05$], an effect also observed in apical dendrites [*Apical CTRL* = 3862 ± 126.7 μm and *Apical DEX* = 4766.0 ± 305.0 μm; $p < 0.05$] reflecting an overall neuronal hypertrophy (Figure 12 – e,f). This hypertrophy was also observed in the Sholl analysis, in which the tendency to a higher number of intersections was visible (Figure 12 – c,d), thus indicating a more ramified dendritic arborization (see Supplementary Table 1 in the Supplementary Data section for more detailed data).

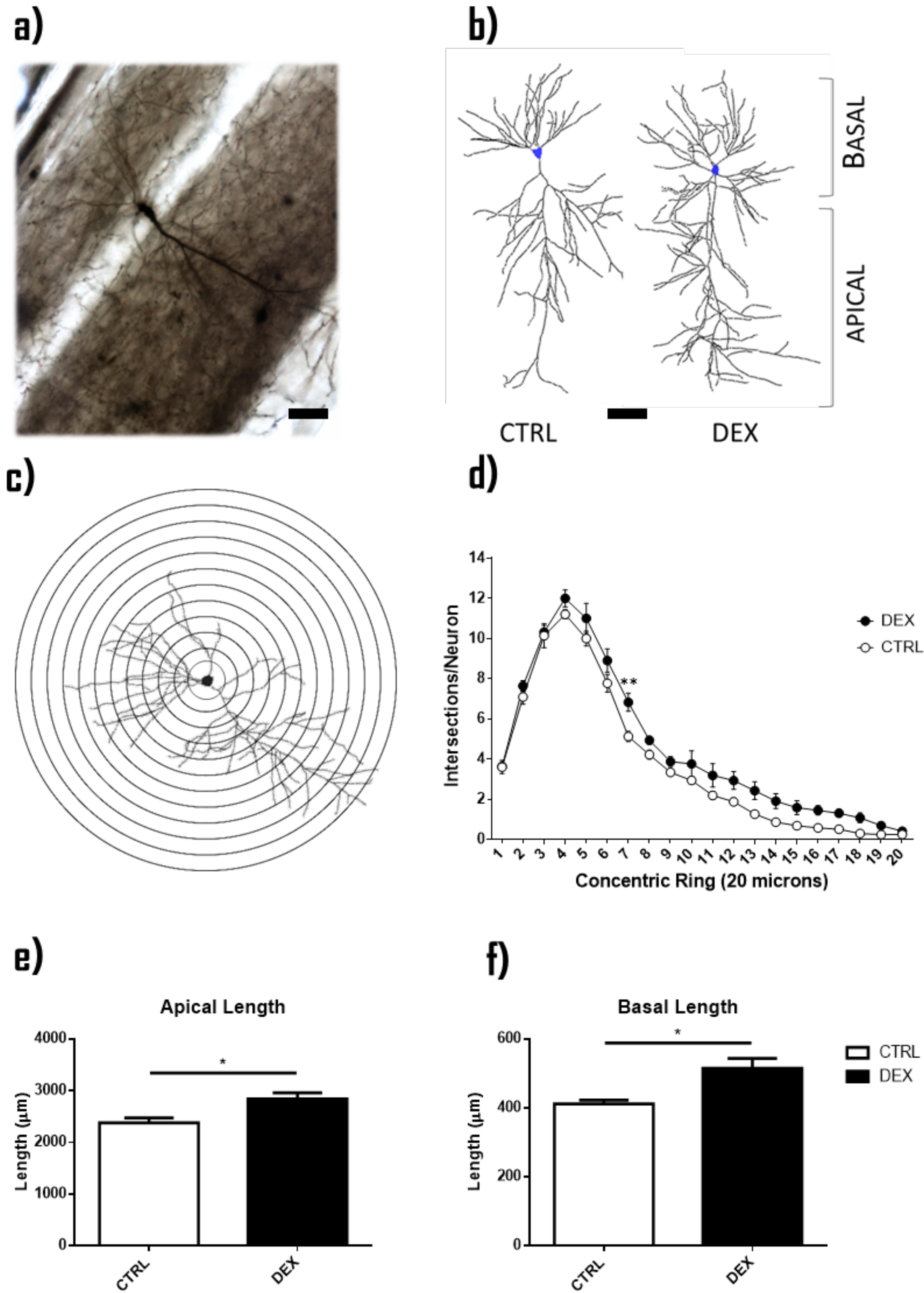


Figure 11/ Effect of prenatal exposure to DEX in the dendritic arborization of hippocampal CA1 pyramidal neurons. Pregnant females were injected with saline (CTRL) or DEX (1 mg/kg s.c) at ED18 and ED19. Adult male offspring were sacrificed at PND90 and then processed for Golgi–Cox staining (**a**). 5-7 cells were randomly selected in the CA1 region of the HIP. Neuronal morphology was assessed by morphometric tridimensional manual reconstruction in Neurolucida Software (**b**) Sholl analysis (**c**, **d**) and dendritic length measurement (apical - **e**; basal - **f**) were performed in from Neurolucida Explorer. Results are expressed as mean ± SEM of 3-4 animals (statistical significance was assessed by t-student: * $p < 0.05$ when comparing CTRL with DEX treated animals)**.** Scale bar = 50 µm.

4.2. ORGANOTYPICAL HIPPOMCAPAL SLICES EXPOSED TO DEX DID NOT SHOW SIGNIFICANT ALTERATIONS IN SPINE DENSITY AND DEVELOPMENT

In addition to the dendritic reshaping that takes place in the HIP upon a stress-like stimuli, these structural adaptations also occur at a dendritic spine level. In overall, studies suggest that high levels of corticosteroid evoke synaptic alterations including spine remodeling in the HIP (Martínez-Téllez et al., 2009; Pawlak et al., 2005), PFC (Bessa et al., 2009; Cerqueira et al., 2007) and amygdala (Vyas et al., 2002), all areas responsible in modulating a stress response.

To determine the extent of prenatal DEX effects concerning neuronal structure in the HIP, we conducted a spine analysis in CA1 pyramidal neurons from organotypic hippocampal slices to address if spine development and number were altered.

Intriguingly, a 24 h DEX stimuli (0.25 μM) didn't elicit any significant alterations concerning spine number. Neither the apical [*Apical CTRL* = 4.419 ± 0.349 and *Apical DEX* = 4.821 ± 0.3550; $p > 0.05$] and basal dendrites [*Basal CTRL* = 4.621 ± 0.3384 and *Basal DEX* = 4.379 ± 0.3630; $p > 0.05$] presented any relevant differences (Figure 13 – e, f).

However, some interesting tendencies were revealed regarding spine maturation. In the apical arborization, we observed a downward tendency in the number of filopodia spines in DEX treated slices [*Apical CTR Thin* = 1.186 ± 0.182 and *Apical DEX Thin* = 0.947 ± 0.147; $p > 0.05$], as well as in stubby [*Apical CTRL Stubby* = 1.045 ± 0.104 and *Apical DEX Stubby*= 0.834 ± 0.098; $p > 0.05$] and bifurcated spines [*Apical CTRL Bifurcated* = 0.563 ± 0.129 and *Apical DEX Bifurcated* = 0.283 ± 0.057; $p > 0.05$]. These results were accompanied by an increasing tendency in thin [*Apical CTRL Thin* = 1.250 ± 0.138 and *Apical DEX Thin*= 1.442 ± 0.189; $p > 0.05$] and mushroom spines [*Apical CTRL Mushroom* = 0.722 ± 0.122 and *Apical DEX Mushroom*= 0.915 ± 0.131; $p > 0.05$]. In basal dendrites, similar tendencies in spine density were observable with reductions in filopodia [*Basal CTR Filopodia* = 1.069 ± 0.133 and *Basal DEX Filopodia* = 0.863 ± 0.126; $p > 0.05$] and bifurcated spines [*Basal CTR Bifurcated* = 0.492 ± 0.068 and *Basal DEX Bifurcated* = 0.306 ± 0.052; $p > 0.05$], followed by an upward tendecy in thin spines number [*Basal CTR Thin* = 1.314 ± 0.152 and *Basal DEX Thin* = 1.421 ± 0.213; $p > 0.05$]. No considerable differences were detected in the density of both stubby [*Basal CTR Stubby* = 1.043 ± 0.068 and *Basal DEX Filopodia* = 1.033 ± 0.128; $p > 0.05$] and mushroom spines [*Basal CTR Mushroom* = 0.703 ± 0.093 and *Basal DEX Mushroom* = 0.757 ± 0.084; $p > 0.05$] (Figure 13 – c, d).

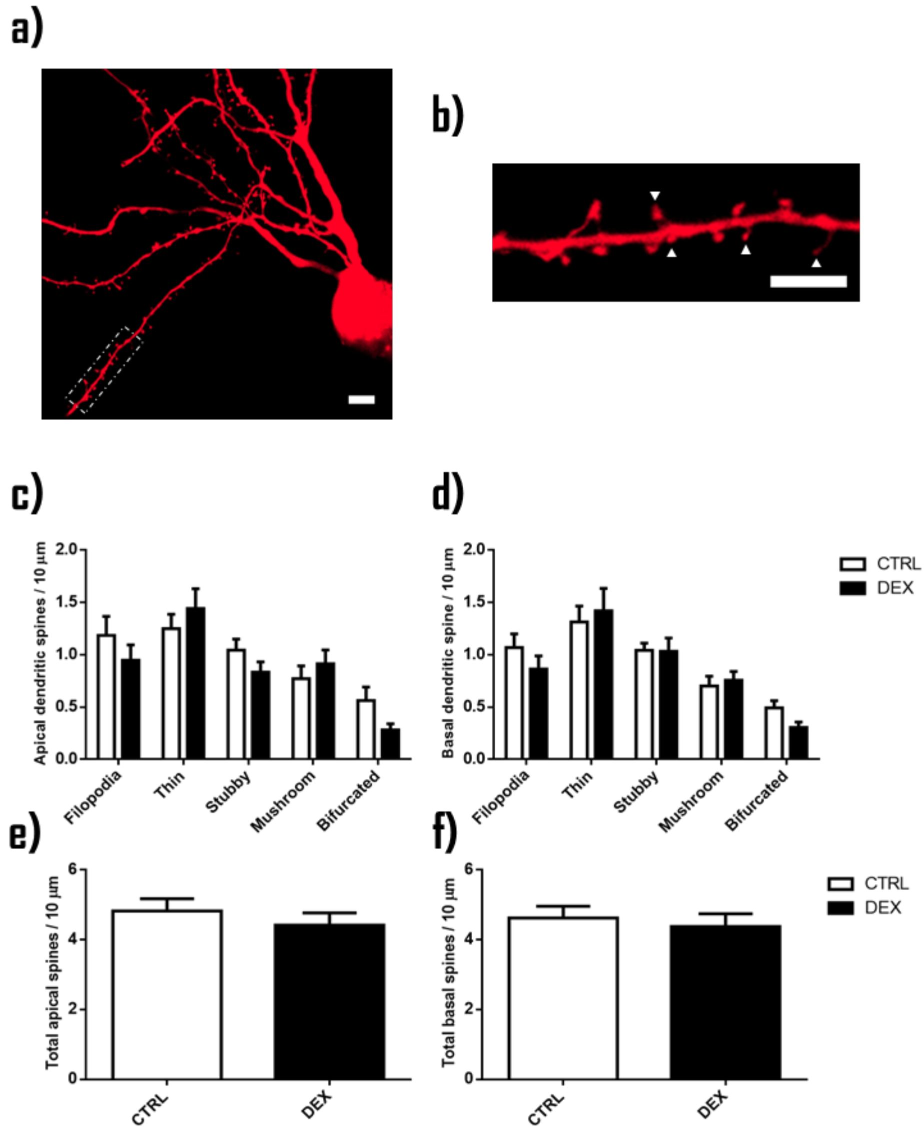


Figure 12| Effect of acute DEX treatment in spine density of organotypic hippocampal slices. Organotypic hippocampal cultures were prepared from P6 male pups and transfected with mCherry at DIV3 through biolistic transfection. At DIV5, slices were treated with DEX (0.25 µM) for 24 H and used for live imaging at DIV 6 (**a**, **b**). Spines were extracted from 1-2 basal or apical secondary and tertiary dendrites per neuron (4-5) and analyzed using Neuronstudio software. Spines were manually counted and classified as filopodia, thin, stubby, mushroom and bifurcated in apical (**c**, **e**) and basal dendrites (**d**, **f**) dendrites from CA1 pyramidal neurons in hippocampal slices. **a** – basal dendrites from a CA1 pyramidal neuron at DIV6; **b** – amplification of the marked area with white arrows pointing to representative spine morphologies (from left to right in order of appearance: mushroom, stubby, thin and filopodia). Total spine density is expressed as mean ± SEM of 4 animals (statistical significance was assessed by t-student: * p <0.05 when comparing CTRL with DEX treated animals). Spine density between types is expressed as mean ± SEM of 4 animals (statistical significance was assessed by two-way ANOVA, followed by Bonferroni post-hoc test). Scale bar = 5 µm.

4.3. ANTENATAL DEXAMETHASONE ALTERED MICROGLIA MORPHOLOGY IN THE HIP OF ADULT MALES

Similar to our previous report, where differences in male PFC microglia morphology were shown in DEX exposed animals, male HIP microglia from the DG also showed a tendency for an overall hypertrophy, marked by a mild increase in the total processes' length [*CTRL* = 585.0 ± 71.76 μm and *DEX* = 681.0 ± 104.3 μm; $p > 0.05$] and number [*CTRL* = 114.6 ± 15.05 μm and *DEX* = 142.1 ± 27.08 μm; $p > 0.05$] (Figure 14 – e, f). Branched structure analysis also revealed this tendency in both processes' length and number. Notably, this increase was only detected past the 3rd order (Figure 14 – g, h). All values concerning the branched structure analysis in rats are summarized in the Supplementary Tables 1 (length of processes) and 2 (number of processes) in the Supplementary Data section.

4.4. CHRONIC TREATMENT WITH $A_{2A}R$ ANTAGONIST SHOWED MILD EFFECTS REGARDING MICROGLIA MORPHOLOGY IN HIP OF ADULT MALES

Considering previous studies reporting the physiological relevance of $A_{2A}R$ in coordinating microglia cytoskeleton (Gyoneva et al., 2016; Orr et al., 2009), including previous work by our group showing an atrophy in microglia in the PFC only in adult female rats subjected to $A_{2A}R$ chronic treatment (Caetano et al., 2016), we ought to confirm if the chronic blockade of $A_{2A}R$ would promote morphological changes in microglia cells in the HIP of adult males.

After analyzing the branched structure in microglia confined to the DG area, we reported that $A_{2A}R$ antagonist treatment induced upward tendencies regarding the length and number of microglial processes. SCH treated microglia presented a slight increase in the total length [*CTRL* = 585.0 ± 71.76 μm and *SCH* = 716.9 ± 82.25 μm; $p > 0.05$] and number [*CTRL* = 114.6 ± 15.05 μm and *SCH* = 134.6 ± 21.85 μm; $p > 0.05$] (Figure 15 –e, f). Contrary to what was observed in microglia from DEX exposed males, this tendency was observed in all orders (Figure 15 – g, h).

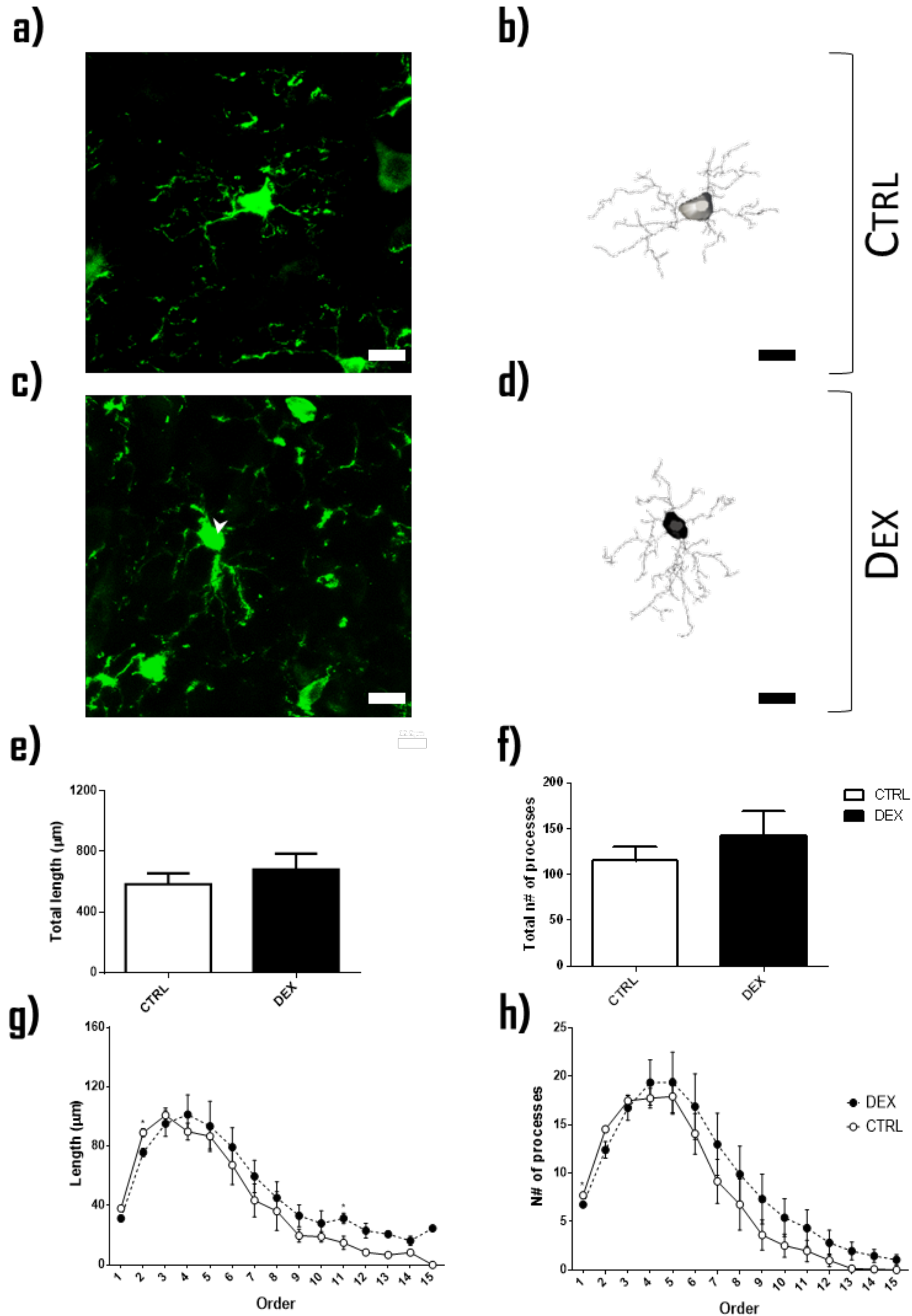


Figure 13| Effect of antenatal exposure to DEX in the number and length of PFC microglia processes. Pregnant females were injected with saline (CTRL) or DEX (1 mg/kg s.c.) at ED18 and ED19. Adult male progeny was sacrificed at PND90 and brain slices were stained with IBA-1 antibody (**a**, **c**). Microglia morphology was assessed by morphometric tridimensional manual reconstruction in Neurolucida Software (**b**, **d**). 10 cells were randomly selected in the DG of the HIP. Morphometric data was acquired in Neurolucida Explorer concerning the length (**e**, **g**) and number (**f**, **h**) of processes. Results are expressed as mean ± SEM of 3-4 animals (statistical significance was assessed by one-way ANOVA, followed by Tukey's Multiple Comparison Test). White arrow is pointing to the soma of the reconstructed microglia (**a,** **c**). Scale bar = 10 µm.

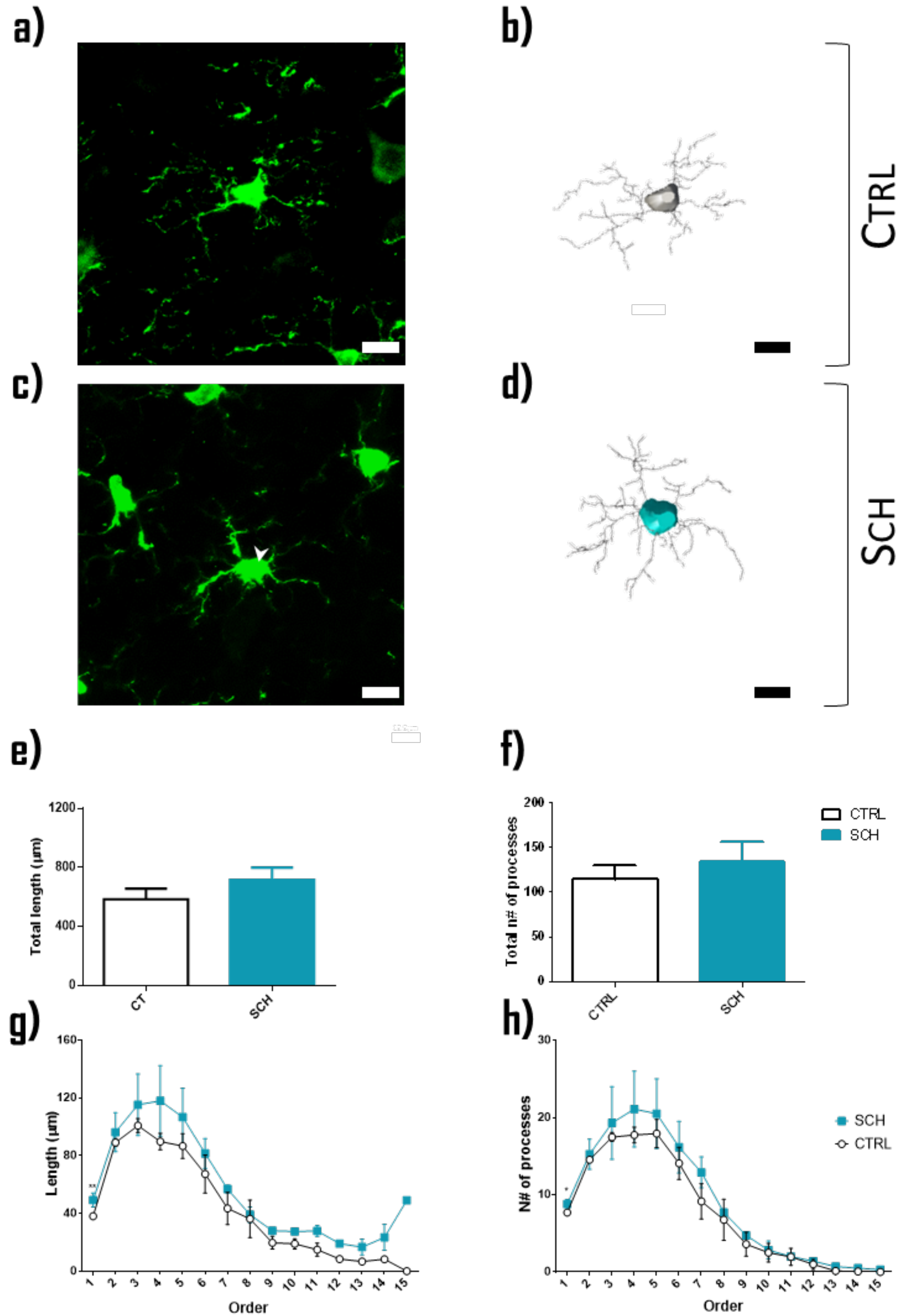


Figure 14| Effect of $A_{2A}R$ chronic blockade on the number and length of HIP microglia processes. Adult male rats were treated for 21 consecutive days with saline (CTRL) or the $A_{2A}R$ antagonist SCH58261 (SCH) and sacrificed at PND90. Brain slices were stained with IBA-1 antibody (**a**, **c**). Microglia morphology was assessed by morphometric tridimensional manual reconstruction in Neurolucida Software (**b**, **d**). 10 cells were randomly selected in the DG of the HIP. Morphometrical data was adquired in Neurolucida Explorer concerning the length (**e**, **g**) and number (**f**, **h**) of processes. Results are expressed as mean ± SEM of 2-4 animals (statistical significance was assessed by one-way ANOVA, followed by Tukey's Multiple Comparison Test). White arrow is pointing to the soma of the reconstructed microglia (**a, c**). Scale bar = 10 µm.

4.5. $A_{2A}R$ PHARMACOLOGICAL BLOCKADE PROMOTED A MICROGLIAL HYPERTROPHY IN THE HIP OF ADULT RATS PRENATALLY EXPOSED TO DEX

Given the anxiolytic effect of $A_{2A}R$ chronic blockade in male adult rats, which positively correlated with a normalization in microglia morphology in the PFC (Caetano et al., 2016), we performed the same morphological analysis in the HIP to validate if this brain region was somehow involved in the aforementioned alterations. In fact, we observed that in DEX prenatally exposed male adult rats, $A_{2A}R$ chronic blockade induced a pronounced microglia hypertrophy. Although the total length only presents an upward tendency in the length [*CTRL* = 585.0 ± 71.76 μm; *DEX* = 681.0 ± 104.3 and *DEX+SCH* = 716.9 ± 82.25 μm; $p > 0.05$] as well as in the number of processes [*CTRL* =114.6 ± 15.05; *DEX* = 142.1 ± 27.08 and *DEX+SCH* = 196.5 ± 27.51; $p > 0.05$] (Figure 16 – e.f), several branched orders revealed significant differences in processes length and number, although only noticeable past the two first branched orders (Figure 16 – g, h).

4.6. $A_{2A}R$ KO MICE REVEALED MINOR STRUCTURAL CHANGES IN HIP MICROGLIA

Due to their presence in the premature brain (Weaver, 1993), we further research the physiological importance of $A_{2A}R$ in configuring microglia morphology in early development. For that purpose we conducted the same morphometric approach above mentioned and analyzed microglia branched structure in the HIP of male adult mice.

$A_{2A}R$ KO male mice presented minor alterations concerning microglia cytoarchitecture when comparing to WT mice, showing mild tendencies towards longer processes length [*WT* = 500.0 ± 20.22 μm and *$A_{2A}R$ KO* = 545.5 ± 27.17 μm; $p < 0,05$] and number [*WT* = 115.6 ± 4.337 and *$A_{2A}R$ KO* = 130.2 ± 13.13; $p < 0.05$] (Figure 17 – e,f). Nonetheless, this increase was detectable in mid-range branch orders (between the 3rd and 11th orders (Figure 17 – g,h). All values concerning the branched structure analysis in mice are summarized in the Supplementary Tables 3 (length of processes) and 4 (number of processes) in the Supplementary Data.

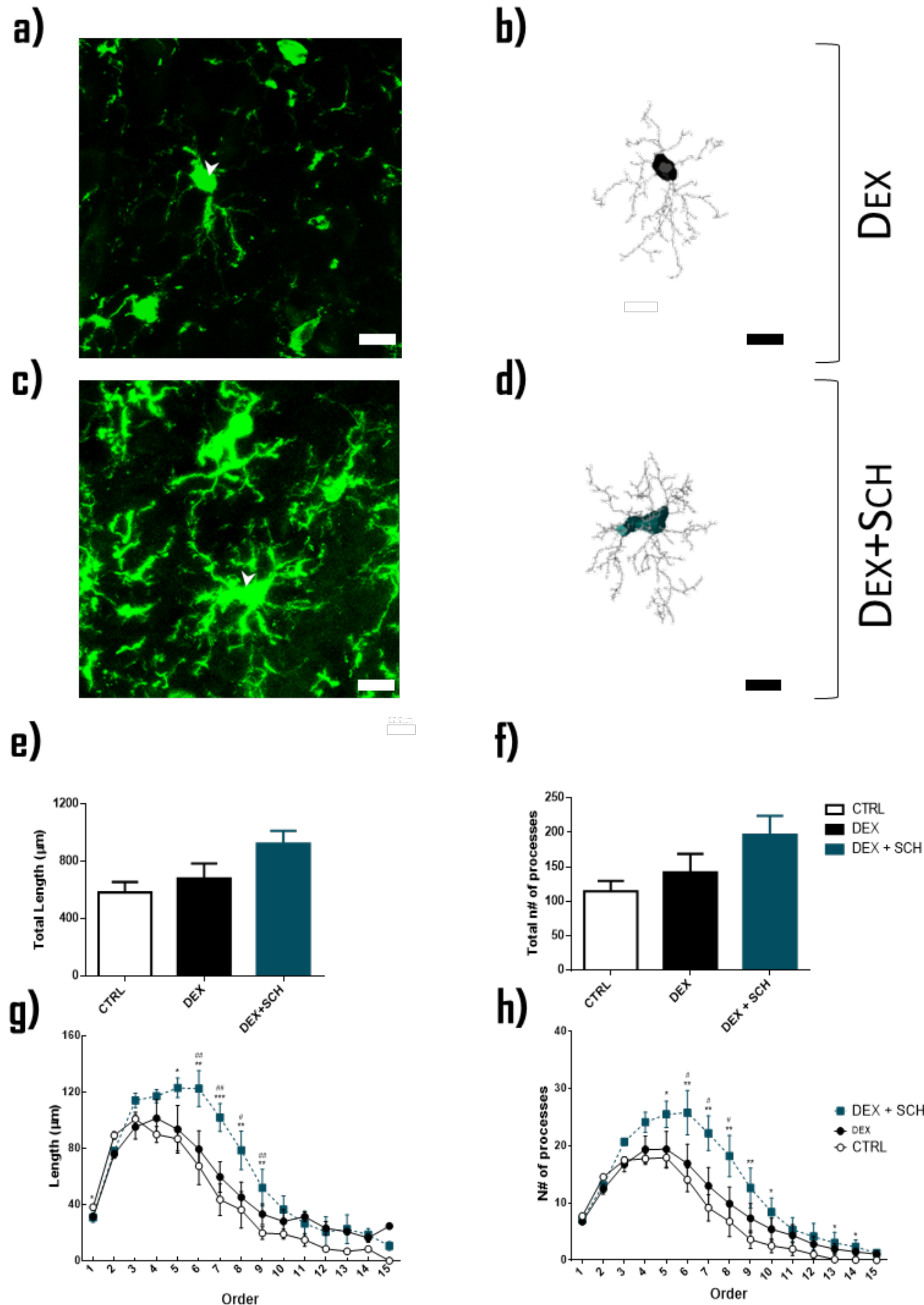


Figure 15| Effect of $A_{2A}R$ chronic blockade on the number and length of HIP microglia processes in prenatally DEX exposed male rats. Pregnant females were injected with saline (CTRL) and/or DEX (1 mg/kg s.c.) at ED18 and ED19 and sacrificed at PND90. Brain slices were stained with IBA-1 antibody (**a**, **c**). Microglia morphology was assessed by tridimensional manual reconstruction in Neurolucida software (**b**, **d**) and morphometric data was acquired in Neurolucida Explorer regarding the length (**e, g**) and number (**f**, **h**) of processes. Results are expressed as mean ± SEM of 3-4 animals (statistical significance was assessed by one-way ANOVA, followed by Tukey's Multiple Comparison Test: * $p < 0.05$; ** $p < 0.01$ when comparing non-treated animals (CTRL) with chronic treatment with $A_{2A}R$ antagonist after DEX prenatal exposure (DEX+SCH); # $p < 0.05$; ## $p < 0.05$ when comparing DEX treated animals with chronic treatment with $A_{2A}R$ antagonist after DEX prenatal exposure (DEX+SCH). White arrow is pointing to the soma of the reconstructed microglia (**a, c**). Scale bar = 10 µm.

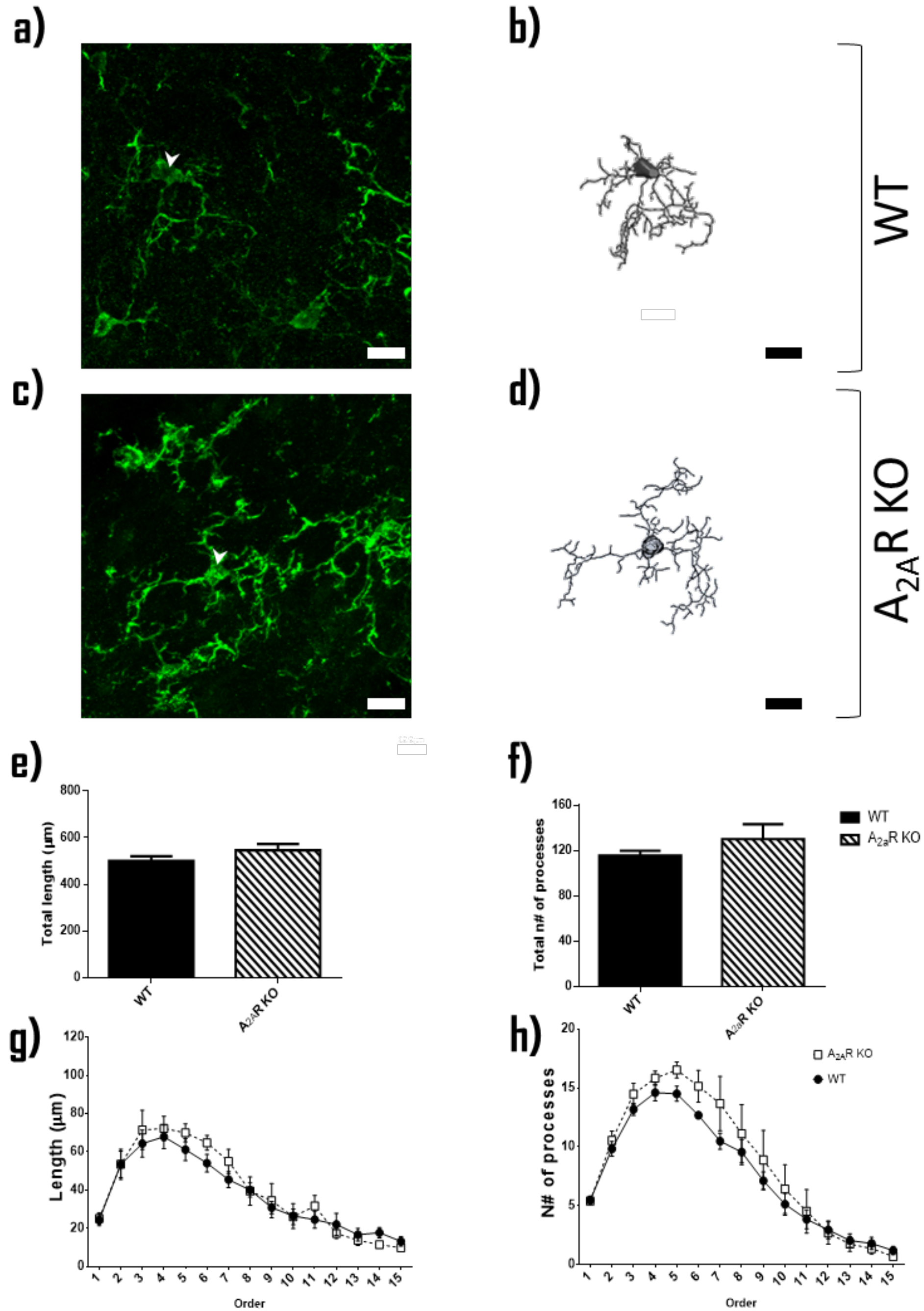


Figure 16| Morphological assessment of microglia structure in A_{2A}R KO male mice. Adult male mice were sacrificed at PND90 and brain slices were stained with IBA-1 antibody (**a**, **c**). Microglia morphology was assessed by morphometric tridimensional manual reconstruction in Neurolucida Software (**b**, **d**). 10 cells were randomly chosen from the DG region of the HIP (**b**). Morphometric data was acquired in Neurolucida Explorer concerning the length (**e**, **g**) and number (**f**, **h**) of processes. Results are expressed as mean ± SEM of 4 animals (statistical significance was assessed by t-student. White arrow is pointing to the soma of the reconstructed microglia (**a, c**) Scale bar = 10 µm.

DISCUSSION

Although having gained substantial knowledge subjacent to the etiology of psychiatric illness, the scientific community still strives to fully understand their causes and pathological mechanisms. This endeavor gains significant weight when epidemiologic studies predict that one out of every three to four youths is estimated to develop a Diagnostic and Statistical Manual of Mental Disorders (DSM) mental disorder (Costello et al., 2003). Having said, it is crucial to extensively dissect the core of neuropsychiatric conditions in order to retaliate this oppressive data.

The intricate strings orchestrating the onset of neuropsychiatric diseases seem to be tightly linked with a neurodevelopmental basis (Bock et al., 2015). The plastic nature of the maturing brain makes it extreme sensitive to both endogenous and exogenous signals that may change the developmental trajectory of cells (Caetano et al., 2016; Uno et al., 1990), neural circuits and associated behavioral outcomes (Caetano et al., 2016; Oliveira et al., 2006; Rodrigues et al., 2012)

In an effort to further understand the alterations in neurodevelopment programming with the neuropsychiatric illness, a model of anxiety has proven to be a reliable tool. In fact, animals prenatally exposed to dexamethasone (DEX) exhibit long lasting behavioral, such as an increase in susceptibility to develop anxiety and stress disorders (Caetano et al., 2016; Oliveira et al., 2006) and brain structural alterations, shown by our research group and others (Caetano et al., 2016; Oliveira et al., 2012).

Our previous work revealed structural differences in PFC microglia from animals exposed to DEX *in utero* (PND90), in both males and females. Regardless of the contrasting morphological alterations between genders, which correlated with an anxiogenic profile, it became clear that antenatal GC exposure compromises the development of an healthy organism (Caetano et al., 2016). Besides the structural cellular alterations, microglia from females exposed to DEX were more ramified comparing to the control animals in the DG of the HIP. Additionally, females also presented also a significant decrease of coherence between the PFC and HIP, suggesting that the connectivity between these areas is affected, possibly contributing to the behavioral alterations (Duarte et. al, unpublished). These data show that along with the PFC, HIP might also be involved in the onset of anxious-like behavior.

Considering the symbiotic crosstalk between neurons and microglia, allowing for physiological adaptations in both cell populations in a chemical (Kettenmann et al., 2011; Paolicelli et al., 2011b; Sheridan and Murphy, 2013) and activity-dependent manner (Tremblay et al., 2010), it is important to account for both cells when evaluating the physiology of one or another. Therefore, it became imperative to execute the same

morphometric analysis in the HIP to explore if either neurons or microglia cell populations were structurally altered in male animals prenatally exposed to DEX.

5.1. Prenatal DEX exposure altered the cellular architecture in CA1 pyramidal NEURONS IN the HIP of male adult rats

Previous reports have already confirmed that exposure to DEX can have long-term effects concerning neuronal structure. These changes have been observed in the medial PFC, where the treatment with the GR-selective agonist DEX and with corticosterone resulted in a significant reduction in the total length of apical dendrites in the pyramidal neurons in lamina II/III of the anterior cingulate/prelimbic and infralimbic cortices (Cerqueira et al., 2005, 2007). The morphological alterations were also accompanied with behavioral deficits, such as impairments in working memory and behavioral flexibility (Cerqueira et al., 2005). The spectrum of DEX effects in brain structure were also found to extend to other brain regions, such as the bed nucleus of the stria terminalis (BNST) and the amygdala. The first presented an increase in volume, more pronounced in the anteromedial subdivision which presented increased dendritic length. Conversely, the amygdala showed opposite effects, with a reduced volume due to a significant dendritic atrophy (Oliveira et al., 2012; Pêgo et al., 2008), demonstrating that brain regions adapt differently when prenatally exposed to corticosteroids, namely DEX.

Accordingly, our study showed that pyramidal neurons from the CA1 region of the HIP are structurally compromised in adult male rats exposed to DEX during gestation. These neurons presented an overall hypertrophy in the apical and basal dendrites, both presenting increased length in their dendritic arborization.

The morphological imprint induced by DEX suggests that cell and/or network function may have been altered. In fact, PS has been shown to decrease spine density in the HIP. Martínez-Téllez and colleagues have reported that prepubertal males (PND35) showed an increase in spine density in the CA1 region of the HIP with a decrease in CA3, whereas adult (PND65) rats showed a decrease in the spine density in both areas. Also, CA1 pyramidal neurons from prepubertal rats presented a retraction in the dendritic filed, whereas adult rats displayed neurons with increased dendritic length (Martínez-Téllez et al., 2009).

This differential response is widely reflected in the current literature. Although the morphological changes in the hippocampal formation induced by corticosteroid hormones are still poorly documented, it is widely established that stress or corticosteroid administration induces neuronal atrophy in the CA3 pyramidal neurons. In the CA1 region,

however, some controversy remains. Prolonged activity-induced stress or corticosterone administration promoted dendritic retraction of pyramidal neurons from CA1 and CA3 regions in both young and adult rats (Lambert et al., 1998; Magariños and McEwen, 1995; Sousa et al., 2000). On the other hand, adult rats chronically treated with corticosteroid present a mild tendency towards neuronal hypertrophy in pyramidal neurons from CA1 (Woolley et al., 1990). This effect was exacerbated in rats subjected to PS (brief exposure (30 min) to bright light in an elevated platform), where the dendritic length in CA1 pyramidal neurons was significantly increased (Mychasiuk et al., 2012). Remarkably, brief exposure to elevated corticosteroids promoted neuronal atrophy in the apical branches of CA1 neurons in brain slices from animals that were submitted to prolonged mild stress (Alfarez et al., 2008), indicating enhanced vulnerability to elevated corticosteroid levels in animals exposed to prolonged stress. These adaptive changes in circuitry functionality could partially explain the mood changes presented by patients exposed to long-term treatment with GCs, as well as the cognitive impairments in both declarative and working memory (Brown, 2009).

The debatable nature of these results could be related to different approaches when analyzing the effects of stress in the brain. Martínez-Téllez and colleagues, beside describing a neuronal atrophy in both CA1 and CA3 pyramidal neurons, observed an increase in the dendritic structure of CA1 neurons of prepubertal rats, implying that PS affects neuronal structure in a time-dependent manner (Martínez-Téllez et al., 2009). Hence, the time points for experimental analysis implicating stress-related features should be taken in consideration. Additionally, the type of stressor must be accounted. The intensity of the stressor is directly implicated in the levels of corticosteroids in the bloodstream. This has been reported in a study where acute (immobilization or cold exposure) and chronic (psychosocial isolation) stressors were compared. While acute stress (2 hours) increased the levels of corticosteroid, rats subjected to psychosocial isolation (individually housing of animals for 21 days), presented a blunted response toward a subsequent acute stress reportedly due to an impairment in the HPA axis activity (Filipović et al., 2013). Ultimately, sex also plays a role in modulating stress responses. It has been reported that repeated restraint stress induced apical dendritic atrophy in male CA3 pyramidal neurons, while not affecting the same neuronal niche in females (Galea et al., 1997). Ergo, it is crucial to distinguish both gender populations when performing stress-related studies to be able to discriminate sex-specific effects.

Nonetheless, it should be taken in consideration that the majority of the aforesaid studies applied stress protocols in postnatal animals. In our animal model, since DEX is administered in the prenatal period, it will affect the development of the organism in a

systemic level, having putative implications in neurodevelopmental programming. Therefore, one most cautiously account for the nature of these results when comparing them.

5.2. DEX DIDN'T SIGNIFICANTLY AFFECT SPINE DEVELOPMENT AND DENSITY IN ORGANOTYPICAL HIPPOCAMPAL SLICES

The structural plasticity of spines can occur spontaneously or in an activity dependent manner. Spine turnover, which refers to the genesis and elimination of spines, has been described to affect a small subpopulation of spines under basal activity conditions (Caroni et al., 2012). In a remarkable manner, behavioral learning or an increase in neuronal activity alters spine dynamics, as well as their morphology. This remodeling in the neural circuitry could mirror alterations in memory and learning processes (Caroni et al., 2012). Ergo, assessing the morphology and number of spines is a conceivable way to characterize spine dynamics.

Regarding the CA1 region, Jafari and colleagues have reported that GRs are indeed localized to dendritic spines of adult CA1 pyramidal cells in both rat and mouse. Curiously, he observed that these receptors were located both in the head and neck of the spines. Suggesting that corticosteroids may be implied in the structural remodeling of dendritic spines. This hypothesis was reinforced by the fact that an acute DEX treatment in hippocampal slices lead to the activation of actin regulatory pathways (Jafari et al., 2012).

In organotypical hippocampal cultures prepared from male rat pups treated with DEX (0.25 μM for 24 hours), CA1 pyramidal neurons displayed tendencies regarding structural changes in the dendritic spines. In the apical dendrites, a tendency to a reduction in the density of filopodia was noticeable, as well as in the number of stubby and bifurcated spines (Figure 13). In contrast, this effect was compensated by a tendency to an increase in the density of thin and mushroom spines. Similar changes regarding filopodia, thin and bifurcated spines densities were also visible in basal dendrites, while no changes were detected in stubby and mushroom spines. Interestingly, neither apical nor basal total dendritic spines densities seemed to be affected, hence suggesting that DEX treatment may affect spine development rather than density. The fact that a decrease in filopodia and stubby spines was concomitant with an increase in both thin and mushroom spines density, could suggest that DEX enhanced spine maturation, promoting the shift from filopodia to thin and stubby spines to mushroom. It becomes apparent in the basal dendrites, since the reduction in filopodia density was only followed by an increase in thin

spines density, not affecting the ratios of both stubby and mushroom spines. Although a decrease in bifurcated type spines was observed, it is worth mentioning that the contribution of these spines towards the sample size was less substantial due to their minor prevalence. Therefore, minor changes in these spines density might not reflect tangible alterations.

Our results regarding the total number of spines seem to contradict the widely accepted thought that stress-like stimuli lead to an overall dendritic spine loss in the HIP (Bessa et al., 2009; Magariños and McEwen, 1995; Vyas et al., 2002; Watanabe et al., 1992). However, Mychasiuk and colleagues showed that both male and female rats subjected to PS actually present increase density of spines in CA1 neurons (Mychasiuk et al., 2012). Another study has reported that chronic stress or subcutaneous corticosterone injections in adult male rats didn't change spine density in CA1 neurons (Sousa et al., 2000). Once again, the nature of the stress-like stimuli along with the experimental timepoints might explain these controversial results and need to be taken in account.

It is documented that the exposure to abnormal corticosteroid levels can elicit cellular restructuration in the brain, explicitly in the HIP. Regarding neurons, this structural reconfiguration is also correlated with synaptic changes, namely in dendritic spines. Male rats subjected to a chronic mild stress protocol have shown neuronal atrophy in the main regions of the hippocampal formation, namely in the CA3 and DG. Concomitantly, this dendritic reduction was accompanied by an equal reduction in the number of dendritic spines. Nonetheless, no alterations in spine density were observed in the granule neurons of the DG (Bessa et al., 2009). This simple approach elegantly depicts the differential neural plasticity across the sub-regions of the HIP and their distinct response towards stress. Hence, region-dependent effects on spine remodeling also need to be considered when assessing the morphometric changes in the HIP upon stress conditions.

The effects of stress in modeling spine morphology, directly correlated with their maturation, have been reported and shown to be gender-based. Using a maternal separation (MS) paradigm, it has been shown that MS females had lower density of thin spines in the PFC while it had no effect on spine density in males. Also, MS females had proportionately more small-headed thin spines while males had more large-headed mushroom spines (Farrell et al., 2016). Exposure to restraint and brief intermittent tail stimulation also affected spine density in a sex-dependent fashion, increasing in the male HIP while decreasing in females (Shors et al., 2001). The distinct response between males and females, reflected in the dissimilar neural adaptions towards stress, might

explain the differential predisposition towards cognitive deficits stress-related pathologies (Bowman and Kelly, 2012; Bowman et al., 2001). Taking in account the role of microglia in synapse turnover (Hong et al., 2016; Parkhurst et al., 2013; Stephan et al., 2012; Tremblay et al., 2010) and the distinct density of microglia cells between male and female rats in the perinatal brain (Schwarz et al., 2012), it's fair to suggest that microglia could influence the distintic synaptic patterning found between genders upon a stress-like insult.

5.3. ANTENATAL DEX AFFECTS MICROGLIA MORPHOLOGY IN THE DG OF MALE ADULT RATS

In our latest study, we reported that prenatal DEX induces morphological changes in microglia in a sex-dependent manner in the PFC. While male adults presented hypertrophic microglia, with increased processes length and number, female microglia showed an opposite response, with fewer and shorter processes (Caetano et al., 2016). Regardless of the contrasting morphological alterations between genders, both presented anxious-like behavior. These results suggest that microglia dysfunction could be implicated in the development of these behavioral alterations.

Regarding the hippocampal formation, our group brought into evidence that prenatal DEX promotes structural alterations in adult female microglia in the DG (Duarte et. al, unpublished). The data analysis performed in the present study validated cytoarchitectural changes in adult males subjected to antenatal DEX. Although both genders displayed microglia reflecting an overall hypertrophy, the structural remodeling presented by these cells differed between them. In females, DEX treatment increased the number of microglia ramifications while not affecting their length. In turn, male HIP microglia present alterations in both parameters, showing more ramifications number and longer processes.

The results obtained in this study corroborate our previous studies, further extending the gender dimorphisms regarding microglia morphology. Considering the vast implication of microglia in the etiology of neuropsychiatric illness (Blank and Prinz, 2013; Prinz and Priller, 2014), the unique microglia identity between sexes can mirror gender vulnerability in developing mood disorders. For instance, a plethora of stress-linked structural and functional alterations in the mPFC have been implicated in psychological disorders (Price and Drevets, 2010), with females being more susceptible than males (Van de Velde et al., 2010). In contrast, males appear to be more prone to cognitive deficits than females in the pathological context of stress (Bowman and Kelly, 2012; Bowman et al., 2001). This data highlights different inter-regional forms of plasticity in the brain upon a stress stimulus, being underpinned with a strong gender imprint.

What underlies these gender dimorphisms still remains elusive. Wang and colleagues observed that depressed female patients had higher GR immunoreactivity in the HIP than depressed male patients (Wang et al., 2012). The differential GR density between sexes can be accounted for the dissimilar morphological responses found in both genders HIP microglia. Moreover, Diz-Chaves et al. conducted two different studies where were described sex differences within the DG regarding microglia density of prenatally stressed rats. While females presented an increased number of Iba-1 (microglia marker) positive cells (Diz-Chaves et al., 2012), males did not show significant changes when compared with non-stressed animals (Diz-Chaves et al., 2013). The higher density of microglial cells in the DG of stressed females might explain why DEX only increased the number of microglia ramifications and not their length. Since the spatial distribution of microglia is mainly repulsive (meaning that most neighboring microglia processes don't overlap between them) (Jinno et al., 2007), it's possible that this increase in cell density evoked by prenatal DEX limited the growth of microglial processes due to repulsive gradients. In turn, as males didn't show alterations in the microglial density upon antenatal DEX exposure, these spatial boundaries wouldn't confine cellular outgrowth, explaining why ramification length was also increased.

5.4. $A_{2A}R$ ANTAGONIST EXACERBATED MICROGLIA HYPERTROPHY IN ADULT MALE RATS EXPOSED TO DEX

The mechanism underlying microglia reshaping in stress models it's still poorly explored and requires further examination. The $A_{2A}R$ is a known regulator of microglia morphology (Gyoneva et al., 2014b, 2014a, 2016; Orr et al., 2009), being widely implied in brain pathology (Batalha et al., 2016; Ingwersen et al., 2016; Kaster et al., 2015; López-Cruz et al., 2017).

$A_{2A}R$ blockade also showed to have a gender-biased effect. DEX exposed males showed improvements in microglia morphology upon chronic blockade of $A_{2A}R$, ameliorating anxious-like behavior. This enhancement was not observed in females. In fact, the $A_{2A}R$ blockade further compromised microglia structure in adult females exposed to DEX, with no behavioral improvements (Caetano et al., 2016). In the HIP, however, females prenatally exposed to DEX and treated with SCH58261 showed a significant reduction in the number of ramifications, reaching levels close to control ones (Duarte et al., unpublished). Nonetheless, a decrease in processes length was also detected. In DEX treated males administered with the $A_{2A}R$ antagonist, microglia morphology was affected in a striking manner. Both ramification length and processes drastically increased, being

well evident in the branch orders towards the periphery (5th and beyond), contrasting with mild shrinkage detected in HIP microglia structure from DEX treated females.

The blockade of $A_{2A}R$ also showed prominent sex-dependent results in healthy animals with differential results according to different brain regions. Adult females displayed an atrophy in microglia branching in the PFC, affecting both processes length and number, whereas males didn't show any structural alterations. In the HIP, the $A_{2A}R$ antagonist treatment *per se* exerted a marked effect (reduction) in the length of microglia processes in adult females, although not affecting the number of processes. In this study, adult males presented similar structural alteration in microglia cytoarchitecture prior to chronic blockade of the $A_{2A}R$ as the ones in DEX treated animals, with a mild increase in processes length and decrease in the ramifications number in comparison with the latter.

The inconsistency of these results could be due to a putative interaction between GR and $A_{2A}R$. Accordingly, rats prenatally exposed to DEX revealed different $A_{2A}R$ levels in a sex-specific manner, with contrasting results. While males showed higher levels of $A_{2A}R$ in the PFC, females revealed lower levels (Caetano et al., 2016), hence suggesting a possible interaction between GR activation and $A_{2A}R$ activity. The availability of adenosine might also clarify these gender differences. A *post mortem* analysis of human brains revealed gender differences in the adenosine levels of several brain regions, reporting lower levels of adenosine in women total cortex (Kovács et al., 2010). This could be explained by the higher activity of monoamine oxidase (MAO) found in women when comparing to men (Robinson et al., 1977).

How stress affects GR density is already described. Karandrea and colleagues elegantly demonstrated that different stress protocols induce contrasting effect in GR density in the HIP and hypothalamus in a gender-dependent manner. While females didn't reveal GR alterations upon exposure to swimming paradigm, the same stressor decreased GR mRNA levels in males' HIP after chronic exposure. Curiously, an acute exposure promoted an up-regulation in both GR in the hypothalamus (Karandrea et al., 2002). Nonetheless, it should be noted that the abundancy in GR in the HIP has species specific patterns. Even though it was shown that GR protein in extensively expressed throughout the main neuronal sub regions of the human HIP (Wang et al., 2013), low levels were detected in the rhesus monkey (Sánchez et al., 2000). In a recent publication, Fourgeaud and colleagues showed that Axl and Mer receptors, important in regulating the innate immune response, also modulate microglia function, namely its morphology (Fourgeaud et al., 2016). Intriguingly, DEX was found to upregulate Mer receptors on macrophages (Zagórska et al., 2014).

The results obtained in this study further validate the idea that microglia have a defined identity based on gender. Furthermore, microglia typology differs between brain regions. The complex individuality of microglia cells and the numerous variables underlying stress make it difficult to portray a holistic panorama concerning the brain adaptive response to stress.

5.5. A_{2A}R KNOCKOUT INCREASED THE NUMBER BUT NOT LENGTH OF PROCESSES IN THE DG OF MALE MICE

Considering the already stated importance of A_{2A}R in defining microglia architecture and their early expression in the developing brain (Weaver, 1993), we further extended our research related to A_{2A}R in microglia morphology. For that purpose, A_{2A}R KO mice were generated in order to understand the developmental relevance of A_{2A}R in defining microglia structure in later adulthood. This part of the work was included in a study in which we aimed to validate inter-gender differences in A_{2A}R adult mice between the mPFC and dHIP (PND90) (Mateus-Pinheiro et al., unpublished).

In this study we have stumbled across gender-biased effects concerning the deletion of A_{2A}R in microglia, also underpinned with differential results across brain regions. Concerning the HIP, microglia structure in adult A_{2A}R KO females didn't show morphological alterations in the DG. In turn, A_{2A}R KO males presented more ramified microglia, which was reflected in a mild upward tendency in the number of processes in some branch orders. In the mPFC, while males didn't display any differences regarding microglia cytoarchitecture, females presented a relevant increase in both number and length of processes, indicating a general hypertrophy.

A_{2A}R function in defining microglia morphology has been already explored. Young adult female mice submitted to caffeine treatment, a known A_{2A}R antagonist, displayed microglia with shorter processes with a decreased branching (Steger et al., 2014). Orr and colleagues described that A_{2A}R activation lead to processes retraction in LPS-primed microglia *in vitro*. Microglia exposed to LPS also showed an increase in the expression of A_{2A}R and a decrease in P2Y12 receptors (Orr et al., 2009), which have been described to mediate microglia processes extension in response to ATP (Haynes et al., 2006). Ergo, a shift in purinergic signaling takes place when an insult takes place, adapting microglia response towards it. Additionally, the blockade of A_{2A}R with the antagonist preladenant in acute brain slices restored microglial response to cellular damage in a mouse model of Parkinson's disease (Gyoneva et al., 2014b), and failed to restore motility toward

damaged cells in slices from an Alzheimer mice model (Gyoneva et al., 2016). Hence, the consequences of $A_{2A}R$ activation or blockade might differ among distinct pathologies. One possible explanation might partially rely in the extracellular levels of glutamate, since local concentrations of glutamate redirects $A_{2A}R$ signaling from antinflammatory (PKA; low concentrations) to proinflammatory (high concentrations) (Dai et al., 2010; George et al., 2015). Results indicate that microglial density throughout the brain is decreased in the caffeine treated groups as compared with control. Caffeine also affected microglia morphology by shortening processes length and decreasing branching.

These results once again implicate $A_{2A}R$ as prominent regulators of microglia morphology. However, it is difficult to explain the causes and consequences of these results. Since the *ADORA2* gene is deleted at the beginning of neurodevelopment, we must account for the possible occurrence of compensatory mechanisms in order to surpass $A_{2A}R$ deficiency. Inducing the genetic knockout in later stages of development would allow us to test this hypothesis. Furthermore, there is evidence that other P1 purinergic receptor modulate microglia morphology, such as the A_1 and A_3 receptors (Luongo et al., 2014; Ohsawa et al., 2012). These receptors might have a more active role in the absence of the $A_{2A}R$, since no adenosine is being sequestered by this receptor.

The expression of the $A_{2A}R$ also involves other types of brain cells, such as neurons and astrocytes (Kaster et al., 2015; Orr et al., 2015). Since $A_{2A}R$ modulates neuronal activity (Rebola et al., 2008), which is regulated by both astrocytes and microglia (Pascual et al., 2012), it is difficult to pinpoint the direct effect from these cells towards these results. An interesting approach would consist in using cell-specific $A_{2A}R$ KO in different developmental time points in order to discriminate the contribution of $A_{2A}R$ from these cell populations in defining microglia during development.

CONCLUSION

The use of synthetic GCs is a common practice among clinics due to their beneficial value in several pathologies. A survey between 2000 and 2001 of members of the European Society for Pediatric Endocrinology (representing 125 institutions), found that DEX treatment in prenatal diagnosis is a common practice in roughly 57% of the centers (Dreger et al., 2012) Ergo, a wider view over the long-term effects of GCs therapy is needed, namely in the premature brain.

In this work, a structural assessment of neuronal and microglial morphology in the HIP revealed that prenatally DEX exposed male rats presented cytoarchitectural alterations in both cell populations.

Regarding neuronal morphology, this experimental paradigm elicit an overall dendritic atrophy in CA1 pyramidal neurons. The dendritic retraction was seen in both apical and basal dendrites. These results are in accordance with the current literature, where stress-like stimuli or corticosterone injections induced a dendritic reduction in several brain areas, including the HIP (Bessa et al., 2009; Lambert et al., 1998; Martínez-Téllez et al., 2009; Sousa et al., 2000), although with some contrasting evidence (Mychasiuk et al., 2012; Woolley et al., 1990). DEX exposure also showed to affect dendritic spine plasticity in CA1 pyramidal neurons *in vitro*, seemingly affecting spine development rather than density. Having said, DEX appears to compromise the neural circuitry integrity in the HIP by remodeling neuronal cytoskeleton, affecting both the dendritic arborization and spine development.

Previous work done by our group confirmed that antenatal DEX exposure can compromise microglia structure in the and PFC in a sex-biased fashion, with both genders presenting anxious like behavior (Caetano et al., 2016). The remodeling effects of DEX were also observable in the DG of HIP female microglia (Duarte et al., unpublished). This work further extended this view by also showing these morphological changes in the HIP of male adults exposed to DEX *in utero*. Microglia from the DG showed a mild hypertrophy as seen by the increase in processes' length and number. Intriguingly, DEX female microglia from the DG only showed alterations in the number of processes, thus showing that microglia structure also has regional-dependent effect.

The blockade of $A_{2A}R$ reversed the morphological and behavioral alteration in males, whereas it aggravated microglia morphology in the PFC females (Caetano et al., 2016). In the HIP from DEX treated males, $A_{2A}R$ pharmacological blockade elicited striking alterations in microglial from DG, increasing the length and number of their processes. In females prenatally exposed to DEX the chronic treatment with SCH58261 restored microglia ramification numbers while decreasing processes' length similar to control

values (Duarte et al., unpublished). The dynamic of the results regarding $A_{2A}R$ blockade among genders and brain regions strongly highlights the differential role of these receptors in modulating microglia structure. Accordingly, the distinct results regarding microglia morphology among $A_{2A}R$ KO adult mice in both PFC and HIP (DG) highlights the importance of these receptors in defining microglia structure.

Thus, when assessing the morphometric effects of DEX in the CNS, all cellular subtypes must be considered due to their different structural plasticity, along with intrinsic gender differences. These cellular dimorphisms among gender might be responsible for the distinct vulnerability to neuropsychiatric disorders between sexes (Van de Velde et al., 2010). Sex influence should also be considered when advancing therapeutic strategies regarding the $A_{2A}R$, since their pharmacological blockade has shown contrasting effects between males and females.

FUTURE PERSPECTIVES

The dynamic profile of microglia morphology across brain regions clearly depicts the brain complex compartmentalization. Microglia gender imprint adds an extra layer in the characterization of these cells and suggests that these cells are physiologically different. Although DEX elicited differential responses between genders, its physiological relevance still remains to be explored. Thus, it would be interesting to analyze other features regarding microglia, namely its cytokine levels expression, which has been described to be altered in rats subject to PS (Diz-Chaves et al., 2012, 2013). Furthermore, it would be worth the effort to address microglia structure in other brain areas implicated in mood disorders, such as the amygdala and the insular cortex, to confirm if these gender imprints also extend to those areas.

Concerning the HIP, the same morphometric approach in the hippocampal formation would give us a more detailed insight towards the structural plasticity of this region, since numerous reports have demonstrated differential adaptations in the neuronal populations towards high corticosteroid levels. In the developing HIP, microglia has an important role in controlling the number of neuronal precursors (Cunningham et al., 2013). Analyzing the phagocytic capability of microglia upon prenatal DEX exposure would allow us to discriminate if other physiological aspects of microglia are compromised.

Having microglia being implicated in the formation and elimination of synapses (Hong et al., 2016; Parkhurst et al., 2013; Stephan et al., 2012; Tremblay et al., 2010), it would be interesting to address if the synaptic alterations induced by elevated GC levels are mediated by microglia. To do so, *in vitro* hippocampal organotypical cultures would allow us to temporally delete microglia populations by treating them with clodronate, which selectively eliminates microglia. This way, we would be able to discriminate DEX effects in dendritic synapses in the absence and presence of microglial cells. If microglia were to be the culprit behind these synaptic alterations, preventive therapies targeting microglia would convey a possible way to circuvent DEX deleterious effects.

Regarding the global knockouts of the $A_{2A}R$, the approach done in this work didn't allow us to pinpoint the exact influence of these receptors between distinct cell populations. Targeting specific cells for the $A_{2A}R$ knockout would enable us to discriminate how each cell type, namely neurons and astrocytes, affects microglia morphology through the activity of the $A_{2A}R$. Since other P1 receptors (A_1 and A_3) (Luongo et al., 2014; Ohsawa et al., 2012) have been implicated in defining microglia morphology, it would be interesting to assess if upon the genetic knockout for the $A_{2A}R$ the expression levels of these receptors would be altered. Inducing the knockout for the $A_{2A}R$ in different timepoints would also allow to study the relevance of these receptors during neurodevelopment.

The material here presented will provide fertile ground for future studies regarding structural changes in the developing brain upon an elevated glucocorticoid surge. This will allow us to further investigate the impact of such alterations in the onset in neuropsychiatric disorders and to investigate new therapeutic targets.

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SUPPLEMENTARY DATA

Supplementary Table 1| Summary of the number of intersections in HIP neurons in male rats (p < 0,05 *; p < 0,01 ** when comparing CTRL to DEX treated animals).

INTERSECTIONS/CONCENTRIC RINGS	CTRL	DEX
20	3,60 ± 0,33	3,63 ± 0,10
40	7,10 ± 0,36	7,64 ± 0,26
60	10,14 ± 0,60	10,32 ± 0,35
80	11,21 ± 0,22	12,01 ± 0,42
100	10,00 ± 0,36	11,01 ± 0,75
120	7,77 ± 0,44	8,91 ± 0,58
140	5,13 ± 0,25	6,83 ± 0,45 **
160	4,22 ± 0,22	4,94 ± 0,20
180	3,34 ± 0,10	3,88 ± 0,24
200	2,94 ± 0,18	3,76 ± 0,65
220	2,20 ± 0,20	3,19 ± 0,59
240	1,88 ± 0,19	2,93 ± 0,44
260	1,26 ± 0,13	2,43 ± 0,44
280	0,86 ± 0,12	1,91 ± 0,37
300	0,69 ± 0,03	1,58 ± 0,35
320	0,57 ± 0,12	1,45 ± 0,23
340	0,51 ± 0,16	1,30 ± 0,19
360	0,29 ± 0,10	1,07 ± 0,24
380	0,242 ± 0,01	0,69 ± 0,16
400	0,25 ± 0,09	0,41 ± 0,161

Supplementary Table 2| Summary of the length of processes (μm) in HIP microglia from male rats (p < 0,05 *; p < 0,01 **; p < 0,001 *** when comparing CTRL with DEX treatment animals; p <0,05 #; p < 0,01 ## when comparing DEX treated animals with DEX+SCH treated animals).

ORDER	CTRL	DEX	SCH	DEX+SCH
1	38,12 ± 1,09	31.36 ± 2,14	49,25 ± 4,83**	30,80 ± 3,34*
2	89,12 ± 2,6	75,77 ± 2,83*	96,29 ± 13,57	78,21 ± 0,38
3	100,87 ± 4,76	95,19 ± 8,61	115,30 ± 21,25	114,03 ± 5,06
4	89,84 ± 5,71	101,35 ± 13,22	117,99 ± 24,45	117,02 ± 4,61
5	86,71 ± 8,58	93,46 ± 16,92	106,73 ±20,14	123,05 ± 6,91*
6	67,32 ± 13,19	79,32 ± 13,18	81,57 ± 10.37	122,40 ± 12,72**;##
7	43,48 ± 11,12	59,61 ± 10,87	56,92 ± 2,65	101,89 ± 9,90 ***;##
8	36,23 ± 13,03	45,20 ± 10,82	39,10 ± 5,42	78,51 ± 13,59**;#
9	19,72 ± 4,41	33,14 ± 7,37	28,01 ± 0,60	51,94 ± 12,99##
10	18,98 ± 3,51	28,04 ± 8,39	27,54 ± 0,17	36,43 ± 9,68
11	14,89 ± 4,48	31,38 ± 3,48*	27,88 ± 3,83	26,56 ± 8,63
12	8,42 ± 0,67	23,08 ± 4,90	19,12 ± 1,59	20,61 ± 10,809
13	6,70 ± 0,00	20,75 ± 1,87	16,73 + 5.43	22,46 ± 10,13
14	8,30 ± 0,00	16,28 ± 3,11	23,45 + 0,00	18,58 ± 4,39
15	NA	24,72 ± 0,97	49,10 + 0,00	10,51 ± 3,06

Supplementary Table 3| Summary of the number of processes in male microglia from male rats. ($p < 0,05$ *; $p < 0,01$ ** when comparing CTRL with DEX treatment animals; $p < 0,05$ # when comparing DEX treated animals with DEX+SCH treated animals).

ORDER	CTRL	DEX	SCH	DEX+SCH
1	7,71 ± 0,25	6.75 ± 0,19*	8,80 ± 0,60*	7,05 ± 0,25
2	14,53 ± 0,29	12,40 ± 0,85	15,33 ± 1,95	13,14 ± 0,18
3	17,47 ± 0,58	16, 75 ± 1,29	19,30 ± 4,70	20,69 ± 0,58
4	17,74 ± 1,01	19,35 ± 2,35	21,10 ± 4,90	24,10 ± 1,76
5	17,07 ± 1,82	19,38 ± 3,12	20,50 ± 4,50	25,49 ± 2,30*
6	14,07 ± 2,09	16,88 ± 3,39	16,15 ± 3,35	25,78 ± 3,87**;#
7	9,15 ± 2,28	12,98 ± 3,22	12,90 ± 2,00	22,18 ± 3,02**;#
8	6,76 ± 2,65	9,88 ± 2,91	7,70 ± 0,10	18,19 ± 3,61**;#
9	3,60 ± 1,55	7,33 ± 2,57	4,75 ± 0,15	12,62 ± 3,47**
10	2,51 ± 1,19	5,40 ± 1,96	2,85 ± 1,15	8,41 ± 2,45*
11	1,96 ± 1,11	4,33 ± 1,89	2,00 ± 0,10	5,34 ± 2,10
12	0,99 ± 0,64	2,80 ± 1,31	1,40 ± 0,00	4,12 ± 2,33
13	0,13 ± 0,13	1,95 ± 0,92	0,70 ± 0,10	3,02 ± 1,86*
14	0,07 ± 0,07	1,45 ± 0,66	0,50 ± 0,20	2,32 ± 1,21*
15	NA	1,05 ± 0,52	0,30 ± 0,30	1,24 ± 0,54

Supplementary Table 4/ Summary of the length of processes (μm) in HIP microglia from male mice.

ORDER	CTRL	$A_{2A}R$ KO
1	24,51 ± 3,13	25,17 ± 2,89
2	53, 51 ± 6,90	53,53 ± 7,89
3	64,33 ± 7,08	71,50 ± 10,25
4	67,91 ± 6,16	72,28 ± 6,35
5	61,16 ± 5,72	69,95 ± 4,72
6	54,08 ± 4,60	64,78 ± 3,86
7	45,51 ± 4,22	55,01 ± 6,31
8	40,06 ± 3,75	39,47 ± 7,42
9	30,83 ± 3,18	34,50 ± 8,88
10	26,48 ± 6,55	26,01 ±4,07
11	24,56 ± 4,49	31,73 ± 5,53
12	22,07 ± 5,79	17,33 ± 2,59
13	16,73 ± 3,30	13,82 ± 2,70
14	17,76 ± 2,67	11,44 ± 1,61
15	13,21 ± 2,40	9,88 ± 1,02

Supplementary Table 5| Summary of the number of processes in HIP microglia from male mice.

ORDER	CTRL	A_{2A}R KO
1	5,48 ± 0,34	5,40 ± 0,18
2	9,83 ± 0,62	10,54 ± 0,79
3	13,18 ± 0,52	14,47 ± 0,92
4	14,60 ± 0,69	15,83 ± 0,60
5	14,50 ± 0,63	16,52 ± 0,67
6	12,68 ± 0,27	15,14 ± 1,35
7	10,48 ± 0,68	13,67 ± 2,30
8	9,55 ± 1,07	11,11 ± 2,46
9	7,13 ± 0,74	8,88 ± 2,52
10	5,13 ± 0,94	6,41 ± 2,07
11	3,85 ± 0,79	4,53 ± 1,84
12	2,95 ± 0,76	2,70 ± 0,95
13	2,05 ± 0,56	1,75 ± 0,63
14	1,80 ± 0,52	1,38 ± 0,47
15	1,20 ± 0,35	0,70 ± 0,35