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Alexandra Filipa Figueiredo Cruz

Microglia Morphology and Behavior upon Testosterone Administration of Females Prenatally Exposed to Dexamethasone

Dissertação no âmbito do Mestrado em Investigação Biomédica
orientada pela Doutora Filipa Isabel Cabaço Baptista e pela
Professora Doutora Catarina Alexandra dos Reis Vale Gomes
e apresentada à Faculdade de Medicina da Universidade de Coimbra

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"To accomplish great things,
we must not only act, but also dream,
not only plan, but also believe."

– Anatole France

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Abbreviations List

A

$A_{2A}R$ – A_{2A} Receptor

APS – Ammonium Persulfate

AR – Androgen Receptor

B

BCA – Bicinchoninic Acid

BSA – Bovine Serum Albumin

C

CAPS – N-Cyclohexyl-3-aminopropanesulfonic acid

CGS21680 - 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride

CNS – Central Nervous System

CTRL - Control

D

DEX – Dexamethasone

DOC – Sodium Deoxycholate

DTT – DL-Dithiothreitol

E

ECL – Enchanced Chemiluminescence

EPM – Elevated Plus Maze

ER – Estrogen Receptor

F

FBS – Fetal Bovine Serum

G

GAPDH – Glyceraldehyde 2-phosphate dehydrogenase

GD – Gestational Day

GC – Glucocorticoid

GR – Glucocorticoid Receptor

GW – Gestational week

H

HEPES – 4-(2-hydroxyethil)-1-piperazineethanesulfonic acid

I

Iba-1 - Ionized calcium binding adaptor molecule 1

M

MR – Mineralocorticoid Receptor

O

OPF – Open Field

P

PBS – Phosphate Buffered Saline

PFA - Paraformaldehyde

PFC – Prefrontal Cortex

PND – Postnatal Day

PVDF – Polyvinylidene Difluoride

R

RIPA – Radioimmunoprecipitation Assay Buffer

RPMI – Roswell Park Memorial Institute

RT – Room Temperature

S

SCH58261 - 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine

SDS - Sodium Dodecyl Sulfate

SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SEM – Standard Error of the Mean

T

TBS – Tris-Buffered Saline

TEMED - Tetramethylethylenediamine

Abstract

Neuropsychiatric disorders are the most common mental illnesses in the world and present a sex dimorphism in prevalence, symptoms and treatment. Sex differences arise since neurodevelopment in which males experience a peak in testosterone that is responsible for brain masculinization, meaning there is an organization in density, connectivity and morphology of cells, including microglia, in several brain regions. This remodeling of the brain will have long term consequences in behavior.

Microglia are the immune cells of the central nervous system that are responsible for sculpting neuronal circuits during neurodevelopment, but also ensuring the homeostasis through screening the parenchyma with their highly dynamic ramifications. The morphology and function of microglia are under the influence of the A_{2A} receptor ($A_{2A}R$), which blockade is known to have anxiolytic effects.

Our group has demonstrated that upon prenatal stress, induced by dexamethasone (DEX) exposure on gestational days 18 and 19, males and females have an anxious-like behavior at adulthood (postnatal day 90 – PND90) and present a sex-specific remodeling of microglia morphology in the prefrontal cortex (PFC), a brain region essential for emotional regulation. Furthermore, when these animals are treated with an anxiolytic (SCH58261, an antagonist of the $A_{2A}R$), only male behavior and microglia morphology return to physiological conditions. The sex dimorphism observed in this study lead to the hypothesis that testosterone could have a protective effect in males.

The aim of this thesis is to assess $A_{2A}R$ ability to retrieve microglia morphology and behavior upon brain masculinization of females prenatally exposed to DEX. In other words, through female masculinization (by mimicking the peak of testosterone in males on PND0), we aim to evaluate if testosterone has a permissive effect for the action of the antagonist of the $A_{2A}R$ in females exposed to DEX.

Neurodevelopmental behavior is assessed between PND5 and 17, through a battery of tests. Regarding sex differences, males and females have a similar performance in physiology therefore, neonatal administration of testosterone did not have major effects on behavior. When DEX is administered, no effect is observed on behavior however, females have reduced strength and an anticipation of eye opening day. In DEX groups, no sex dimorphism is observed, but testosterone increases strength and delays eye opening day in females. Furthermore, testosterone induces a delay in cliff avoidance,

which is reverted by DEX exposure. Previous results from our group show that males and females prenatally exposed to DEX have changes in neurodevelopmental tests performance and anxious-like behavior at adulthood. In this cohort of animals DEX did not induce changes in the progeny performance in neurodevelopmental tests nor an anxious-like behavior, suggesting that alterations in neurodevelopmental behavior might be used as predictive factors for the development of neuropsychiatric disorders.

When DEX females are neonatally masculinized with testosterone, no anxious-like behavior is observed with or without SCH58261 administration, suggesting a protective effect of testosterone in behavior. However, when we analyze masculinized females' microglia from the PFC at adulthood, we observe testosterone was not able to revert DEX-induced atrophy even with anxiolytic treatment. Furthermore, when comparing CTRL+T and DEX+T females we observe no association between behavior and microglia morphology. These results suggest that testosterone was not permissive to the action of SCH58261, and also that testosterone has a mechanism to modulate behavior that is independent of microglia morphology.

Neonatal testosterone administration did not induce changes in peripheral levels of corticosterone at adulthood. Hence, when evaluating testosterone levels from serum we observe a tendency for SCH58261 to reduce levels of this hormone in CTRL+T females, but when this anxiolytic is administered in DEX+T females the pattern of action changes, and there is a tendency for an increase in testosterone levels. These results lead us to suggest that the blockage of $A_{2A}R$ modulates testosterone levels, peripherally. *In vitro* studies were conducted aiming to unravel a possible interaction between androgen receptors (AR) and $A_{2A}R$ specifically in microglial cells. We show that testosterone has an impact on $A_{2A}R$ density that is dependent of time of exposure and concentration. On the other hand, activating or blocking $A_{2A}R$ does not have an impact on AR density.

This work highlights the potential anxiolytic properties of testosterone, and also its role in modulating $A_{2A}R$, namely in microglia, which could account for the sex dimorphism observed in microglia morphology and in behavior. Nonetheless, additional studies are needed to further unveil the possible protective and organizational effect of testosterone in this model of chronic anxiety.

Keywords: Dexamethasone; Testosterone; A_{2A} Receptor; Behavior; Microglia

Resumo

As doenças neuropsiquiátricas, são as enfermidades mentais mais comuns no mundo e apresentam dimorfismo de sexo na sua prevalência, sintomatologia e tratamento. Estas diferenças iniciam-se no neurodesenvolvimento, pois os machos têm um pico de testosterona que induz masculinização cerebral, ou seja, reorganização na densidade, conectividade e morfologia das células, como a microglia, em várias regiões cerebrais. A remodelação cerebral irá ter repercussões a longo-prazo no comportamento.

A microglia é a célula imune do sistema nervoso central que é responsável por esculpir os circuitos neuronais durante o desenvolvimento cerebral, mas também por manter a homeostasia no parênquima, através das suas ramificações altamente dinâmicas. A morfologia e função da microglia estão sob a influência dos recetores A2A (A2AR), cujo bloqueio foi demonstrado como tendo efeito ansiolítico.

O nosso grupo mostrou que numa situação de stress pré-natal, induzido por dexametasona (DEX) nos dias gestacionais 18 e 19, machos e fêmeas têm um comportamento do tipo ansioso na idade adulta (dia pós-natal 90 – PND90) e remodelação da morfologia da microglia dependente do sexo no córtex pré-frontal (PFC), uma região cerebral essencial para a regulação emocional. Adicionalmente, quando os descendentes são tratados com um ansiolítico (SCH58261; antagonista dos A2AR), apenas o comportamento e a morfologia da microglia dos machos regressa à fisiologia. O dimorfismo de sexo visto neste estudo levou à hipótese de que a testosterona poderia ter um efeito protetor nos machos.

O objetivo deste estudo é perceber a capacidade dos A2AR recuperarem a morfologia da microglia e comportamento após masculinização cerebral de fêmeas prenatalmente expostas a DEX. Noutras palavras, através da masculinização feminina (mimetização do pico de testosterona que ocorre a PND0 em machos), objetivámos avaliar se a testosterona tem um efeito permissivo para a ação do antagonista dos A2AR em fêmeas expostas a DEX.

O comportamento no neurodesenvolvimento foi estudado entre PND5 e 17 através de uma bateria de testes. Relativamente às diferenças de sexo, machos e fêmeas têm uma performance semelhante fisiologicamente, como tal a administração neonatal de testosterona não teve efeitos major no comportamento. A exposição a DEX não teve efeito no comportamento, contudo, as fêmeas têm uma redução na força e uma antecipação do dia de abertura do olho. Nos grupos DEX, não se observou dimorfismo de sexo, mas a testosterona aumentou a força e atrasou o dia de abertura do olho em

fêmeas. Além disso, a testosterona induziu um atraso na aversão ao precipício, que foi revertida pela DEX. Estudos prévios do grupo mostraram que machos e fêmeas prenatalmente expostos a DEX têm mudanças no comportamento do neurodesenvolvimento e um comportamento do tipo ansioso a PND90. Nestes animais a DEX não induziu mudanças comportamentais em nenhuma idade, sugerindo que as alterações no comportamento do neurodesenvolvimento poderão ser usadas como fatores preditivos para o desenvolvimento de doenças psiquiátricas.

Quando as fêmeas DEX são masculinizadas neonatalmente com testosterona, não se observa comportamento do tipo ansioso, com ou sem administração de SCH58261, sugerindo um efeito protetor da testosterona no comportamento. No entanto, quando analisamos a microglia do PFC de fêmeas a PND90, observamos que a testosterona não é capaz de reverter a atrofia induzida pela DEX mesmo com a administração do ansiolítico. Adicionalmente, quando comparamos as fêmeas CTRL+T e DEX+T vimos que não há associação entre o comportamento e a morfologia da microglia. Estes resultados sugerem que a testosterona não teve um efeito permissivo à ação do SCH58261, mas também que o mecanismo de ação da testosterona no comportamento é independente da morfologia da microglia.

A administração neonatal de testosterona não induziu alterações nos níveis periféricos de corticosterona a PND90. Todavia, quando avaliamos os níveis de testosterona vemos uma tendência para o SCH58261 reduzir os níveis desta hormona em fêmeas CTRL+T, mas em fêmeas DEX+T o ansiolítico induz uma tendência para o aumento de testosterona.

Estes resultados levam a sugerir que o bloqueio dos A2AR modula os níveis de testosterona periféricos. Estudos in vitro tiveram o objetivo de perceber a possível interação entre recetores de androgénios (AR) e A2AR na microglia. Mostramos que a testosterona modula a densidade dos A2AR, de modo dependente do tempo de exposição e da concentração. Por outro lado, a ativação ou bloqueio dos A2AR não teve impacto na densidade dos AR. Este trabalho mostra as propriedades ansiolíticas da testosterona, mas também o seu papel modulador dos A2AR, nomeadamente na microglia, o que poderá explicar o dimorfismo de sexo na morfologia da microglia e comportamento. No entanto, estudos adicionais são necessários para melhor perceber o possível efeito protetor e organizacional da testosterona neste modelo de ansiedade crónica.

Palavras-Chave: Dexametasona; Testosterona; Recetores A_{2A}; Comportamento; Microglia

1 Introduction

1.1 Neurodevelopment Overview

Neurodevelopment is a complex process that consists in the formation of the nervous system architecture. Specifically, in the Central Nervous System (CNS), brain development comprehends the formation of synapses, neuronal circuits and programming behavior (Rice & Barone, 2000). This process begins in the third week of gestation in humans (gestational day 7- GD7; in rodents) and continues throughout life (Meredith, 2015). The development of the brain has five main events: cellular proliferation; migration; differentiation; synaptogenesis, and refinement through elimination of synapses and neurons (**Figure 1**) (Kolb & Gibb, 2011).

Brain development begins with neurulation, which implies the formation of progenitor cells. Neuronal progenitors are mitotic stem cells that can maturate and differentiate into any type of neuron. The process by which new neurons are formed, also called neurogenesis, begins in the third week after conception and is complete by the middle of gestation in humans (neurulation occurs between GD7 and GD11 in rodents), except for certain areas such as the hippocampus, which maintain a pool of progenitor cells throughout life (Eriksson et al., 1998; Gould et al., 1999). Once formed, neurons migrate radially to the cortical plate where, influenced by genes, hormones and environmental signals, differentiate and begin axonal and dendritic growth. The migration process begins shortly after the end of neurogenesis and is completed until birth both in humans and rodents (Chen et al., 2017; Stiles & Jernigan, 2010).

After the formation of branches, neurons interact with each other to form synapses. In humans, synaptogenesis begins in gestational week 20 (GD15 in rodents) and consists of biochemical and morphological changes in neurons (Semple et al., 2013). During this developmental stage there is an exuberant production of synapses, followed by a period of synaptic pruning and cell death (Rice & Barone, 2000). Apoptosis begins after neurogenesis, but the pruning of synapses is predominantly a postnatal event in both humans and animals (Kuan et al., 2000). Both mechanisms refine the CNS, since not all neurons reach the right location and not all synapses are correctly formed. These processes explain the need of an excessive production of both neurons and synapses in order to ensure the correct formation of circuits and cell populations. During neurodevelopment the formation of synapses is based on received signals; however, at adulthood synapses result of experiences and are important for memory and learning (Semple et al., 2013; Stiles & Jernigan, 2010).

The refinement of neuronal circuits during development is orchestrated by microglial cells. These cells derive from the yolk sac myeloid progenitors and infiltrate the CNS at embryonic day 10.5 in rodents (GW3 in humans) (Ginhoux et al., 2013). Once in the parenchyma, microglia start proliferating throughout the CNS. Microglial cells are responsible for the pruning of synapses and phagocytosis of newborn cells, highlighting the importance of the immune system in brain development and function (Konishi et al., 2019; Paolicelli et al., 2011; Schafer et al., 2012; VanRyzin et al., 2020)

All these stages have a specific period of action called critical window, which is crucial for adult brain and behavior programming. During these windows the brain acquires plasticity making it responsive to intrinsic and extrinsic factors. However, this plasticity is dependent on brain region, temporal action and if the signal reaches the CNS. Brain plasticity is modified by experience and has an important role in synaptic refinement and therefore, in CNS development. Neurodevelopmental events occur throughout the brain in different time points, since not all regions are formed simultaneously, meaning that the vulnerability period is different between regions. In the absence or presence of environmental cues, the brain will have different neuronal circuits, which will modulate brain function and, consequently, behavior (Meredith, 2015).

Since all brain regions establish connections with each other, any alteration in an area may affect the entire neuronal circuit, resulting in behavioral abnormalities. For example, enriched environments have a positive impact on function and morphology of the limbic system, which is responsible for social and emotional functions. On the other hand, early life stress or neurotoxic molecules can be responsible for the impairment of brain function and behavioral phenotype, and also for the emergence of psychopathologies at adulthood (Baud & Berkane, 2019; Kolb et al., 2017; Patel et al., 2019; Soares-Cunha et al., 2018).

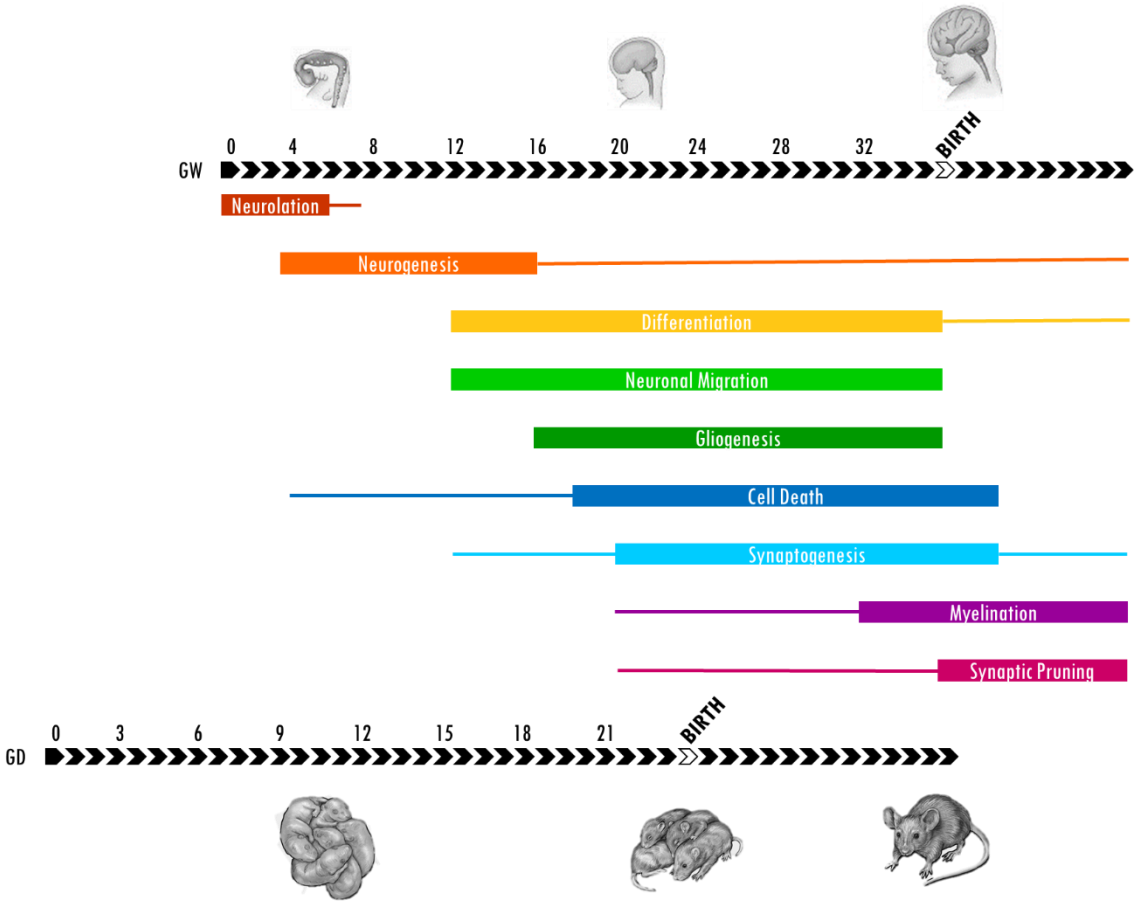

Figure 1: Timeline of Neurodevelopment Events in Humans and in Rodents. The brain undergoes several processes during neurodevelopment, which are similar in humans and rodents. (GW – Gestational Week; GD – Gestational Day) The figures were adapted and modified from Rice & Barone, 2000; Semple et al., 2013; https://sites.duke.edu/apep/module-5-alcohol-and-babies/explore-more/normal-brain-development/ ; https://www.the-scientist.com/features/prenatal-exposure-to-cannabis-affects-the-developing-brain-65230

1.2 Prenatal Stress and Glucocorticoids

i. Early Life Stress and Long-Term Consequences

As mentioned in the previous section, early life stress can have a severe impact in the neurobiology and development of CNS, but also on adulthood behavior (Lupien et al., 2009; Weinstock, 2001, 2017).

There are several types of early-life stress, such as maternal separation, physical and emotional abuse, traumatic experiences and pharmacological administration of substances, namely glucocorticoids (GC) (Hodes et al., 2015).

Many studies have already reported the crosslink between maternal stress (during or after gestation) and the increased risk of the offspring developing neuropsychiatric disorders such as anxiety, depression and addiction at adulthood (Chapman et al., 2004; Heim & Nemeroff, 2001; Van den Bergh et al., 2008).

ii. Respiratory Distress Syndrome and Treatment

Lung development is a process that starts in the first weeks of gestation and continues postnatally (DiFiore & Wilson, 1994). During the third trimester (last gestational week in rodents), endogenous GC stimulate the production of surfactant, important to decrease air-liquid tension in the alveoli (Bolt et al., 2001; Mesquita et al., 2009). However, in the case of preterm pregnancy, the quantity of surfactant is not enough for the lungs to adapt to the extra-uterine life, so the fetus becomes in risk of respiratory distress syndrome development or death (Bolt et al., 2001). In these cases, one of the drugs administered is Dexamethasone (DEX), an exogenous GC, that is given to the mothers, in the third trimester, to promote lung maturation and surfactant production in the offspring (Crane et al., 2003).

Under normal circumstances, the placenta expresses the enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) that metabolizes cortisol into cortisone, reducing the amount of stressors that reach the offspring (Clark, 1998). Since DEX is a small, lipophilic, synthetic GC, it is not metabolized by 11β-HSD2 and can easily reach the fetus' brain of and induce life-long alterations (Clark, 1998).

The use of exogenous glucocorticoids during gestation has an impact on the progeny neurodevelopment and behavior by increasing the risk of neuroendocrine, neurobiological and behavioral alterations at adulthood (Lupien et al., 2009; Mesquita

et al., 2009). Developmental programming through stress is dependent on the time and duration of exposure, but also the type and dosage of GC used (Mesquita et al., 2009).

Long exposure to GCs leads to overactivation of glucocorticoid receptors (GRs), that will permanently affect the Hypothalamus- Pituitary- Adrenal (HPA) axis function, and in turn have an impact on other brain regions such as the paraventricular nucleus (PVN), prefrontal cortex (PFC), hippocampus and amygdala in humans (Charmandari et al., 2005; Diorio et al., 1993) therefore, increasing the susceptibility for mood disorders.

iii. HPA Axis and Glucocorticoids Mechanism of Action

HPA axis activation is a fundamental stress response system (Juruena, 2014), that starts in the PVN of the hypothalamus. Here, corticotropin-releasing hormone (CRH) is formed and released to the blood stream. CRH acts in the anterior pituitary gland by stimulating the production of adrenocorticotropic hormone (ACTH). In turn, ACTH stimulates the adrenal glands to produce corticosteroids (mineralocorticoids or glucocorticoids) that are released systemically.

Corticosteroids are adrenal steroid hormones, that modulate the negative feedback of the HPA axis, in order to stop the stress response (**Figure 2**) (Charmandari et al., 2005; Herman et al., 2012; Ramamoorthy & Cidlowski, 2016), having two types of receptors: mineralocorticoid receptors (MR) and GRs. Both MR and GR can be found in the cytoplasm, bound to the membrane or in mitochondria. MR are expressed mainly in the hypothalamus and can bind glucocorticoids (cortisol in humans or corticosterone in rodents) and mineralocorticoids (aldosterone) (Koning et al., 2019). On the other hand, GRs have less affinity for cortisol and are ubiquitously distributed in the brain, being more predominant in the hippocampus, hypothalamus, nucleus accumbens and cerebral cortex (Fietta et al., 2007; Matthews, 2001).

In the cytoplasm, corticosteroid receptors are bound to heat shock proteins (hsp) to maintain stability, but when GCs enter the cell and bind to the receptors, the hsp dissociate. The receptors then dimerize and translocate to the nucleus to bind to the GC response elements (GRE), in the DNA, and act as transcription factors (**Figure 3**) (Ramamoorthy & Cidlowski, 2016). However, GRs can also have non-genomic effects by binding to factors that mediate anti-inflammatory responses such as Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB), a protein complex that

mediates DNA transcription and cytokine production (Gray et al., 2017; Herman et al., 2012). Besides molecular effects, GCs' impact on neuronal differentiation, growth and survival can also modulate behavior, mood, cognition and memory (Fietta et al., 2007).

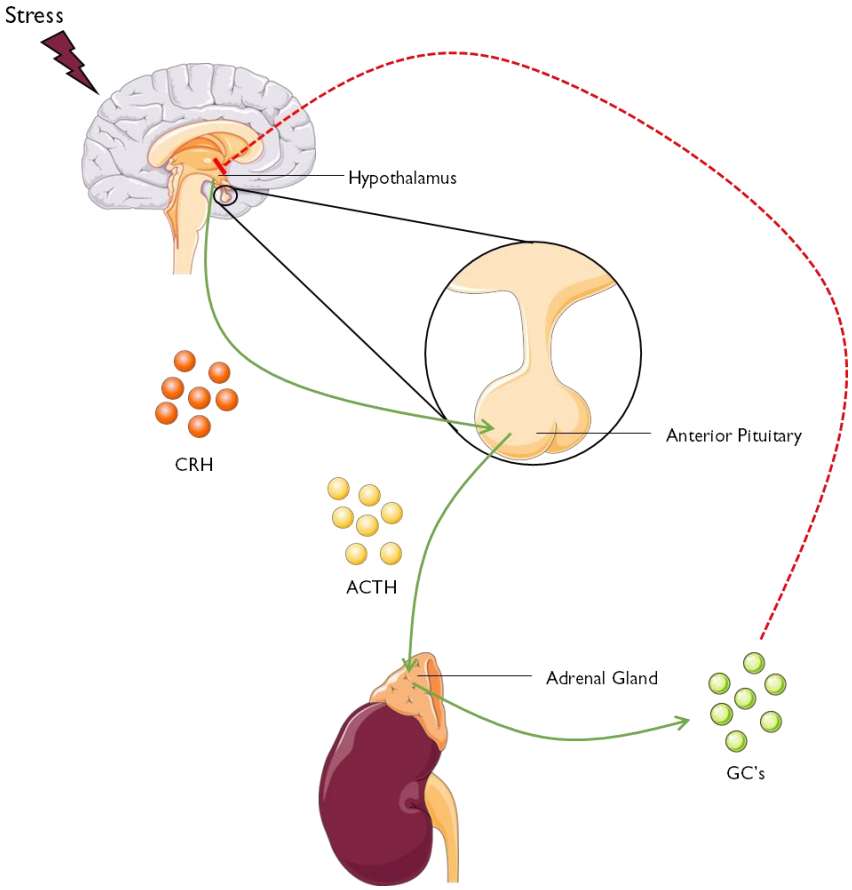

Figure 2: Hypothalamic-Pituitary Axis. A stressor activates the production of CRH by the PVN, which in turn activates the production and release of ACTH by the anterior pituitary gland into the blood stream. The adrenal glands are activated by ACTH and release cortisol that by negative feedback inhibits the PVN response. (CRH - Corticotropin-Releasing Hormone; ACTH - Adrenocorticotropic Hormone; GCs – Glucocorticoids) The figure was constructed using Servier Medical Art (https://smart.servier.com/)

Synthetic GCs have a similar mechanism to natural GCs. However, they have more affinity for GRs, different levels of efficacy, potency and response duration (Fietta et al., 2009). It is thought that early life stress caused by DEX modulates the fetus' HPA axis, through increased levels of GCs, that impair the negative feedback, and lead to alterations in CNS normal function, in both humans and animal models (Charil et al, 2010; Moisiadis & Matthews, 2014).

A dysfunction in the HPA axis has been correlated with mood, cognitive and neuropsychiatric disorders in humans (Moisiadis & Matthews, 2014; Wardenaar et al.,

2011). Studies in humans, have shown that treatment with synthetic GCs induces molecular and cellular alterations, such as a decrease in neurogenesis, reduced volume of certain brain areas (e.g. PFC), and anxiety at adulthood (Fietta et al., 2009; Pryce et al., 2011).

Studies in rodents showed that antenatal corticotherapy with DEX displayed an impact on behavior and HPA axis responsiveness (Oliveira et al., 2006). Furthermore, DEX modulated male sexual behavior, and also induced neurochemical, morphological, and molecular changes in the bed nucleus of stria terminalis (BNST; region that controls neuroendocrine and behavioral responses) (Oliveira et al., 2011, 2012). Furthermore, our group, showed that in utero exposure to DEX, causes anxious-like behavior at adulthood in both sexes and cognitive deficits in females, which are paralleled with a sex specific remodeling of microglia morphology in the PFC (Caetano et al., 2017) and in the dorsal hippocampus (Duarte et al., 2019).

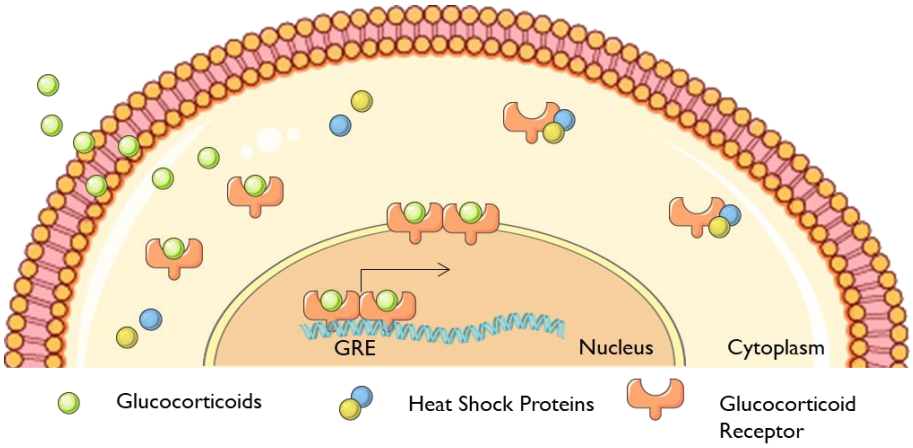

Figure 3: Glucocorticoids mechanism of action. Glucocorticoids enter the cell by diffusion and bind to GR. Once dimerized, the receptor translocates to the nucleus and bind to GRE to induce epigenetic responses. The figure was constructed using Servier Medical Art (https://smart.servier.com/)

1.3 Prefrontal Cortex and Neuropsychiatric Disorders – Impact of Sex and Stress

i. Prefrontal Cortex – Development, Function and impact of Stress

The PFC is a brain region responsible for executive functions such as working memory, planning, regulation of emotions, decision making and attention shifting (**Figure 4**) (Kesner & Churchwell, 2011; Tsujimoto, 2008). The maturation process of this region starts *in utero* and ends at early adulthood, meaning the PFC is highly susceptible to early-life stress events. Consequences in the PFC include structural, neurochemical and functional abnormalities in neuronal circuits, that ultimately lead to behavioral alterations at adulthood (Kolb et al., 2012).

The PFC establishes connections with other limbic areas like the hippocampus and amygdala; together they form neuronal circuits that regulate the negative and positive feedback of the HPA axis, and therefore, promoting or dampening anxiety (Patel et al., 2019; Sigurdsson & Duvarci, 2016; Tovote et al., 2015).

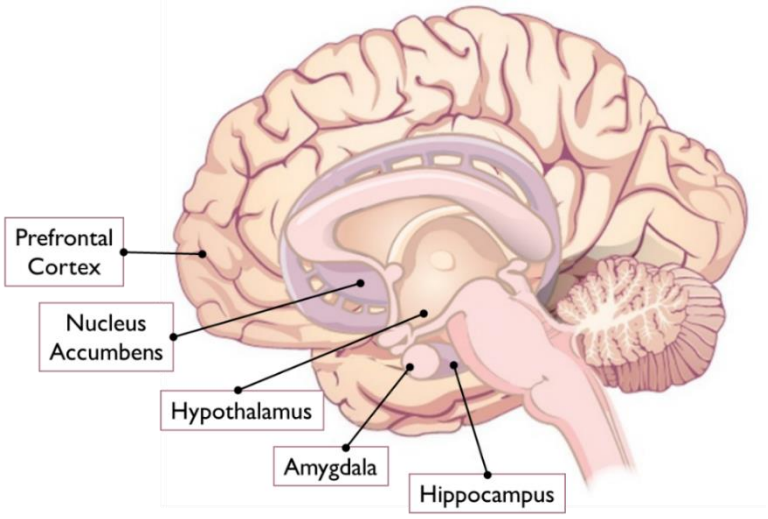

Figure 4: Location of the PFC and other relevant limbic structures for anxiety and depression in the human brain. The figures were adapted and modified from https://courses.lumenlearning.com/wmopen-psychology/chapter/reading-the-limbic-system-and-other-brain-areas/

Under stress, the GCs released by the adrenal gland bind to GRs present in the amygdala, thereby activating this region to perform positive feedback directly on the PVN, making this a crucial response for survival. The high content GCs will also activate the GRs located in the PVN, medial prefrontal cortex (mPFC) and ventral hippocampus

(vHip), which will trigger the HPA axis negative feedback (Diorio et al., 1993; Herman et al., 2012; McEwen & Morrison, 2013). Under chronic stress, the feedback mechanisms become dysfunctional and the response is delayed, causing several impairments throughout the CNS (Jankord & Herman, 2008).

An increase in GC content, similar to what happens in anxiety and depression, is associated with PFC dysfunction, which might be correlated with the emotional dysregulation seen in neuropsychiatric patients (Diorio et al., 1993; Herman et al., 2012). Besides, these patients also show a hyporesponsive PFC with decreased volume and a hyperactivated HPA axis (Arnsten, 2009; Godsil et al., 2013; McEwen & Morrison, 2013; Shin et al., 2006).

Hormones have an impact on the cytoarchitecture and function of developing PFC, hence programming the infant brain and adult behavior to differently respond to stimuli (Baudin et al., 2012; Godsil et al., 2013; Kolb et al., 2012; McEwen & Morrison, 2013).

ii. Influence of Sex on Brain Development and Testosterone Mechanism of Action

Endogenous or exogenous signals can change the brain in a similar way as sexual differentiation during brain development (Mesquita et al., 2009). Sexual differentiation will modulate structure, connectivity and neuronal circuits (morphology and neurochemistry) and also program adult behaviors (copulatory, parental and territorial) (Bao & Swaab, 2010; McCarthy et al., 2017). Sex differences begin at conception, by the establishment of the fetus' chromosomes, which determine gender, but also hormone production (Lenz et al., 2012). In rodent males, sexual programming of the brain begins in late gestation (E18), when the first surge of androgens occurs, and ends on the day of birth, with a peak in circulating testosterone (McCarthy et al., 2017).

During critical windows of neurodevelopment, neuronal circuits are organized through sexual hormone exposure and their receptors activity (organizational effects). Moreover, in puberty, gonadal hormones will activate those programmed circuits (activational effects) and induce alterations on adulthood behavior (Phoenix et al., 1959). Any interference during these critical windows will impair brain structure, function and consequently behavior, thereby increasing the risk for the development of neuropsychiatric disorders (Bao & Swaab, 2010).

Testosterone is a steroid hormone, produced in the testis, ovaries or in the adrenal gland cortex (Burger, 2002; Dohle et al., 2003). However, synthesis can also happen in the brain, either by *de novo* production through cholesterol, or by classic steroids that might be present in the CNS (Mellon et al., 2001). Moreover, testosterone can either be reduced by 5-α reductase in Dihydrotestosterone, or metabolized by aromatase in estradiol (Durdiakova, Ostatnikova, & Celec, 2011).

Peripheral testosterone can be found bound to stabilizing hormones (globulins or albumin), or in a free form, which can cross the blood brain barrier and enter the brain. Once inside the cytoplasm, testosterone binds to androgen receptors (AR), which disconnect the hsp that previously ensured its stability (Bennett et al., 2010). To induce genomic effects, AR have to dimerize and translocate to the nucleus, where they bind to androgen responsive elements (ARE) and induce or repress transcription of genes involved in proliferation, differentiation, metabolism and apoptosis (Durdiakova et al., 2011; Harris et al. 2009). Androgens can also modulate brain structure, cognition and emotion through non-genomic mechanisms (cell birth, death and migration; differentiation of circuits) (Matsuda et al., 2012; McCarthy et al., 2017).

Initially, it was thought that estrogen receptors (ER) were responsible for brain masculinization, due to testosterone aromatization (Lenz et al., 2012). This theory was supported by the presence of α-fetoprotein in the placenta, which binds maternal and fetal estrogens to prevent their presence in the brain; however, they do not have high affinity for estrogens (Konkle & McCarthy, 2011; McCarthy et al., 2017). Therefore, ER activity could not be accounted for several sex differences in brain development and behavior, highlighting the role of AR in this process (Sato et al., 2004; Wallen, 2005; Zuloaga et al., 2008).

Feminization, is a default process, meaning it occurs in the absence of testosterone. Nonetheless, females are highly sensitive to the action of sexual hormones and have a broader critical window of action (Konkle & McCarthy, 2011; McCarthy et al., 2018). Authors have already shown that injecting female mice with testosterone during brain development will masculinize volume and density of cells in the BNST, but also male behavior in guinea pigs, regardless of the chromosomes (Hisasue et al., 2010; Phoenix et al., 1959). This androgenization in females can be a key factor for explaining the sex dimorphism observed in neuropsychiatric disorders.

iii. Neuropsychiatric Disorders: Effect of Sex and Stress

Unveiling sex differences and how they influence brain development and behavior is very important to understand the gender dimorphism observed in prevalence, symptoms and treatment of neuropsychiatric and neurodevelopmental disorders (Turano et al., 2018).

Mood and anxiety disorders are the most common mental health problems in the modern world (Craske & Stein, 2016; Schmidt et al., 2018) and studies showed that patients with anxiety have a high probability of developing depression (Bekker & van Mens-Verhulst, 2007). Developmental neuropsychiatric disorders like autism, cerebral palsy or schizophrenia are more prevalent in men, which might reflect the programming effects of hormones during brain development. However, late onset disorders, such as anxiety and depression are twice more common in women, probably due to the effects of circulating hormones or the impact of the environment (Bao & Swaab, 2010; Steel et al., 2014).

Endogenous or exogenous exposure to testosterone, in mice, was showed to decrease anxious-like behavior, in a time and dose dependent manner, highlighting the protective effect of this hormone (Aikey et al., 2002). It was also demonstrated that a single injection of testosterone at adulthood was not able of reverting anxiety, but continuous administration had an anxiolytic effect (Fernández-Guasti & Martínez-Mota, 2005). Furthermore, Hodosy and colleagues found that when flutamide, an antagonist of the AR, was administered in male Wistar rats, it also had an anxiolytic effect (Hodosy et al., 2012). These studies demonstrate that the protective role of testosterone still remains to be fully elucidated.

A dysregulation in the hormonal balance, for example induced by prenatal stress, causes abnormalities in fetal development, increasing the risk of neuropsychiatric disorders (Baud & Berkane, 2019). This highlights that the equilibrium of the hormonal milieu is a key factor for proper brain development and behavior programming. Many neuropsychiatric disorders as chronic anxiety (Chapter 1.2), have been linked to sex differences (Chapter 1.3), but also with changes in microglia cells (Lenz et al., 2012; Turano et al., 2018), which will be addressed in the next chapter (Chapter 1.4).

1.4 Microglia and Behavior – Impact of Stress and Sex

i. Microglia Function: Effect of Sexual Differentiation and Stress

As previously mentioned, throughout neurodevelopment microglia participate in several processes such as neuronal maturation, survival and death, synaptogenesis, synaptic pruning, neuronal circuit programing, phagocytosis of cellular debris and apoptosis (**Figure 5**) (Cristovão et al., 2014; Paolicelli et al., 2011; Schafer et al., 2012; Schafer & Stevens, 2015).

A hallmark of microglia is their ability to change morphology, which in turn is associated with their function (Kreisel et al., 2014). In a resting/surveilling state, microglia scan the CNS through their highly dynamic ramifications, which are constantly extending and retracting their processes to ensure homeostasis (Nimmerjahn, 2005). When responding to injury, microglia ramifications become small and stout, changing to an amoeboid phenotype (Salter & Stevens, 2017).

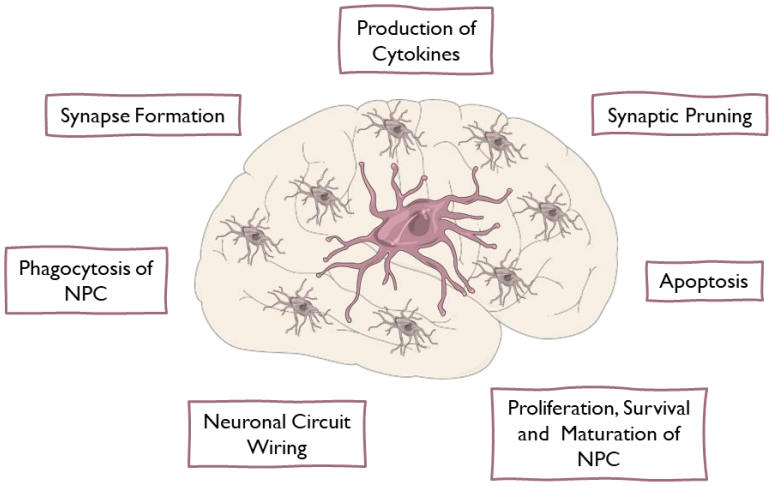

Figure 5: Microglia functions during development in the CNS. These immune cells are responsible for proper CNS development and homeostasis maintenance by surveilling the parenchyma. (NPC – Neuronal Precursor Cells) The figure was constructed using Servier Medical Art (https://smart.servier.com/)

Microglia phenotype is dependent on brain region, age, sex and homeostatic conditions (Schwarz et al., 2012). In physiological situations, Schwarz and colleagues showed that, at postnatal day 0 (PND0) the CA1 region and dentate gyrus of the hippocampus, parietal cortex and amygdala of males and females presented no differences in microglia morphology; however, at PND4 the organizational male peak of testosterone induces an increase in the number of male microglia in the same regions.

During adolescence (PND30), microglia develops ramifications and a shift occurs in the number/morphology of microglia, meaning females present more microglia with an amoeboid shape, an effect maintained until the beginning of adulthood (PND60). Our group also showed that, at PND90 females present microglia with longer ramifications and higher number of processes, when compared with males, in the PFC (Caetano et al., 2017).

These sentinels are influenced by chromosomes, gonadal hormones and environmental cues, highlighting their importance in establishing sex differences in brain development and behavior (VanRyzin et al., 2020). Microglia are both targets and modulators of sexual differentiation, since sexual hormone exposure during development changes microglial function, number and morphology. These alterations will consequently modulate neuronal circuit formation, synaptic plasticity, brain architecture and connectivity in a sex-specific manner (Lenz et al., 2013; Lenz & McCarthy, 2015; Sierra et al., 2008).

Due to microglia's function and plasticity, they are very vulnerable to stress and hormonal imbalance (Bilbo & Schwarz, 2012; Bollinger et al., 2016; Thion & Garel, 2017). Stress can impair microglia morphology and function, but also change behavior and circuits, which contribute to cognitive, memory and learning impairments (Hanamsagar & Bilbo, 2017; Tay et al., 2017; Zhan et al., 2014), as well as increased risk of neuropsychiatric disorders (Mesquita et al., 2009) such as depression (Yirmiya et al., 2015) and anxiety (Caetano et al., 2017). Studies from our group suggest that there is a link between microglia morphology/function and behavior, in both PFC (Caetano et al. 2017) and hippocampus (Duarte et al., 2019) that are sex-specific and can be modulated by a A_{2A} receptor ($A_{2A}R$) antagonist.

ii. Microglia Remodeling by A_{2A} Receptors

Adenosine is a neuromodulator that controls neuronal circuit response (Ribeiro et al., 2002). Its effects are mediated via four widespread metabotropic G-protein-coupled receptors (Borea et al., 2018), that present different densities during development and between brain regions (Ribeiro et al., 2002). Adenosine is produced and released by all cells of the CNS, including microglia, and its action relies on the balance between inhibitory A1 receptors and enhancing $A_{2A}Rs$, which mainly act on excitatory glutamatergic synapses (Fredholm et al., 2005). These receptors are involved

in controlling microglia dynamics, since their activation promotes microglia atrophy (**Figure 6**) (Gyoneva et al., 2009; Orr et al., 2009). Activation of the A_{2A}Rs occurs in the presence of high levels of adenosine and due to its prime synaptic location, it plays a role in synaptic plasticity and transmission (Sebastião & Ribeiro, 2009).

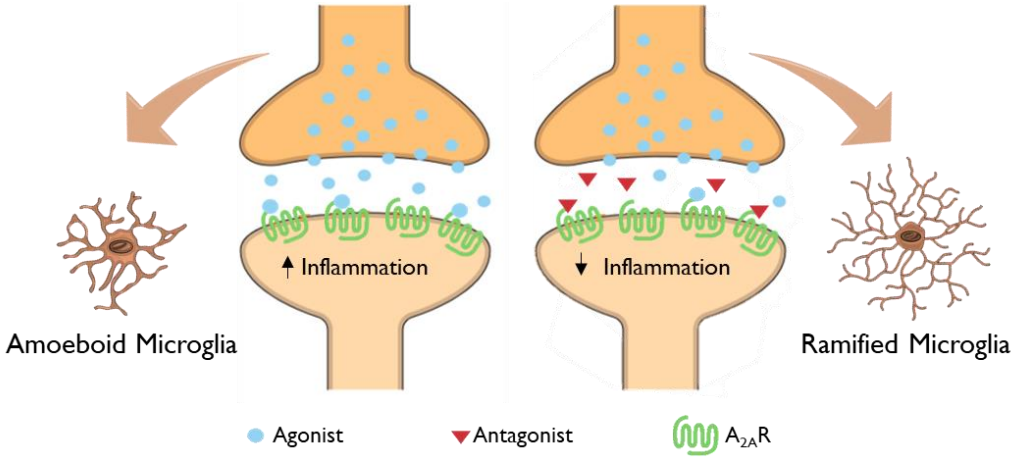

Figure 6: Microglia Modulation by Adenosine A_{2A} Receptor. Adenosine activates A_{2A}R, which induces a change of microglia morphology from ramified to amoeboid. However, when A_{2A}R are blocked, microglia activation is reduced. The figure was constructed using Mind the Graph pictures (https://mindthegraph.com/)

A_{2A}Rs are described has been involved in several brain disorders such as epilepsy, depression, anxiety and schizophrenia (Gomes et al., 2011). Neuropsychiatric disorders are correlated with glial activation and synaptic dysfunction in brain regions such as the PFC and hippocampus (Duman & Aghajanian, 2012; Rial et al., 2016). Adenosine receptors arise as possible candidates to normalize these effects, due to their synaptic location, but also because the modulation of these receptors was shown to have an impact on behavior of animal models (Cunha, 2008; George et al., 2015; Rebola et al., 2011).

Pharmacological blockade of A_{2A}R was demonstrated to have antidepressive effects, observed by an increased escape time in the tail suspension and forced swimming tests (El Yacoubi et al., 2001). Caffeine, an A_{2A}R antagonist, when consumed in moderate doses, reduces anxious and depression-like behaviors, but also normalizes synaptic function, in rodents subjected to chronic unpredictable stress (Kaster et al., 2015). This study demonstrated that A_{2A}R blockade had a prophylactic as well as therapeutical effect (Kaster et al., 2015) and therefore, reduced the risk of neuropsychiatric disorders.

The impact of A_{2A}Rs on anxiety was showed by our group, through the administration of the experimental anxiolytic SCH58261 (A_{2A}R antagonist), which

recovers cognitive deficits in females (Duarte et al., 2019), but also the anxious-like behavior of male rats prenatally exposed to DEX (Caetano et al., 2017).

Stress is a factor that modulates microglia function and morphology (Sierra et al., 2008). The blockage of $A_{2A}R$ in microglia, appears as a candidate to normalize microglia morphology, controlling its proliferation and neuroinflammatory profile (George et al., 2015; Gomes et al., 2013). A study showed that repeated stress in rodents induced morphological changes in microglia, which in turn modulated the response to stress and anxious-like behavior (Kreisel et al., 2014). Furthermore, our group showed that prenatal stress, induced by DEX exposure during pregnancy, causes both anxious-like behavior and microglia morphology remodeling in a sex-specific manner in the PFC at adulthood (female microglia is atrophied when exposed DEX, whereas males have a hyper ramified microglia), but treatment with SCH58261 reverts these effects only in males (Caetano et al., 2017). We also showed that knockout of the $A_{2A}R$ in healthy mice does not change the sex differences seen in physiology, but instead increases the complexity of female microglia from the PFC (Simões-Henriques et al., 2020).

Knowing that microglia is essential for brain sexual differentiation and environmental signals response (Bollinger et al., 2016; VanRyzin et al., 2020), we hypothesize that these cells under stress could impair CNS development in a sex specific manner and therefore, underlie anxious-like behavior at adulthood. Furthermore, we suggest the different response to DEX and SCH58261, is due to the permissive effect of androgens. The present thesis will address this premise.

2

Impact of Neonatal Testosterone Injection on Females Prenatally Exposed to DEX: Focus on Behavior And Microglia Morphology

2.1 Rationale and Aims

Hormones, such as GCs or androgens are known to have an impact on the CNS, namely by programming brain development and behavior (McCarthy et al., 2017). In this work we focused on two steroid hormones, DEX and testosterone.

Previous studies from our group have shown that both male and female rats exposed to DEX *in utero* present an anxious-like behavior at adulthood (PND90), and differences in the morphology of microglia in the PFC and hippocampus (Caetano et al., 2017; Duarte et al., 2019). However, when an experimental anxiolytic (SCH58261) is administered at adulthood, only males revert both behavior and microglial morphology to physiological conditions.

Animals exposed to glucocorticoids during the third trimester of the pregnancy are prone to develop chronic anxiety. Since females have a higher prevalence of mood and anxiety disorders, sex differences must be considered. Sexual differentiation begins in development, when males experience a peak of testosterone on the day of birth, which is responsible for programming the male brain and adult behavior. In the present study, we aim to dissect if masculinizing DEX female offspring with testosterone, makes females permissive to the anxiolytic treatment.

Taking these factors into account, the main aims of this study are:

- ➔ To assess neurodevelopmental behavior and evaluate if any early alteration induced by DEX could be used as a predictive factor for anxiety at adulthood;
- ➔ To unravel if testosterone administration at birth makes females prenatally exposed to dexamethasone permissive to anxiolytic treatment at adulthood;
- ➔ To appraise if testosterone can reshape microglia morphology of females prenatally exposed to DEX and treated with SCH58261, making them resemble males;
- ➔ To consider sex dimorphism in all tasks.

2.2 Experimental Work

i. Animals

All procedures with animals were approved by the Animal Welfare Committee of the Faculty of Medicine of the University of Coimbra and performed in agreement with the EU guidelines (EU Directive 2010/63/EU) and Portuguese laws (Decreto-Lei 113/2013; Decreto-Lei 1/2019). Pregnant Wistar Han rats were purchased from Charles River (Barcelona, Spain). Animals were housed with controlled temperature, humidity, light and *ad libitum* access to food and water.

A. Pharmacological Treatment

Wistar Han dams with 3 months of age, were arbitrarily assigned to CTRL or DEX groups. The dams were injected subcutaneously with 1mg/kg Dexamethasone (in sesame oil) at GD18 and 19 to induce an anxious phenotype in the progeny, whereas control animals were injected with vehicle. This dose of GC was already reported and demonstrated to induce an anxious-like behavior in the offspring at adulthood (Caetano et al., 2017). At PND0 female offspring were injected subcutaneously with 100μg of Testosterone Propionate in 25μL of peanut oil (or only with vehicle, depending on the group), to mimic the peak of testosterone that occurs in males (Hisasue et al., 2010). Weight was monitored from PND0 until PND90.

From PND5 until PND17 animals were subjected to a battery of behavior tests to assess proper neurodevelopment such as vestibular system development, locomotion, discriminatory ability, strength and physical milestones of development as auditory response and day of eye opening.

Weaning was made at PND21 and rats were distributed into 5 groups: CTRL, CTRL+TEST, DEX+TEST, CTRL+TEST+SCH, DEX+TEST+SCH.

Twenty-one days before the progeny reached PND90, offspring were administered daily with the experimental anxiolytic SCH58261 (0,1mg/kg/day) via intraperitoneal injection, whereas the other groups were injected with vehicle (DMSO in PBS). At PND90 the open field test (OPF), was performed to evaluate locomotion, and elevated plus maze test (EPM) to analyze anxious-like behavior. After the behavioral analysis rats were sacrificed. **Figure 7** summarizes the attainment of the model, the time points of pharmacological administrations and behavioral assessment.

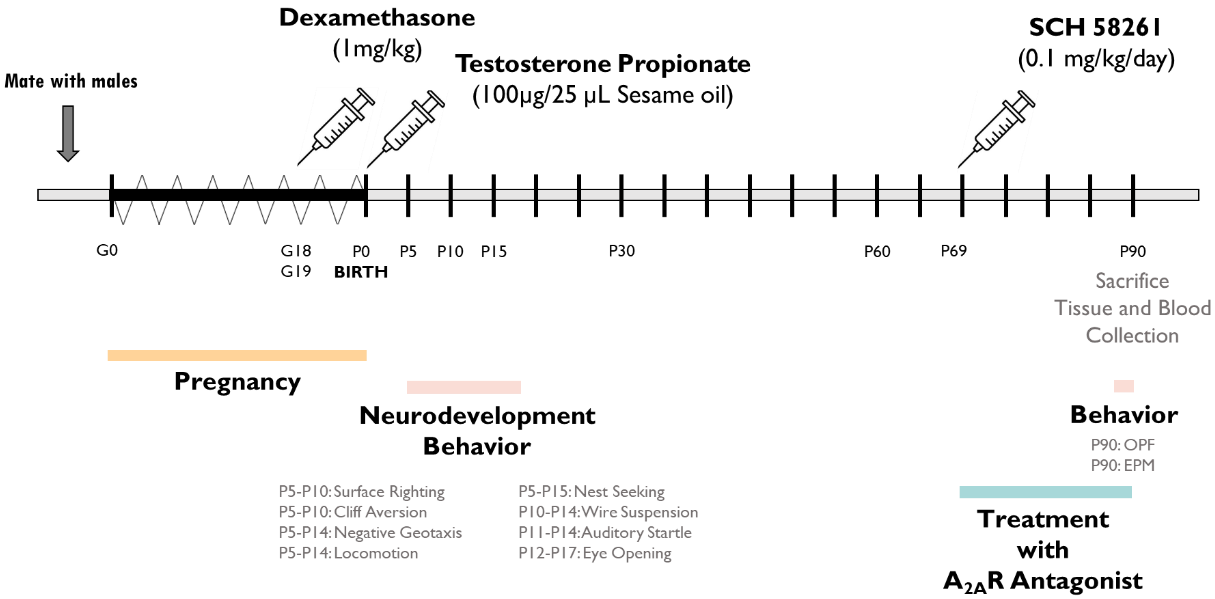

Figure 7: Timeline of the procedures with the animal model. Pregnant Wistar dams were injected with DEX (1mg/g) during the third trimester of gestation. Female offspring were injected with 100 μg of Testosterone to induce brain masculinization. From PND5 to PND17 neurodevelopmental behavior was analyzed. For 21 consecutive days, before PND90, animals were administered with 0,1 mg/kg of SCH58261. At PND 90 locomotion and anxious-like behavior were evaluated after which animals were sacrificed.

ii. Behavior

A. Neurodevelopmental Tests

Between PND5 and PND17 offspring neurodevelopment was assessed in CTRL, DEX, CTRL+T and DEX+T groups (**Figure 8**). These tests comprise: Surface Righting Reflex, Cliff aversion and Negative Geotaxis Reaction to evaluate the vestibular system development; Locomotion for motor development; Nest seeking for olfactory and discriminatory ability; Wire Suspension for upper limb strength; Auditory Startle and Eye Opening as physical milestones of neurodevelopment. Pups were separated from their mothers for a brief period of time in order to avoid low body heat or prolonged stress. All tests were performed between 9:00h and 16:00h.

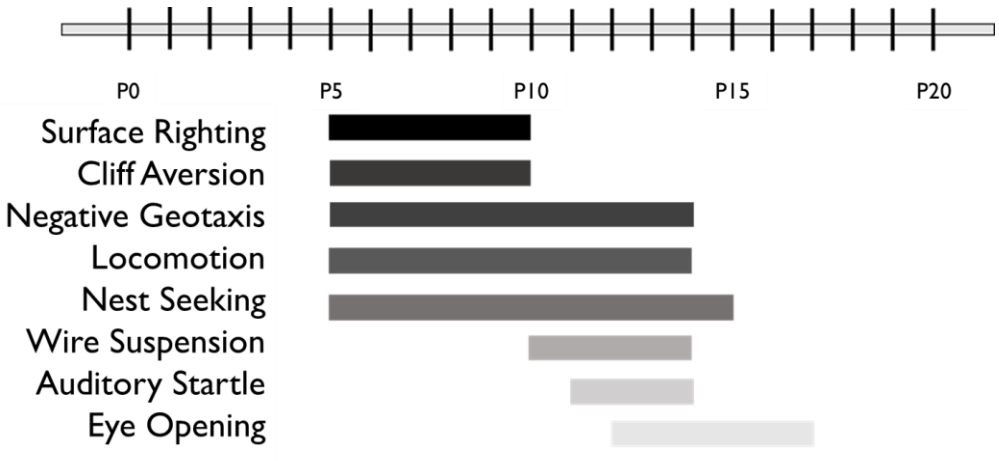

Figure 8: Timeline of Neurodevelopment tests. A battery of tests was performed from PND5 until PND17. In order to evaluate physical milestones, upper limb strength, the vestibular system development and olfactory discrimination.

a. Surface Righting Reflex

The Surface Righting was performed between PND5-PND10 and consists on placing the pup in supine position and counting the amount of time (seconds-s) the pup takes to return to prone position (**Figure 9**). The time limit of the test was 5 s. Since this test aims to the evaluate spatial orientation, a delayed response might suggest vestibular system impairments (Baharnoori et al., 2012; VanRyzin et al., 2016).

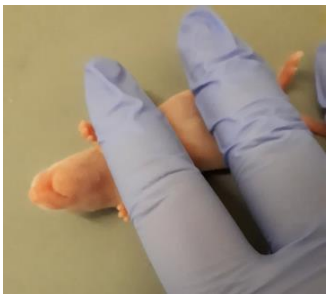

Figure 9: Surface righting reflex test. Pups are placed on their back and the time(s) spent to turn over is evaluated.

b. Cliff Aversion

The Cliff Aversion test is performed from PND5 to PND10. In this test the pup is placed on the edge of a cliff with their snout and forepaws hanging down and the time (s) it takes the animal to go to a safe position is counted (**Figure 10**). The cut-off time

of the test was 30 seconds. The aim of the test is to analyze spatial orientation and vestibular imbalance (Baharnoori et al., 2012; VanRyzin et al., 2016).

Figure 10: Cliff Aversion test. Pups were placed on the edge of an elevated flat surface and the time (s) the animal takes to retract with its snout and forepaws was assessed.

c. Negative Geotaxis Reaction

In the Negative Geotaxis Reaction, the pup is placed on a hill with a 35° inclination with their head facing down. The time (s) the animal takes to rotate 180° is evaluated, within a maximum of 30 s (**Figure 11**). The aim of this test is to evaluate spatial orientation, balance and head position; features related to the vestibular system (Baharnoori et al., 2012).

Figure 11: Negative Geotaxis Reaction test. Animals were placed on a hill facing down and the time (s) taken to perform a 180° turn was evaluated.

d. Nest Seeking

The Nest Seeking test is performed from PND5 to PND15 and it evaluates the animal's ability to discriminate beddings through olfaction. (**Figure 12**). The arena is divided into three sections: the middle zone where the animal is placed, and the sections of home and fresh beddings located on opposite sides. The pup must complete the goal within a maximum of 120 s. The test is performed twice to ensure the animal's choice is based on smell, meaning the latency to goal score is the mean of both attempts.

Between trials animals are facing opposite sides of the arena, but always perpendicular to the beddings. A negative score was given when the animal chooses the fresh bedding instead of the mother's nest (VanRyzin et al., 2016). Between tests the arena is cleaned with 10% ethanol.

Figure 12: Nest Seeking test. **Nest Seeking test**. The pup is placed in the middle of an arena and must choose between their mothers' nest or fresh bedding. The test is performed twice and the time(s) it takes the animal to reach the bedding is evaluated.

e. Locomotion

Locomotion test is performed between PND5 and PND14 and analyzes motor development. The pup is placed in the middle of a circle (flat surface), with 13cm of diameter and the animal must leave the circle with their four paws within 30 s (**Figure 13**) (VanRyzin et al., 2016).

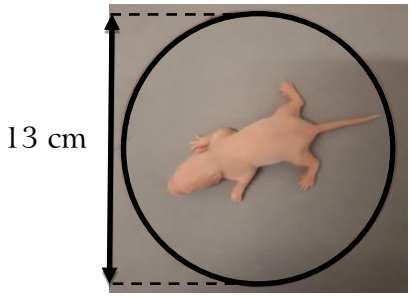

Figure 13: Locomotion. The offspring is put in the middle of a circle and the time the animal takes to exit the circle with its four limbs is recorded.

f. Wire Suspension

The Wire Suspension test is performed between PND10 and PND14. In this test the animal is paced on a wire, which he can only grab with his front paws (**Figure 14**). The time (s) the animal is able to hold on to the wire is assessed within a maximum of 10 s. The goal of this test is to evaluate upper limb strength. (VanRyzin et al., 2016).

Figure 14: Wire Suspension test. The pup was placed on top of a wire, only holding with its front paws. The time(s) the animal was able to hang on was counted.

g. Auditory Startle

The auditory startle test goal is to analyze the development of the auditory system. The animal is subjected to a loud noise (e.g. finger snap) and a positive auditory response is counted if the pup startles. The test was performed between PND11 and PND14 (Baharnoori et al., 2012).

h. Eye Opening

The day of pup eye opening was assessed (**Figure 15**). The pup is observed and the day in which the eye is completely open is recorded. This test was performed between PND12 and PND17.

Figure 15: Eye Opening analysis. The analysis of the opening of the eyes was made between PND12 and PND17.

B. Behavior at Adulthood

Females from CTRL, CTRL+T, CTRL+T+SCH, DEX+T and DEX+T+SCH were subjected to behavior analysis at adulthood (PND90). On the day of the test the animals were left in the behavior room for 1 hour before behavioral assessment

(habituation phase). All experiments were performed and recorded between 9:00h and 16:00h and under dim red light. The arena was cleaned with 10% ethanol between each animal.

a. Open Field

The open field test was performed on PND90 and consisted on placing the animals in the center of an arena and leave them to explore it for 5 minutes (**Figure 16**). The animals are placed in the arena always facing the same side. This test was used to assess locomotion in order to avoid bias on the EPM test. The total distance travelled(m), and mean speed(m/s) were the parameters analyzed with the ANY-Maze software.

This test was also used to assess offspring anxious-like behavior, by evaluating time spent in the center of the arena. On the test day the animals in SCH58261 group were not injected with the anxiolytic, since it likely modulates behavior.

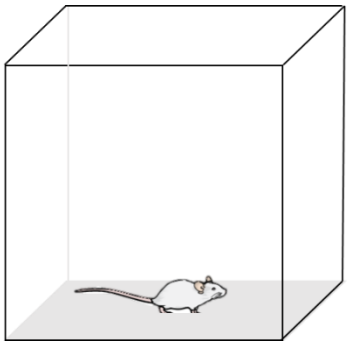

Figure 16: Open Field Test. The animal is placed in the center of an arena and is left to explore for 5 minutes. Locomotion is evaluated through the assessment of the distance travelled (meters-m) and the mean speed(m/s).

b. Elevated Plus Maze

The Elevated Plus Maze was performed at PND90 to evaluate rat anxious-like behavior. The test consists on placing the animal in the center of an arena with two closed and two open arms, allowing the animal to explore the maze for 5 minutes (**Figure 17**). Although rodents prefer closed and dark places, they also have an innate ability to explore new environments, meaning that animals without an anxious behavior are driven to explore the arena, instead of remaining still in one place. The time (s) spent in open arms and the number of open arm entries were evaluated through the Observer software.

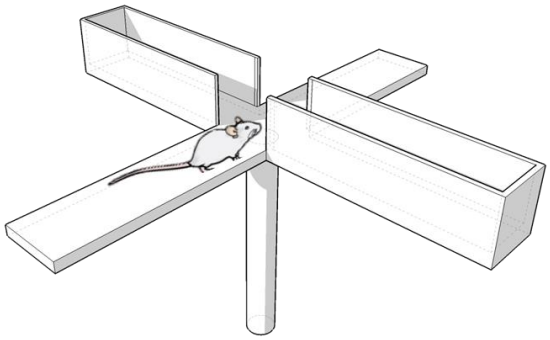

Figure 17: Elevated Plus Maze. Test to evaluate anxious-like behavior by placing an animal in the middle of an elevated arena with two closed and two open arms. Figure adapted from: http://neurobau.com/behavior/elevated-plus-maze.html

iii. Estrous Cycle

Female rodents are spontaneous ovulators with a 4-6 day cycle. In humans, the peak of estradiol and progesterone do not overlap; however, in rodents there is a synchronization of both peaks, leading to a smaller cycle. The rat estrous cycle is composed of 4 stages: Estrus, Proestrus, Metestrus and Diestrus. These phases result on concomitant cellular, behavioral and hormonal alterations. Cellular modifications are based in the absence or presence of certain types of cells, their density and morphology (Cora et al., 2015; Westwood, 2008). Drugs and chemicals are known to interfere with the reproductive function of rodents, and several authors consider the evaluation of the estrous cycle a measure of the functional status of the HPA axis (Goldman et al., 2007; Yuan & Foley, 2002).

The Estrus stage is considered the nonbreeding season, where the epithelial cells are non-nucleated, cornified, flat and irregular. During Proestrus females are receptive to males and vagina's cell morphology is composed of round proliferating epithelial cells. Metestrus is the stage where the reproductive organs begin to change and the cells stop proliferating, however there are a lot of cell debris. The Diestrus is considered the end of the breeding season where the organs prepare for the reception of the embryo, this phase is characterized by lymphocyte infiltration (Westwood, 2008).

Female Wistar Han offspring aged 90 days were subjected to a vaginal smear, made with an inoculating loop soaked in phosphate buffer saline 1x (PBS 1x) (137 mM NaCl, 2.1 mM KCl, 1.8 mM KH_2PO_4 and 10 mM Na_2HPO_4, at pH 7.4). After collecting the cells, they were placed in microscope slides, which were then fixed and stored in

96% Ethanol until observation in the light microscope LEICA DM 4000B (Leica, Wetzlar, Germany) with the 10 x objective lens (Plan 10x/0.25 PH1).

iv. Celular and Molecular Procedures

A. ELISA kit

a. Sample Preparation

Female offspring aged 90 days were anesthetized with 3% Isoflurane and a heart puncture was performed. Blood was then centrifuged at 16,000xg for 10 minutes at 4°C. The supernatant was collected and stored at -80°C until use. Before initiating the ELISA protocol, reagents were equilibrated in room temperature for at least 30 minutes. All reagents were prepared in conformity with the protocol provided by the manufacturer's (Abcam, Cambridge, United Kingdom). All reagents used are listed in **Table 3**.

b. Corticosterone Analysis

Samples were diluted 1:100 in 1x Diluent M. The standard curve was made with the Corticosterone Standard vial, which was then diluted 5 times to obtain different concentrations. All standard and serum samples were placed into the wells after which the Biotinylated Corticosterone was added. The multiwell was incubated for 2 hours at room temperature (RT). Each well was washed five times with 1x Wash Buffer. The 1x SP Conjugate was added and left to incubate for 30 minutes at RT. Another set of five washes was made. The wells were left to incubate for 25 minutes with the Chromogen Substrate after which Stop solution was added and absorbance measured at 450 nm and 570 nm.

c. Testosterone Analysis

Samples were defrosted and stirred gently for 5 minutes. Standards, controls and samples were placed in the wells after which Testosterone-HRP conjugate was added and left to incubate in the dark for 1 hour at 37°C. The wells were aspirated and washed 3 times. The samples were incubated with TMB substrate solution for 15 minutes at 37°C in the dark. Stop solution was added and absorbance was measured at 450 nm and 620 nm.

B. Immunohistochemistry

a. Sample Preparation

Rats with 90 days were anesthetized with 100mg/mL Ketamine (80 mg/kg) and 20 mg/mL Xylazine (5 mg/kg). Transcardiac perfusion was made with PBS 1x followed by 4% paraformaldehyde (PFA) in PBS 1x. Brains were dissected and preserved in 4% PFA for 24 hours. After this period, PFA was removed and the brains placed in 30% Sucrose for 48 hours. Subsequently, the solution was discarded and the brains were freezed in dry ice and stored in -80°C until use.

b. Cryosections Preparation

The PFC cryosections with 30 μm thickness, were obtained with a cryostat (Leica CM3050S, Germany) and stored in cryopreservation buffer (30% sucrose, 30% ethylene glycol, 10mM PBS, pH=7.2). Slices were maintained in 4°C until use.

c. Free- Floating Immunohistochemistry

Cryosectioned slices from the PFC, located at stereotaxic region interaural 6.02mm and bregma 2.22mm (Paxinos & Franklin, 2001) were washed 3x with PBS 1x for 10 minutes each, in mild agitation. Cryosections were incubated with blocking solution (5% BSA 0,1% Triton X in PBS 1x) for 2 hours at RT. The cryosections were incubated with the primary antibody (**Table 1**) for 48 hours at 4°C. Another set of 3 washes was made with PBS 1x, followed by incubation with the secondary antibody (**Table 1**) for 2 hours at RT, in mild agitation. During the incubation, slices were maintained in the dark covered with aluminum foil. After washing the slices three times, they were mounted using DAKO glycergel mounting medium (DAKO, Glostrup, Denmark). All reagents used are listed in **Table 3**.

Table 1: List of antibodies used in immunohistochemistry.

Antibody	Animal	Dilution	Company
Anti- Iba-1	Rabbit	1:1000	Wako
Alexa Fluor 488 Anti-Rabbit	Goat	1:500	Life Technologies

d. Image Acquisition

Images of microglial cells were obtained with the Confocal microscope (Zeiss Confocal Microscope LSM 710 META) connected to ZEN software (Zeiss, Germany). Five Z-stack images of the PFC were obtained per hemisphere per animal (**Figure 18**), with the 63x objective lens (oil immersed, Plan-Apochromat 63x /1.40 Oil DIC M27).

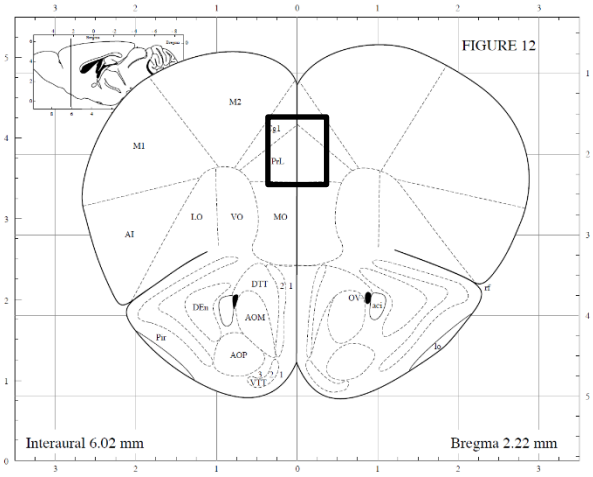

Figure 18: Rat brain section with the PFC region analyzed. Confocal images were taken at the medial PFC represented by the black rectangle (interaural 6.02mm and bregma 2.22mm). Image adapted from (Paxinos & Franklin, 2001).

e. Microglia Tridimensional Reconstruction

Microglia was tridimensionally reconstructed with the Neurolucida software (MBF Biosciences), from the z-stack images previously taken in the Confocal microscope. This allowed to reconstruct microglia processes in all planes and consequently perform quantitative analysis. Five microglia from the PFC were drawn per hemisphere, per animal; however, two slices were analyzed for each animal, meaning 60 cells were reconstructed per condition. Microglia was analyzed according to the

number and length of branches in each order (branched analysis), and also the number and length of processes that intersected consequent radius of 10 μm (sholl analysis). This evaluation was performed with NeuroExplorer software (MBF Biosciences).

v. Statistical Analysis

Statistical analysis was made with the GraphPad Prism 8 (GraphPad, USA). Between groups, differences were analyzed with One-Way ANOVA or with Two-Way ANOVA. Results are represented as mean±standard error of the mean (SEM), and statistical significance was set for $p<0.05$. Outliers were identified and removed when found.

Disclosure:

At adulthood (PND90), data regarding DEX and DEX+SCH experimental groups are not represented in the graphs presented in the Results Section. The animal model of chronic anxiety induced by prenatal exposure to DEX is an unanimously validated model. Nevertheless, the cohort of DEX animals used specifically in this study, did not present the expected anxious-like behavior at adulthood. Possible explanations for this fact will be presented at Discussion section, and the results obtained in this work will be discussed considering previous works from the group.

2.3 Results

i. Animal weight characterization

A. Prenatal DEX and neonatal testosterone have no major impact on offspring weight

To simplify animal nomenclature, from now on, control offspring will be named CTRL, offspring exposed to dexamethasone *in utero* will be designated as DEX, whereas female control offspring exposed to testosterone will be named as CTRL+T and the females exposed to both dexamethasone and testosterone as DEX+T.

Several studies have described a crosstalk between metabolic dysfunctions and neuropsychiatric disorders (Kaidanovich-Beilin et al., 2012), highlighting the importance of weight monitoring. The male and female offspring from CTRL and DEX groups as well as females from CTRL+T and DEX+T groups were weighted (g) between PND0 and PND90.

Analyzing CTRL groups, males show a significant increase in weight from PND35 until PND90 when compared with females; moreover, between females, testosterone increases female weight since PND77 (**Figure 19 A**). In DEX groups, males were heavier than females from PND42 until PND90 and testosterone increased DEX females weight since PND70, when compared with the respective controls (**Figure 19 B**).

Prenatal administration of DEX did not induce changes in weight except for specific time-points in females (P28; P35; P42) (**Figure 19 C**) and masculinized females (P63) (**Figure 19 E**), but not in males (**Figure 19 D**). Discriminated values for the offspring's weight are presented in supplementary data (**Table S1**).

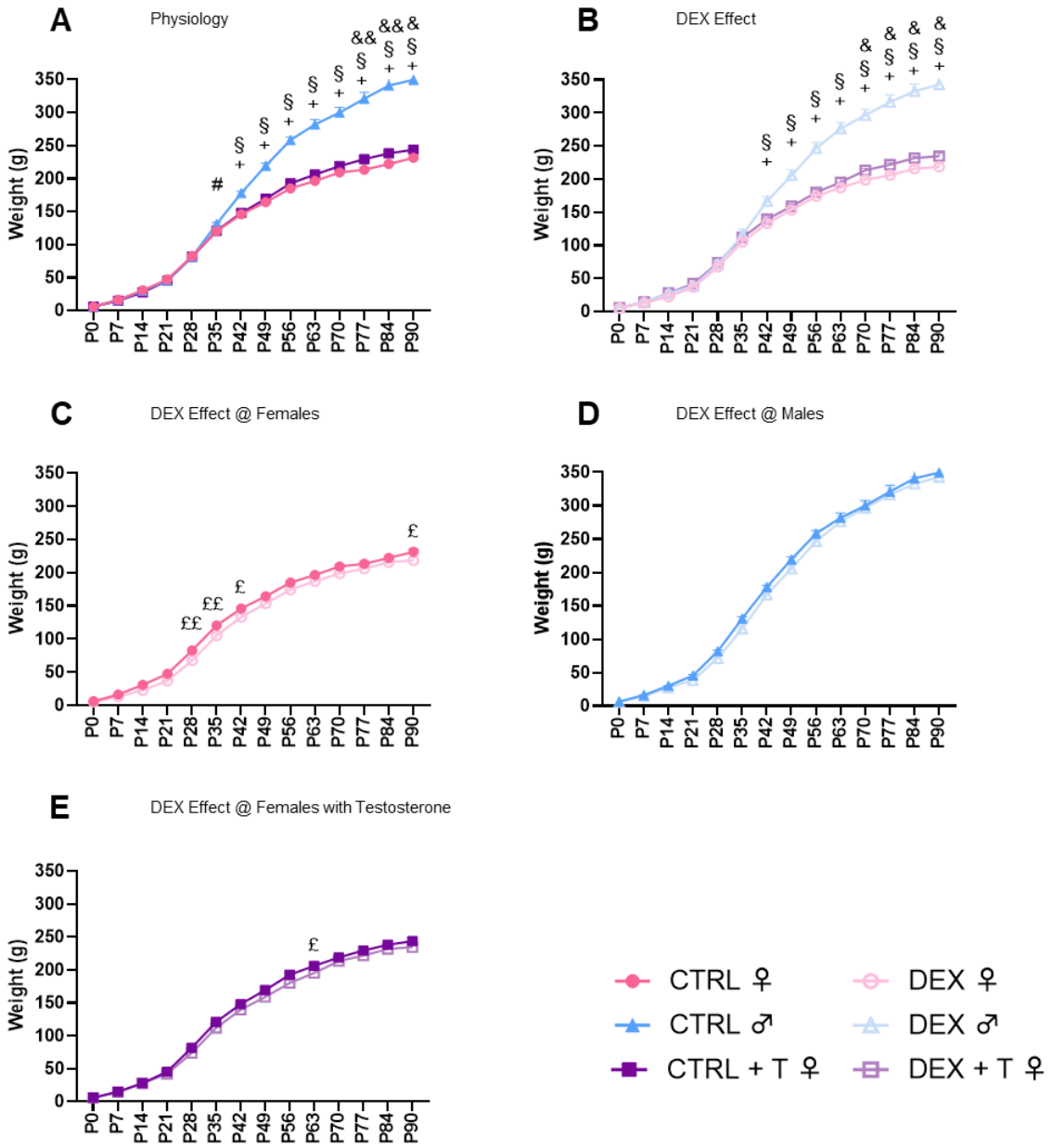

Figure 19: Administration of DEX does not induce weight alterations from PND0 to PND90. The weight (g) of males, females and masculinized females of CTRL and DEX groups are displayed in graphs **A** and **B** respectively. The offspring's weight from CTRL and DEX groups are compared in separate graphs for females (**C**), males (**D**) and masculinized females (**E**). Results are expressed as mean±SEM (n=7-36). Statistical analysis was assessed with the two-way ANOVA test. #p<0.05 males versus females with testosterone; &p<0.05, &&p<0.01 females versus females with testosterone; £p<0.05, ££p<0.01 CTRL versus DEX; +p<0.0001 males versus females; §p<0.0001 males versus females with testosterone. (P – Postnatal Day)

ii. Neurodevelopmental Behavior

Some studies have already showed that early-life stress caused by GC exposure is associated with the development of neuropsychiatric disorders at adulthood (Drozdowicz & Bostwick, 2014; Huang, 2011). Previous studies from our group have shown that both male and female rats exposed to DEX *in utero* present an anxious-like behavior at adulthood (PND90) (Caetano et al., 2017). Aiming to evaluate if any neurodevelopmental alteration induced by DEX could be used as a predictive factor for anxiety at adulthood, we performed a battery of tests to analyze locomotion, vestibular system development, strength, discriminatory ability and physical milestones of neurodevelopment. A group of CTRL and DEX masculinized females was also subject to neurodevelopmental tests, to assess testosterone's effect on programming brain and behavior. Behavioral analysis from male and female CTRL and DEX offspring, but also from females CTRL+T and DEX+T pups was performed between PND5 and PND17.

B. Neither DEX nor Testosterone exposure during development impairs offspring motor development

Locomotion assessment was performed from PND5 until PND14 to evaluate motor development (VanRyzin et al., 2016). In this test, the time a pup takes to exit a circle with all four limbs, within 30 seconds, is analyzed. Regarding CTRL offspring, males and females overall show no locomotor abnormalities, despite of testosterone inducing a delay in females, but only in specific days (PND10 – CTRL♂: 22.13±1.7s; CTRL♀: 23.86±3.8s; CTRL+T♀: 26.69±1.3s| PND12 – CTRL♂: 11.70±1.3s; CTRL♀: 13.71±2.2s; CTRL+T♀: 17.13±1.1s| PND13 – CTRL♂: 9.52±1.6s; CTRL♀: 10.43±2.0s; CTRL+T♀: 15.19±2.0s; #$p<0.05$), as masculinized females were slower exiting the circle (**Figure 20 A**). In DEX groups no differences were observed, except for PND12, where DEX+T female pups were significantly slower than DEX males (**Figure 20 B**). However, these differences were transient, since by PND14 they were no longer detected.

DEX pups have a similar performance to controls; nonetheless, these animals have a tendency to take a longer time completing the task (**Figure 20 C**, **Figure 20 D**, **Figure 20 E**). Discriminated values of locomotion test are in the supplementary data table (**Table S2**).

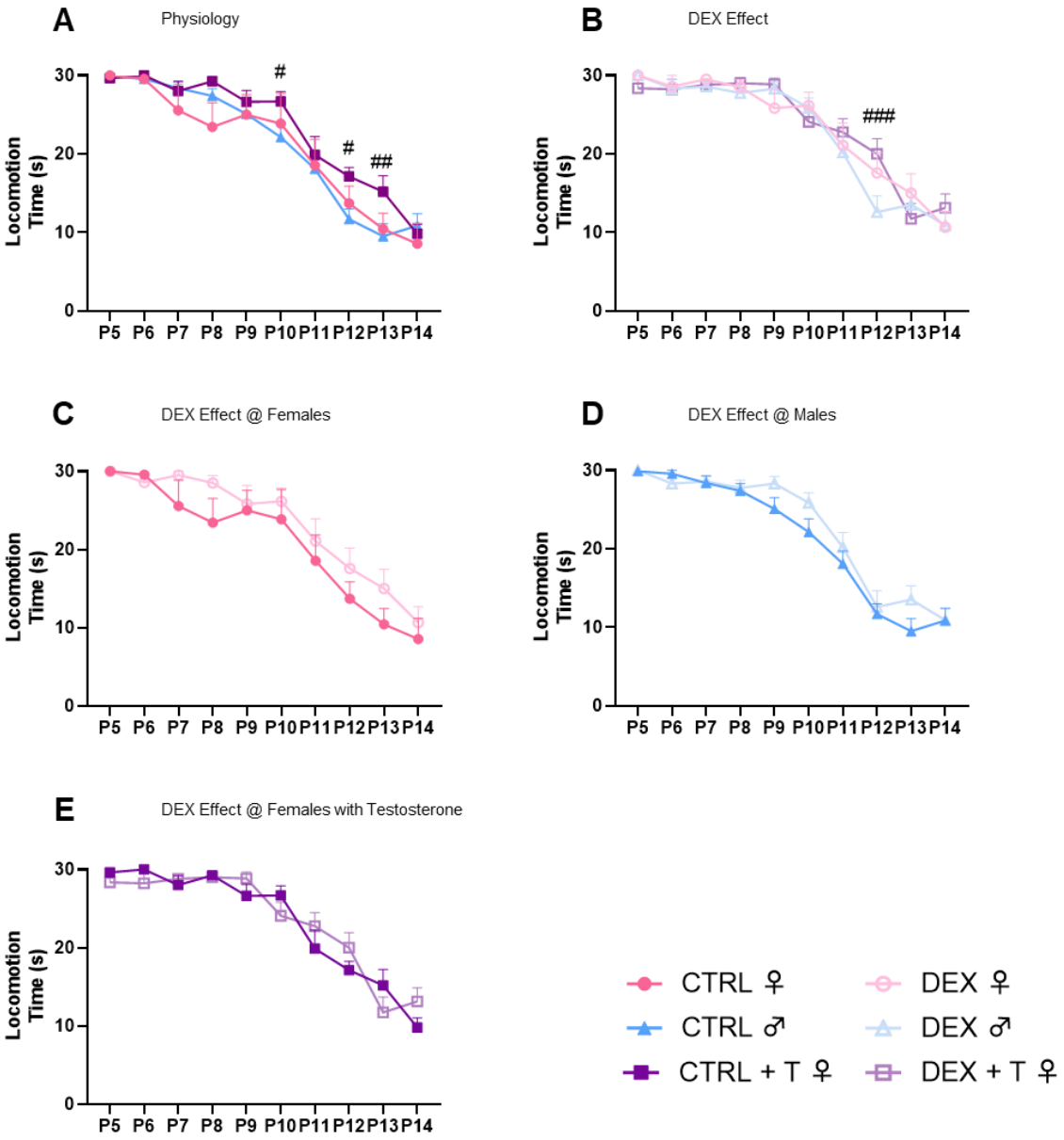

Figure 20: DEX and Testosterone do not induce changes in locomotion. Pups were evaluated in the time(s) they took exiting a defined circle with four limbs from PND10 to PND14. Locomotion from CTRL (**A**) and DEX (**B**) pups was assessed to evaluate motor development. Analysis between CTRL and DEX groups was also made for females (**C**), males (**D**) and females with testosterone (**E**). Results are expressed as mean±SEM (n=7-36). Statistical analysis was assessed with the two-way ANOVA. #p<0.05, ##p<0.01, ###p<0.001 males versus females with testosterone. (P – Postnatal day)

C. Prenatal DEX does not impair vestibular system development whereas neonatal testosterone exposure does not have a masculinization effect

The vestibular system is related to the maintenance of balance and the transmission of this information to the brain. However, some authors have already described an association between vestibular system dysfunction and developmental impairments (Van Hecke et al., 2019). The development of this system can be assessed through several tests, such as surface righting, cliff aversion and negative geotaxis reaction.

In the surface righting reflex, the time (s) pup takes to rotate from supine to prone position is evaluated (Baharnoori et al., 2012). Throughout the days analyzed, no differences were detected between males and females in physiology (**Figure 21 A**). In DEX groups, at PND5 and PND6, females with testosterone had a tendency for delayed performance when compared with males (**Figure 21 B**) (PND5 – DEX♂: 1.4±0.1s; DEX♀: 1.71±0.2s; DEX+T♀: 1.7±0.2s| PND6 – DEX♂: 1.3±0.1s; DEX♀: 1.4±0.1s; DEX+T♀: 1.6±0.1s; #p<0.05). At PND5, DEX females had a tendency to be slower than CTRL females, which was then normalized (**Figure 21 C**). DEX males (**Figure 21 D**) and DEX+T females (**Figure 21 E**) presented no alterations comparing with respective controls.

The cliff aversion test consists on evaluating the time (s) pups take retracting when faced with a cliff (Baharnoori et al., 2012). At PND6, CTRL females were faster than males retracting from the cliff, a behavior delayed by testosterone administration (**Figure 22 A**) (PND6 – CTRL♂: 14.9±2.8s; CTRL♀: 7.4±3.8s; CTRL+T♀: 22.8±2.8s; #p<0.05; &p<0,05). When DEX is administered prenatally, the three groups have a very similar behavior throughout the days analyzed (**Figure 22 B**). Comparing CTRL with DEX groups, no differences were observed in females (**Figure 22 C**), neither in DEX males, even though they have a tendency to be quicker (**Figure 22 D**). In masculinized females, DEX reverted the effect observed in CTRL+T females (**Figure 22 E**) (PND6 – CTRL+T♀: 22.8±2.8s; DEX+T♀: 7.82±7.8s; $p<0.0001).

The negative geotaxis reaction evaluates the latency (s) of the rat to reverse its orientation and face up-wards on an inclined plane within a maximum of 30 seconds (VanRyzin et al., 2016). In CTRL pups, males and females had no differences in behavior, but masculinized females were transiently slower at reversing their orientation at PND7 (**Figure 23 A**) (PND7 – CTRL♂: 14.9±1.5s; CTRL♀: 13.1±3.3s; CTRL+T♀: 19.3±1.9s; #p<0.05; &p<0,05). Similarly to control groups, testosterone in DEX

females induces a delayed response at PND6, when compared with males (**Figure 23 B**). When analyzing CTRL and DEX groups no differences were found between females (**Figure 23 C**), males (**Figure 23 D**) and females administered with testosterone (**Figure 23 E**). Discriminated data from surface righting test (**Table S3**), cliff aversion (**Table S4**) and negative geotaxis reaction test (**Table S5**) are presented in supplementary data.

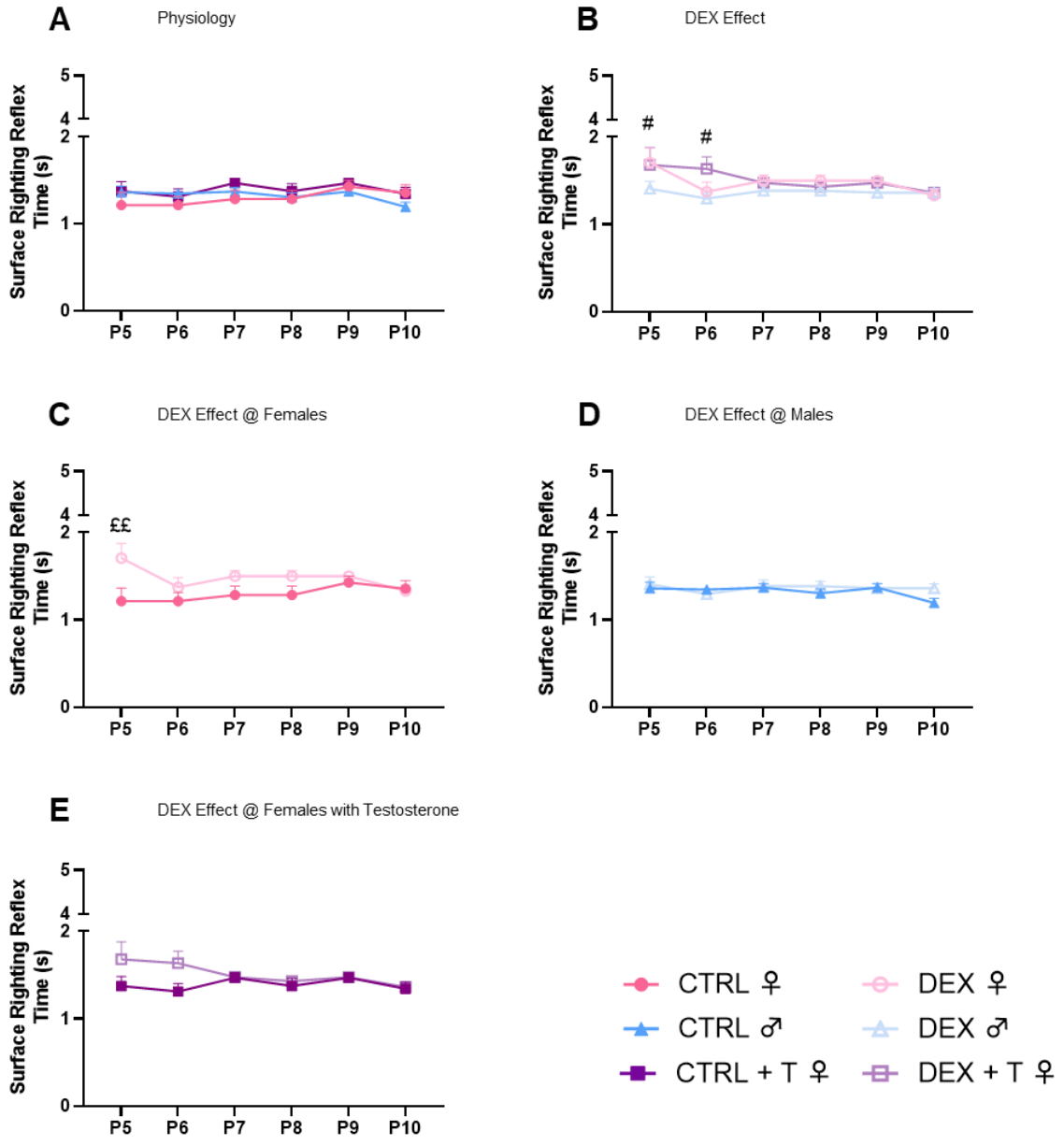

Figure 21: Prenatal DEX and neonatal testosterone administration have no major impact in righting reflex. Surface righting test was assessed from PND5 until PND10 and results represent the time(s) spent to successfully rotate from supine to prone position. In graph **A** CTRL males, females and CTRL+T were analyzed and in **B** the same analysis was made, but for

DEX groups. In graphs **C**, **D**, and **E** there is a comparison between CTRL and DEX females, males and females with testosterone, respectively. Results are expressed as mean±SEM (n=7-36). Statistical analysis was assessed with two-way ANOVA. #p<0.05 CTRL male versus CTRL+T females; ££p<0.01 CTRL versus DEX. (P – Postnatal Day)

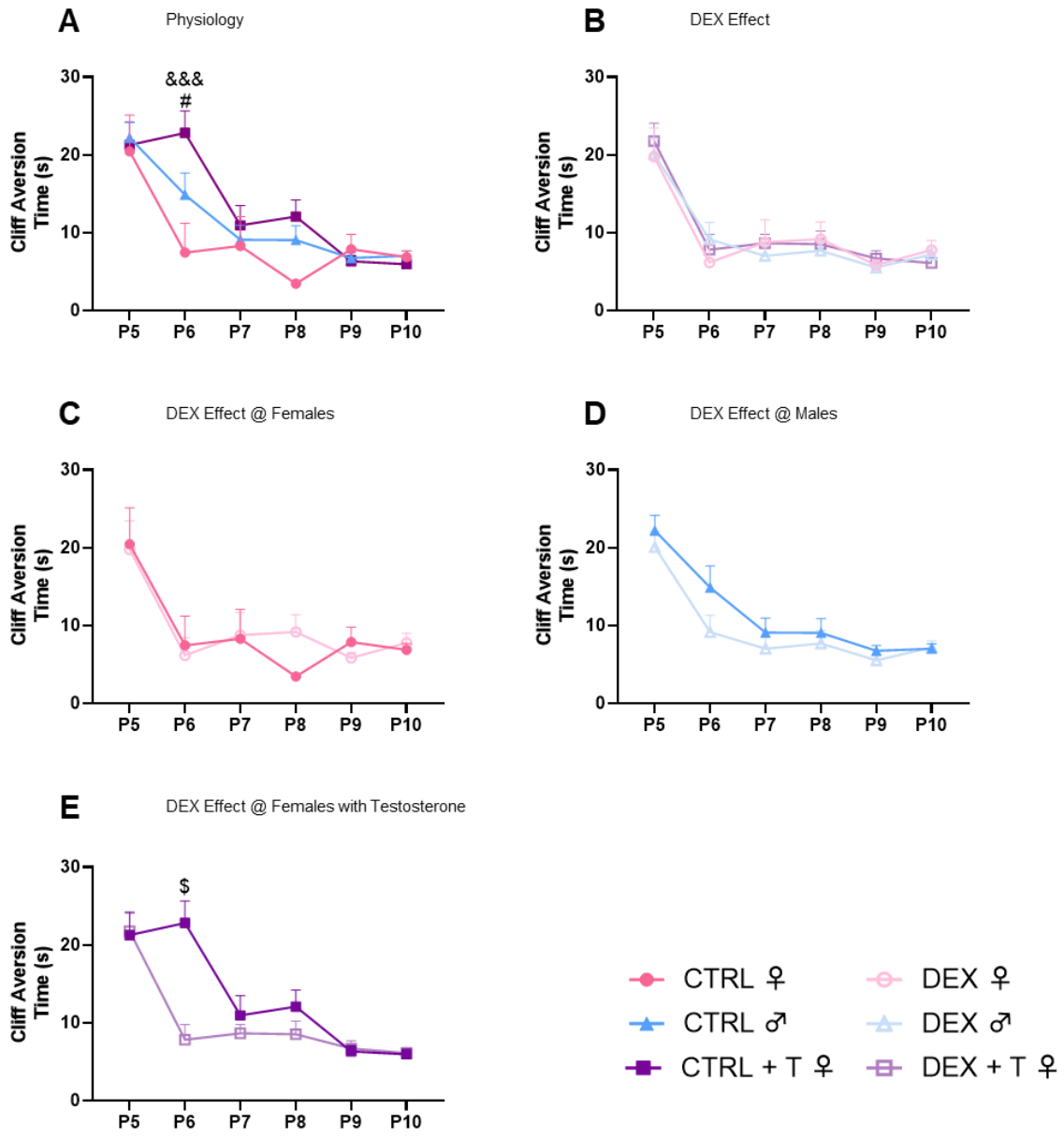

Figure 22: Offspring from DEX groups have no alterations in cliff retracting performance, but DEX reverts the delay induced by Testosterone in females. The cliff aversion test was made between PND5 and PND10, and it evaluates the time(s) pups took to retract from an elevated cliff. CTRL group's results are represented in graph **A**, and DEX group's performance are in graph **B**. CTRL versus DEX group's analysis was made in females (**C**), males (**D**) and masculinized females (**E**). Results are expressed as mean±SEM (n=7-36). Statistical analysis was assessed with two-way ANOVA test. #p<0.05 CTRL male versus CTRL+T females;

&&&p<0.001 CTRL females versus CTRL+T females; $p<0.0001 CTRL versus DEX. (P – Postnatal Day)

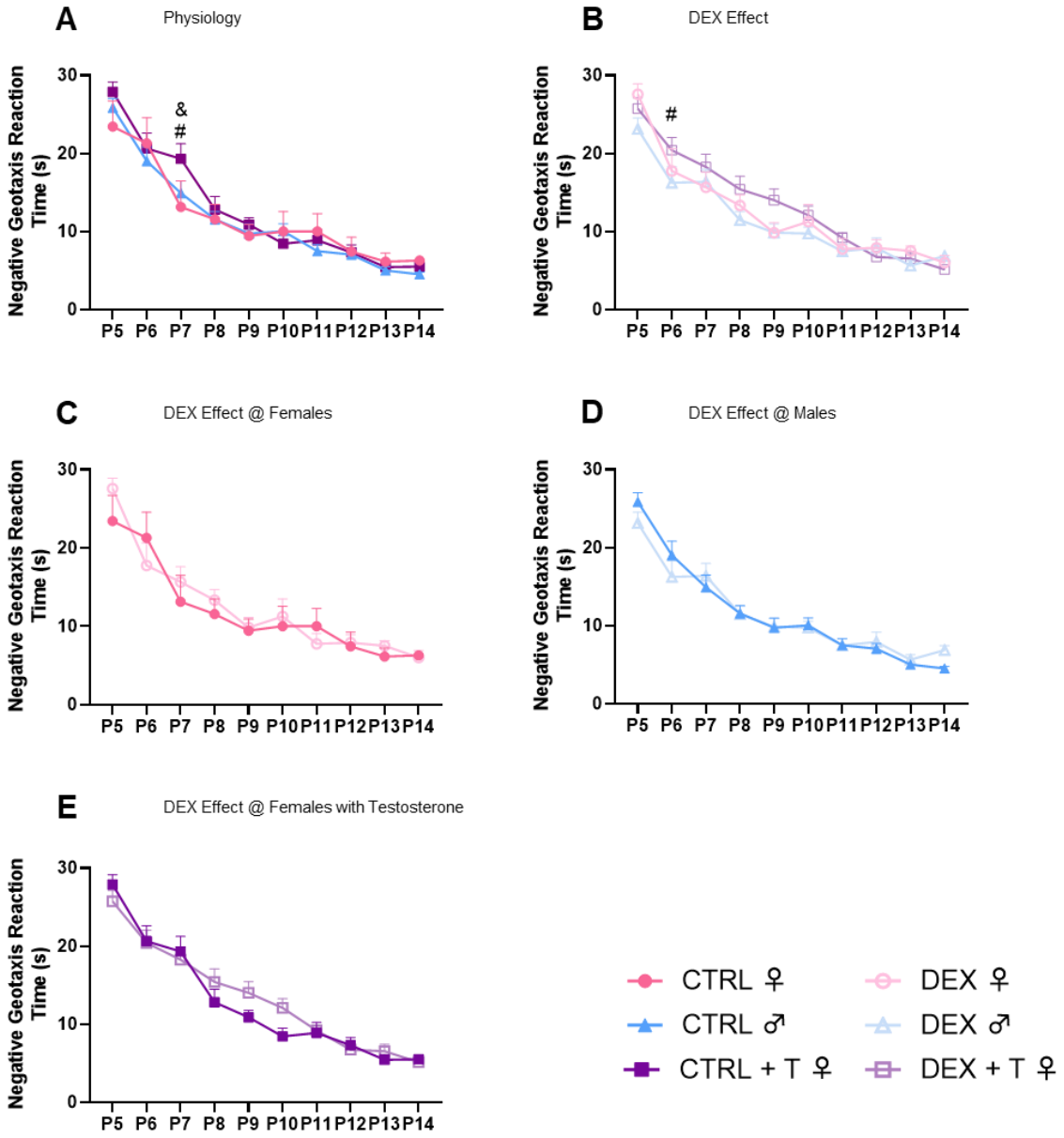

Figure 23: Prenatal exposure to DEX does not impair male and female geotaxis reaction. The negative geotaxis reaction test evaluates the time(s) pups take to rotate 180°on an inclined plane, between PND5 and PND14. In graph **A** and **B** are the CTRL and DEX group's results respectively. In graphs **C**, **D** and **E** a comparison between CTRL and DEX groups was made for females, males and females with testosterone, correspondingly. Results are expressed as mean±SEM (n=7-36). Statistical analysis was assessed with two-way. #p<0.05 CTRL male versus CTRL+T females; &p<0.05 CTRL females versus CTRL+T females. (P – Postnatal Day)

D. Prenatal DEX administration induces transient changes in female offspring discriminatory capacity

In the nest seeking test, the time (s) pups take to choose the mother's nest oppose to new nest is evaluated (VanRyzin et al., 2016). The main aim of this analysis is to understand the discriminatory capacity of the pups, between PND5 and PND15. In CTRL groups transient variations in performance were observed between males and females, but at PND5, testosterone increased time pups took reaching goal bedding (mother's nest), whereas CTRL females were faster (**Figure 24 A**) (PND5 - CTRL♂: 55.11±6.7s; CTRL♀: 29.9±8.3s; CTRL+T♀: 73.78±10.2s; &p<0,05). Contrastingly, at PND6 CTRL+T females reduced the latency to goal and in contrast CTRL females had a worse performance.

When analyzing DEX groups, behavior was very alike, but at PND9 DEX males were slower than DEX+T females (**Figure 24 B**) (PND9 - DEX♂: 54.82±8.7s; DEX♀: 43.46±8.5s; DEX+T♀: 24.84±5.6s; #p<0.05). Comparing DEX and CTRL females, at PND10, CTRL pups were slightly faster however, this response changed throughout the days analyzed (**Figure 24 C**). No differences between CTRL and DEX were found in this test regarding males (**Figure 24 D**) nor females exposed to testosterone (**Figure 24 E**). Raw data are presented in supplementary data (**Table S6**).

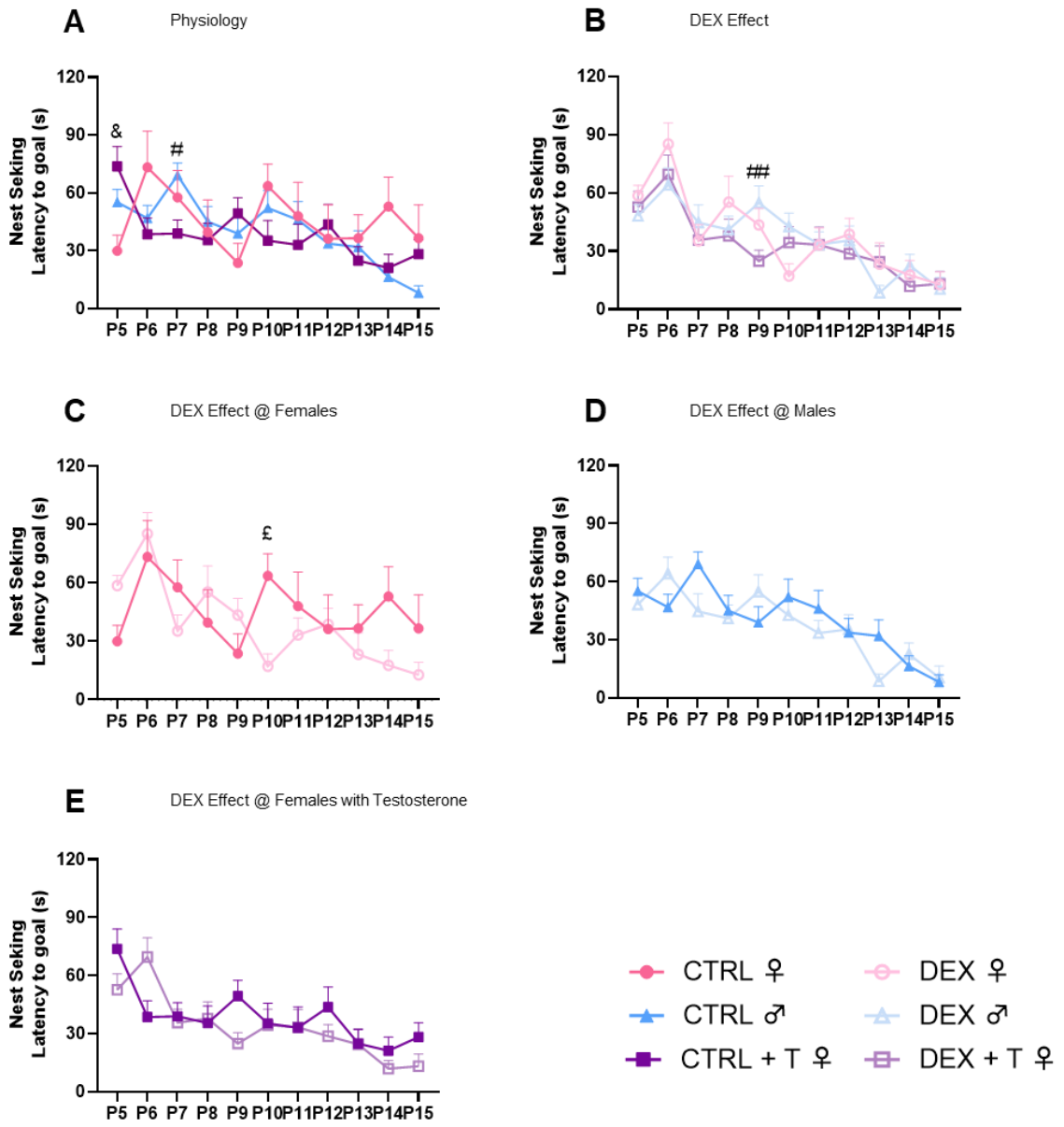

Figure 24: Discriminatory capacity is transiently changed with prenatal Testosterone and DEX administration in both sexes. The discriminatory capacity was evaluated by the nest seeking test, which assesses the time(s) a pup takes to choose familiar nest. This analysis was made between PND5 and PND15 on CTRL (**A**) and DEX groups (**B**). In graphs **C**, **D** and **E** are represented the performance of CTRL and DEX females, males and females with testosterone, respectively. Results are expressed as mean±SEM (n=7-36). Statistical analysis was assessed with two-way ANOVA. #p<0.05, ##p<0.01 males versus females with testosterone; &p<0.05 females versus females with testosterone; £p<0.05 CTRL versus DEX. (P – Postnatal Day)

E. Prenatal DEX impairs male and female strength, whereas neonatal testosterone masculinizes DEX females

In the wire suspension test pups are timed for how long they can stay suspended in a wire only holding with their forepaws (VanRyzin et al., 2016). This test was made between PND10 and PN14 and evaluates upper limb strength. At PND10, both CTRL male and CTRL+T females were able to hold on for a superior period of time when compared with CTRL females, nevertheless at PND11 CTRL females ameliorated their performance when compared with males (**Figure 25 A**) (PND10 - CTRL♂: 8.73±0s; CTRL♀: 8.57±0s; CTRL+T♀: 9.19±0s; *p<0.05; &p<0.05).

In DEX groups, females endured less time than DEX males and DEX+T females on the first days of the test (**Figure 25 B**) (PND10 - DEX♂: 8.73±0s; DEX♀: 6.0±0.0s; DEX+T♀: 9.55±0.0s| PND12 - DEX♂: 9.4±0.3s; DEX♀: 8.08±0.6s; DEX+T♀: 9.77±0.2s| PND13 - DEX♂: 8.86±0.4s; DEX♀: 8.58±0.6s; DEX+T♀: 9.82±0.1s; *p<0.05; &p<0.05; #p<0.05). Between females CTRL and DEX, CTRL pups were capable of staying suspended much longer throughout the days of the test (**Figure 25 C**) (PND10 - CTRL♀: 8.57±0.0s; DEX♀: 6.0±0.0s| PND11 - CTRL♀: 10.0±0.0s; DEX♀: 8.25±0.0s| PND12 - CTRL♀: 10.0±0.0s; DEX♀: 8.08±0.6s; *p<0.05). Regarding males (**Figure 25 D**), DEX pups also presented impaired strength on PND13 (PND13 - CTRL♂: 9.83±0.1s; DEX♂: 8.86±0.4s; £<0.05). Females with testosterone (**Figure 25 E**) had a very similar performance, nonetheless on PND11 the DEX+T group stayed less time suspended than respective CTRL (PND11 – CTRL+T♀: 9.63±0.0s; DEX+T♀: 8.91±0.0s £p<0.05). Discriminated data can be found in the supplementary data section (**Table S7**).

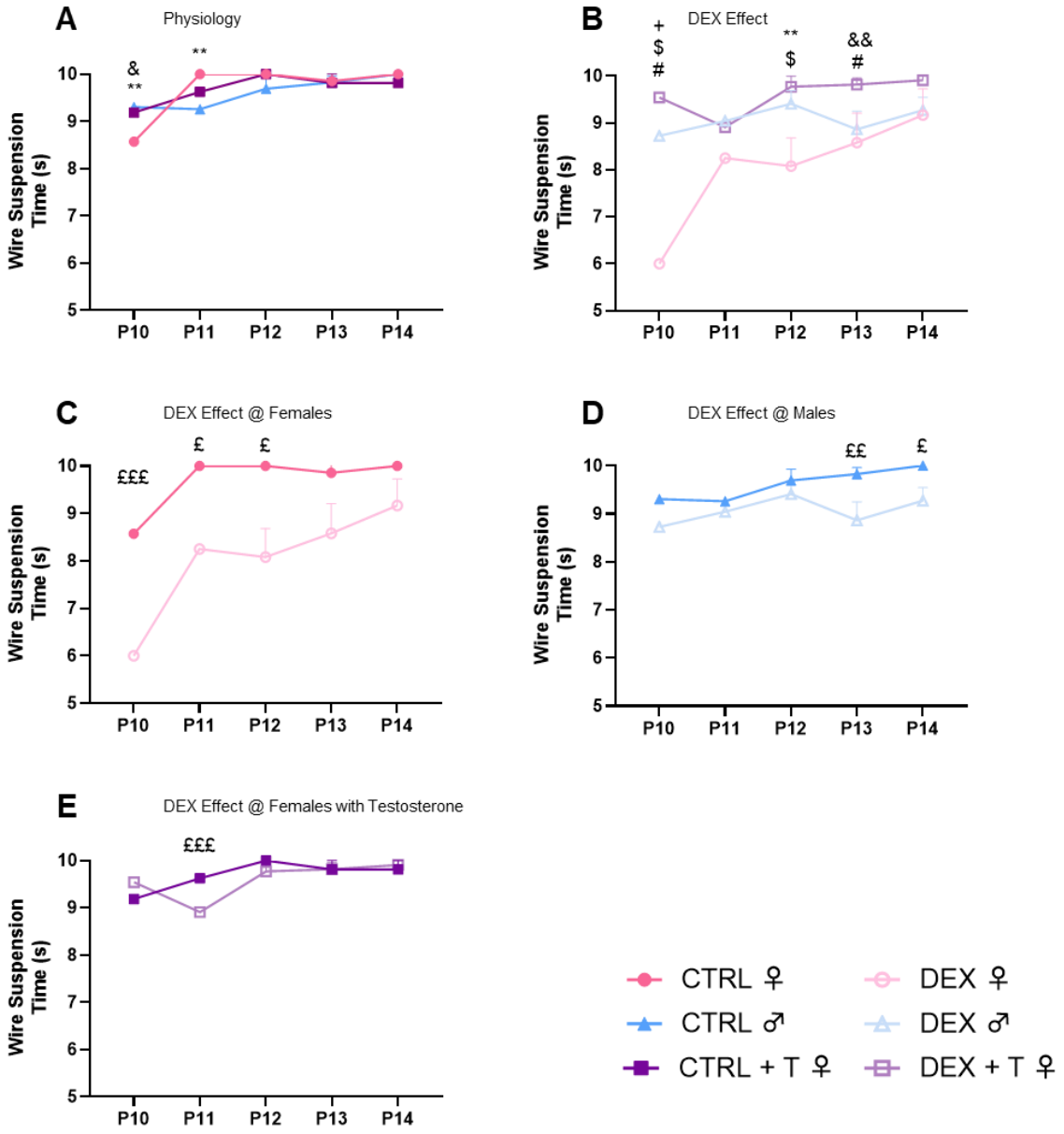

Figure 25: Exposure to DEX during gestation impairs strength of both sexes, an effect reverted by testosterone administration in females. The upper limb strength was assessed through the wire suspension test between PND10 and PND14, by analyzing the time(s) a pup can hold suspended. In graph **A**, CTRL groups are compared and in graph **B** DEX group's performance is represented. The analysis between CTRL and DEX groups between females, males and masculinized females are in graphs **C**, **D** and **E**, respectively. Results are expressed as mean±SEM (n=7-36). Statistical analysis was assessed with two-way ANOVA. **p<0.01 males versus females; +p<0.0001 males versus females; #p<0.05 males versus females with testosterone; &p<0.05, &&p<0.01 females versus females with testosterone;$p<0.0001 females versus females with testosterone; £p<0.05, ££p<0.01, £££p<0.001 CTRL versus DEX. (P – Postnatal Day)

F. Prenatal exposure to DEX has no effect on the acquisition of auditory response, but delays eye opening day

To study physical milestones of neurodevelopment the auditory startle response and eye opening day were evaluated. In the auditory startle a positive response is considered when the animal startles upon a loud sound. This analysis is performed between PND11 and PND14 (Baharnoori et al., 2012). Throughout the days of the test no differences were detected between CTRL groups, and overall, at PND13 most of the pups started reacting to the stimuli (**Figure 26 A**). In **Figure 26 B**, analysis between DEX groups is shown, however, no differences were found and the performance was very similar to CTRL groups. Between CTRL and DEX groups, no differences were observed within females (**Figure 26 C**), males (**Figure 26 D**) nor females administered with testosterone (**Figure 26 E**).

The eye opening test consists of observing the pups between PND12 and PND17. No differences were observed between CTRL groups (**Figure 27 A**) and in general pups start to open their eyes on PND13. When DEX is administered the percentage of pups with open eyes increases. At PND13 testosterone delayed eye opening day when comparing with DEX females (**Figure 27 B**) (PND13 - DEX♂: 18.18±8.4s; DEX♀: 25.0±13.1s; DEX+T♀: 0.0±0.0s; &p<0.05). Comparing CTRL and DEX females no differences were found (**Figure 27 C**), nor between masculinized females (**Figure 27 E**). At PND14, there were significantly more DEX male pups with their eyes opened than CTRL males (**Figure 27 D**) (PND14 - CTRL♂: 30.43±9.8s; DEX♂: 68.18±10.2s; $p<0.0001). Overall, DEX has a tendency for anticipating eye opening day in both males and females, whereas testosterone administration extinguishes this effect. Discriminated values concerning auditory startle response (**Table S8**) and eye opening day (**Table S9**) are presented in the supplementary data section.

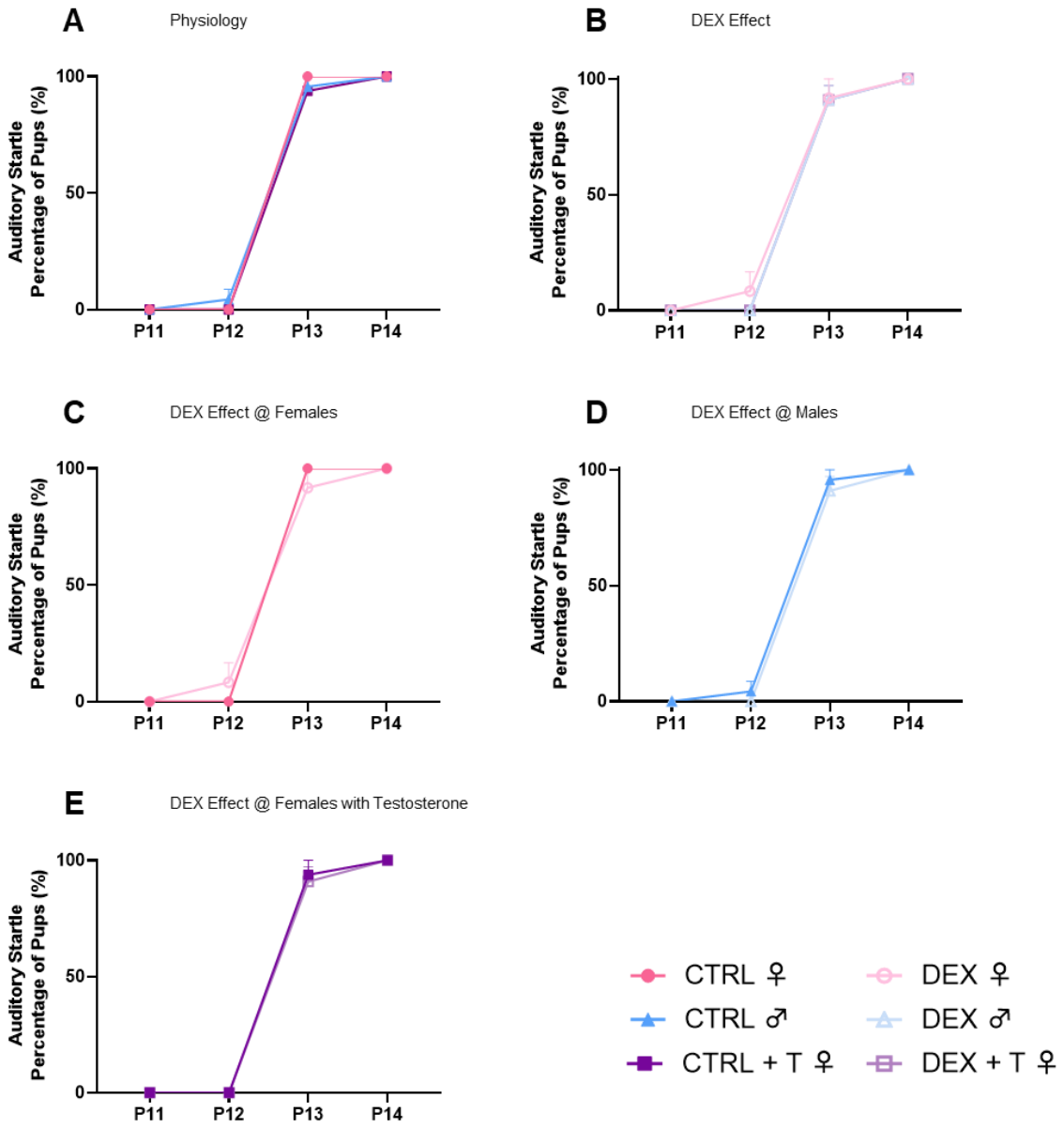

Figure 26: Testosterone administration does not influence Auditory Startle response, neither does prenatal DEX. The auditory startle test evaluates the percentage (%) of pups, which startle due to a loud sound. This analysis was made between PND11 and PND14 on CTRL (**A**) and DEX pups (**B**). The performance of pups from CTRL and DEX was compared between females (**C**), males (**D**) and masculinized females (**E**). Results are expressed as mean±SEM (n=7-36). Statistical analysis was assessed with two-way ANOVA test. (P – Postnatal Day)

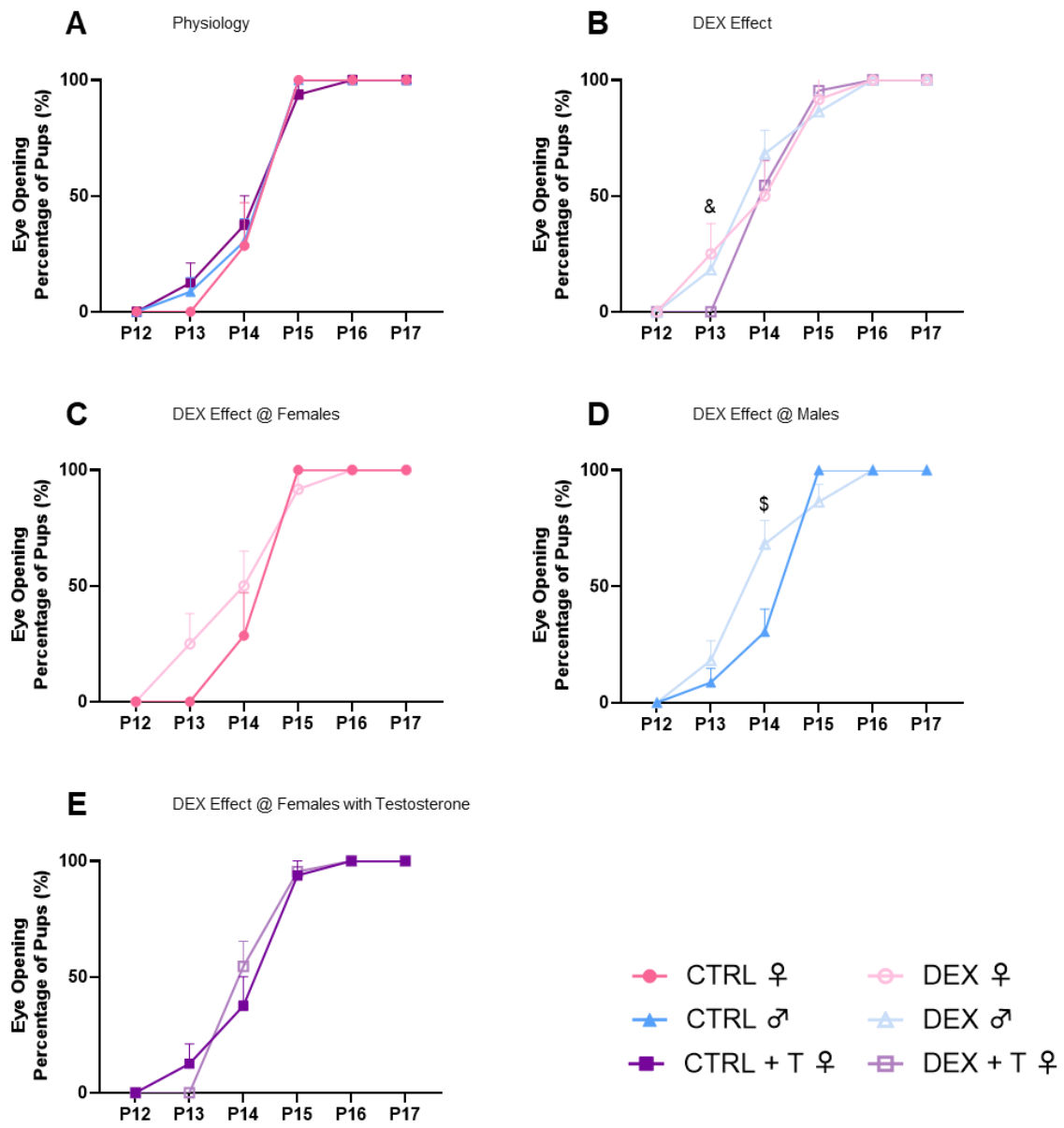

Figure 27: DEX anticipates eye opening day in males and females, but Testosterone exposure during pregnancy delays eye opening day in DEX females. The eye opening was evaluated by observation between PND12 and PND17. Results show the percentage (%) of pups with eyes completely open. In graph **A** there is a comparison between CTRL groups and in **B** the same analysis was made, but for the DEX pups. In **C**, **D** and **E**, CTRL versus DEX performance is represented in females, males and females with testosterone, respectively. Results are expressed as mean±SEM (n=7-36). Statistical analysis was assessed with two-way ANOVA. &$p<0.05$ females versus females with testosterone; \$$p<0.0001$ females versus females with testosterone. (P – Postnatal Day)

Overall, in physiological conditions CTRL males and females had similar performance on neurodevelopmental tests, and likewise neonatal testosterone had no major effect, making it difficult to dissect if testosterone had a masculinizing effect. Only regarding weight, a considerable sex-difference was observed, being males heavier than females. Nevertheless, neonatal testosterone only had a minor effect in increasing weigh towards male values. DEX did not have an impact on offspring performance in neurodevelopmental tests, but reduced female strength and anticipated eye opening day. No sex differences were observed between DEX groups however, DEX+T females were stronger than DEX females, but had a delayed eye opening day compared also with DEX females. On the other hand, the delay induced by testosterone in cliff avoidance was inhibited by DEX administration.

iii. Behavior at Adulthood

Previous studies from our group showed that prenatal exposure to DEX induced an anxious-like behavior at adulthood in both males and females, and treatment with an experimental anxiolytic (SCH58261), could revert anxiety, but only in males (Caetano et al., 2017). Aiming to evaluate if masculinized DEX female offspring could respond similarly to males when treated with A_{2A} receptor antagonist, a group of CTRL and DEX masculinized females was subjected to behavioral assessment in the present study.

The behavior analysis at adulthood (PND90) was performed with the Open Field test (OPF) and Elevated Plus Maze test (EPM). From PND68 until PND 88 the animals were administered with the anxiolytic, meaning the experimental groups were subdivided into vehicle or SCH58261. Therefore, the groups analyzed were CTRL♀, CTRL+T♀, CTRL+T+SCH♀, DEX+T♀ and DEX+T+SCH♀.

A. Neonatal testosterone administration and SCH58261 treatment do not affect female locomotor activity

To exclude effects of the treatments in locomotor activity, which could impact female performance in the EPM test (gold standard test to evaluate anxious-like behavior in rodents), the mean speed (m/s) and distance travelled (m) were assessed by open field test. Here animals were left to explore an open arena for 5 minutes.

No differences were detected neither in distance travelled (**Figure 28 A**) nor in mean speed (**Figure 28 B**), however CTRL+T females tend to travel faster and greater distances than CTRL animals. Overall, both analyses' allow us to infer that these groups have no locomotor impairments and therefore, allow the performance of the EPM test without any bias associated.

The analysis of the time in the center of the arena (**Figure 28 C)** may be an indicative of anxious-like behavior, since anxious rodents tend to not cross the central area. Here we do not observe significant changes between groups regarding the time spent at the central area. Discriminated values of this test are in supplementary data (**Table S10**).

B. Testosterone has a protective effect regarding anxious-like behavior of females prenatally exposed to DEX

The elevated plus maze test consists of an arena shaped like a cross (two open arms and two closed arms) and is commonly used to assess anxiety-like behavior in rodents. Analyzing the ratio of time (s) females took exploring the open arms per total time (**Figure 29 A**), no differences were found between experimental groups. Previous studies from our group show that both DEX males and females have an anxious-like behavior, observed by decreased time spent in open arms (Caetano et al., 2017). However, DEX+T females spent similar time in open arms as CTRL pups, suggesting that neonatal testosterone administration prevents the development of anxious-like behavior in DEX females. Previously, our group showed that SCH58261 alone has anxiogenic effects in females (Caetano et al., 2017). In the present study CTRL+T+SCH females present a tendency to enter open arms less times than CTRL offspring, also suggesting an anxiogenic effect.

Nonetheless DEX+T females with or without SCH58261 present no differences towards CTRL entries (**Figure 29 B**). Discriminated values of this test are in supplementary data (**Table S11**).

Since female hormones can have an impact on behavior, a cytology was performed to analyze the estrous cycle stage. Females within the same experimental groups were in different stages of the cycle, meaning female hormones did not influence behavior (**Figure 29 C**). These results suggest no correlation between EPM performance and estrous cycle, confirming what was previously described (Caetano et al., 2017; Schwarz et al., 2012).

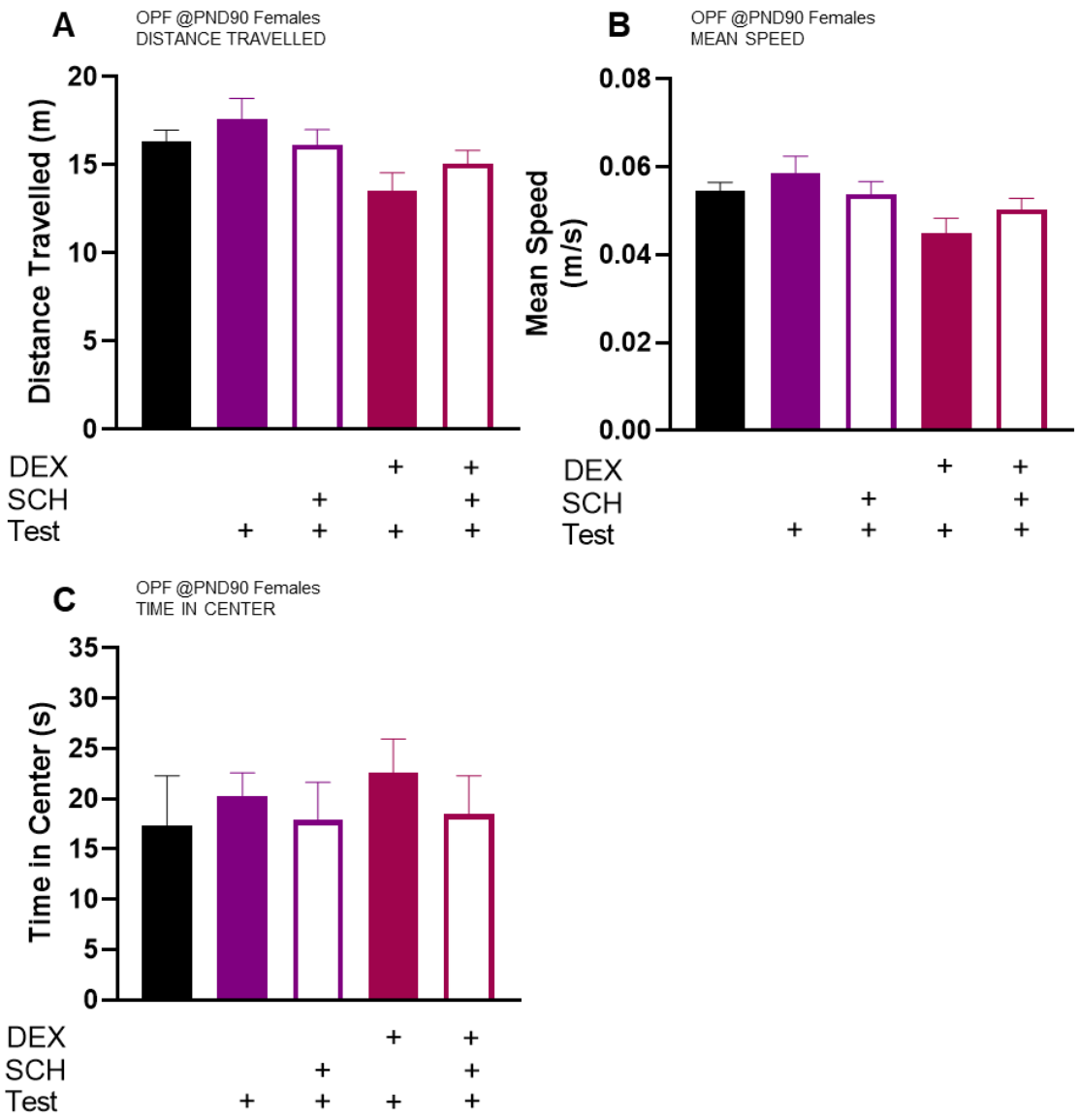

Figure 28: Females administered with DEX and/or testosterone do not have their locomotion affected at adulthood. In OPF test, animals with 90 days are placed in the center of an open arena and left to explore for 5 minutes. The distance travelled (m)(**A**), mean speed (m/s) (**B**) and time in center (s)(**C**) were assessed in females to ensure good locomotor skills. Results are expressed as mean±SEM (n=7-11). Statistical analysis was assessed with One-way ANOVA with Tukey's multiple comparison test.

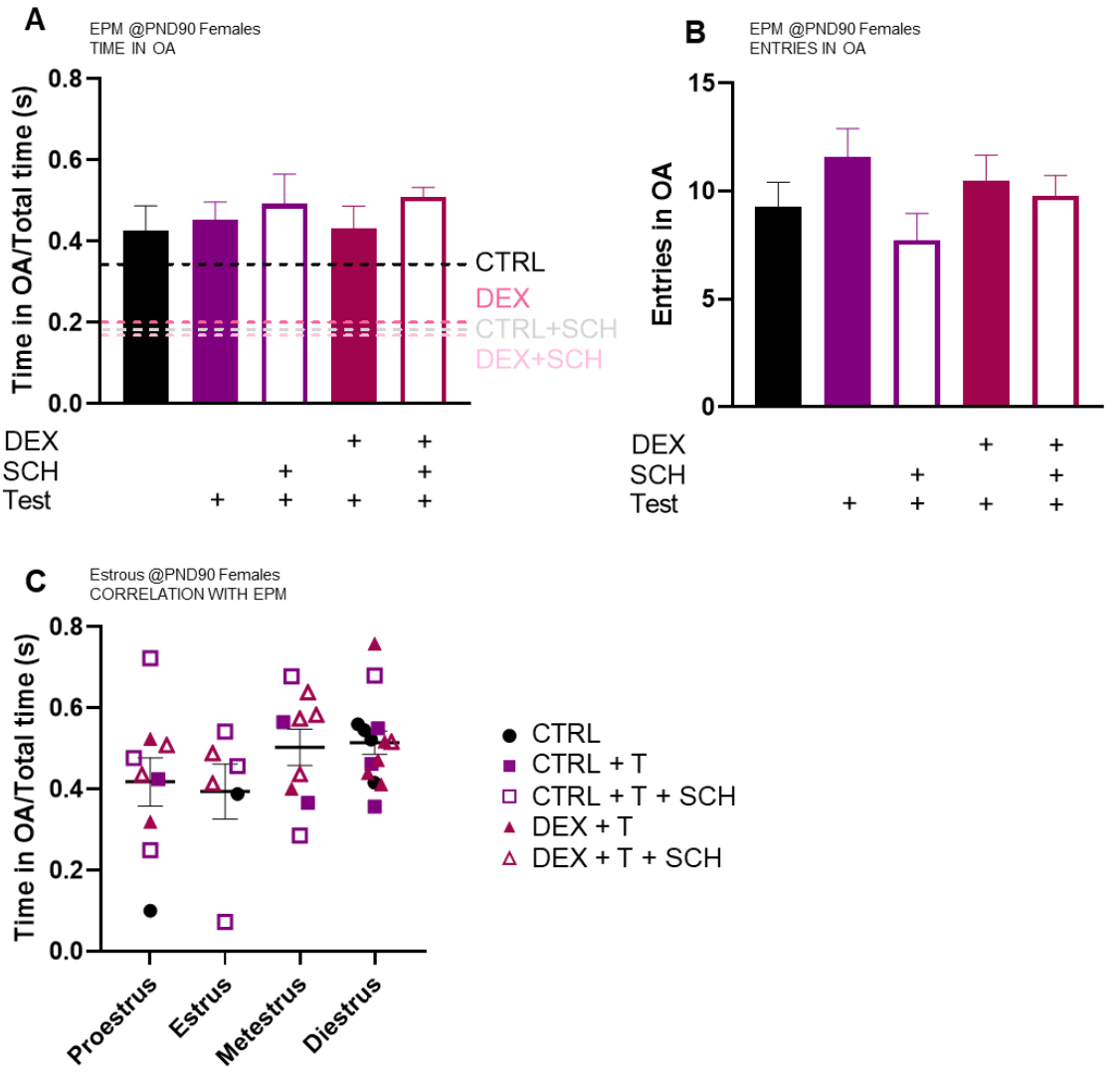

Figure 29: Neonatal testosterone administration has a protective effect in anxious-like behavior of females prenatally exposed to DEX. In the EPM test, animals were placed in the center of a cross-shaped arena, with two open and two closed arms, and left to explore for 5 minutes. A ratio between the time (s) spent in open arms and the total time of the test was made to assess anxious-like behavior in females (**A**), but also open arms entries (**B**). To ensure that the female hormones were not affecting behavior, the analysis of the estrous cycle was also performed (**C**). Results are expressed as mean±SEM (n=7-11). Dotted lines refer to the ratio of time spent in OA/total time of the test, by CTRL (black), CTRL+SCH (gray), DEX (pink) and DEX+SCH (light pink) groups obtained in a previous study (Caetano et al., 2017).Statistical analysis was assessed with One-way ANOVA with Tukey's multiple comparison test. (OA – Open Arms)

iv. Microglia Reconstruction and analysis

A. Neonatal testosterone does not recover the atrophy of microglia from the PFC of DEX females at PND90 even after treatment with SCH58261

Our group showed that prenatal exposure to DEX induced morphological alterations in microglia at adulthood, which were sex-specific. The exogenous glucocorticoid caused a hyper-ramification of microglia in males and an atrophy in females, in the PFC. However, treatment with the anxiolytic SCH58261, reverted microglial morphology in males, but not in females (Caetano et al., 2017).

In the present study microglial morphology analysis was performed at PND90 in the PFC of CTRL+T+SCH♀, DEX+T♀ and DEX+T+SCH♀ pups. The study was only made in these three groups, since the other ones were already well characterized in published (Caetano et al., 2017) and unpublished works of the group. Here our aim was to compare CTRL+T+SCH with CTRL+T females (unpublished data) to further understand the impact of SCH58261 in microglia morphology; DEX + T females with DEX males to assess if microglia morphology was masculinized, but also compare with CTRL+T, to assess if masculinization of microglia reversed DEX-induced morphological alterations; and most importantly compare DEX+T+SCH females with DEX+SCH females to study if testosterone had a permissive effect for the action of the anxiolytic in females (Caetano et al., 2017).

Branched and sholl analysis were performed from the PFC microglia reconstructions obtained through Neurolucida software. Branched analysis consists of analyzing the number (**Figure 30 A**) and length of processes of microglia (**Figure 30 B**) in each branch order. Sholl analysis consists of studying the total length and number of intersections of microglia with predefined 10μm radius. No differences were found in both total length (**Figure 30 C)** per radius and number of intersections (**Figure 30 D**). Representative images of each group's microglia are in **Figure 30 E**, as well as an illustrative reconstruction of these cells.

When comparing microglia from CTRL+T+SCH group with CTRL+T females previously drawn, we observe an atrophy of this cell, which is probably due to the action of SCH58261, an effect previously observed in females (Caetano et al., 2017). Microglia from DEX+T have a tendency for smaller and less ramifications then DEX+T+SCH females however, both are atrophied, similarly to DEX+SCH females (Caetano et al.,

2017), which suggest testosterone was not capable or reverting the atrophy induced by DEX, even after anxiolytic administration. Lastly, DEX+T+SCH females are atrophied contrarily to DEX males previously drawn, thus testosterone did not have a permissive effect of SCH58261 action.

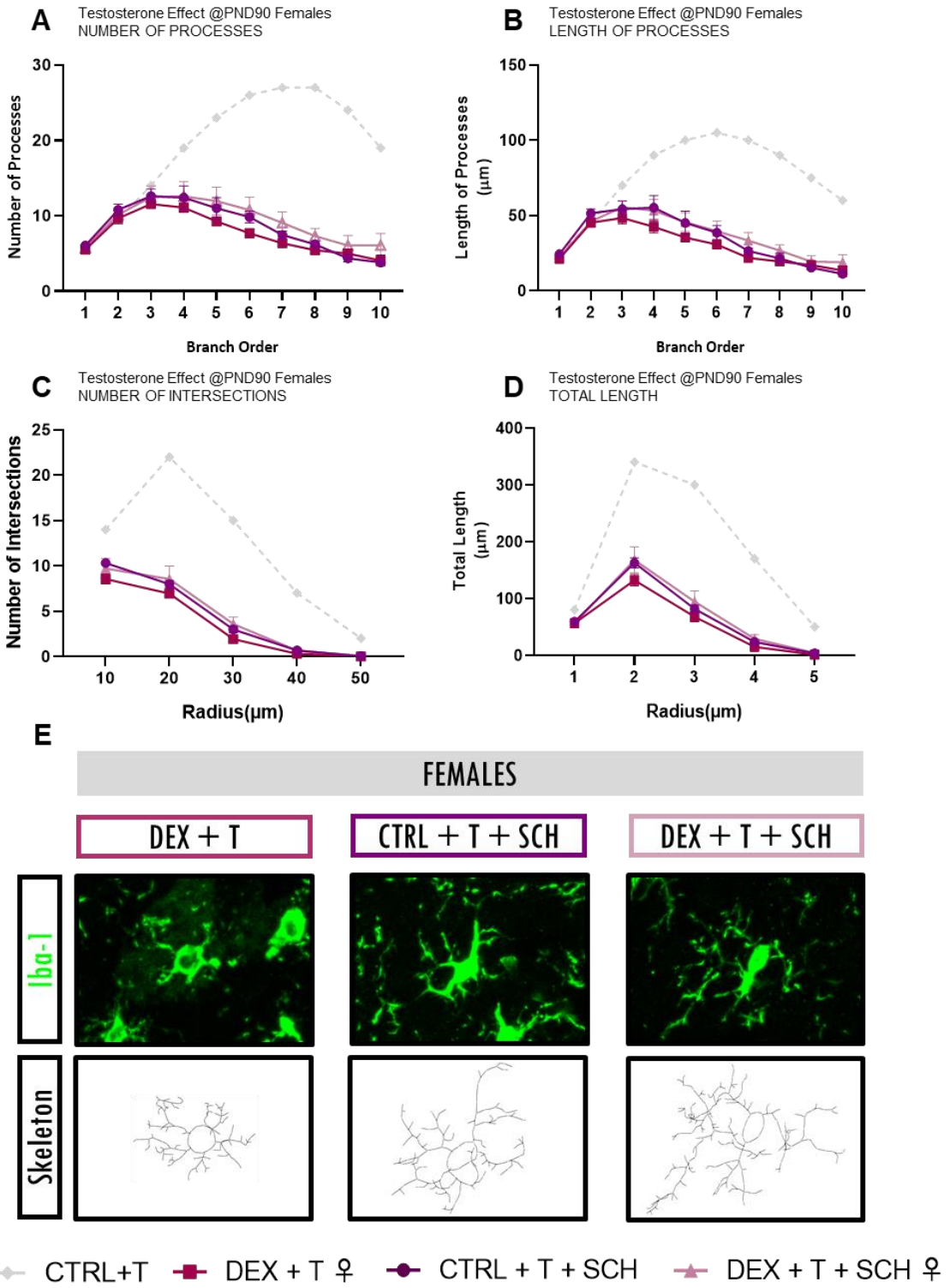

Figure 30: Neonatal Testosterone does not recover the atrophy of microglia from DEX females at neither has a permissive effect upon SCH58261 administration. Microglia from the PFC was tridimensionally reconstructed. Branched (**A**, **B**) and sholl analysis (**C**, **D**) were

performed and representative images from the Confocal microscope and microglia skeleton obtained with Neurolucida software are also represented (**E**). In light gray are represented CTRL+T females drawn in a previous unpublished group study. Results are expressed as mean±SEM (n=3-4). From each animal's PFC 6-15 microglia were reconstructed. Statistical analysis was assessed with two-way ANOVA.

v. Serum levels Analysis

At PND90 blood was collected to analyze peripheral levels of endogenous corticosterone and testosterone. Hormone levels were quantified to evaluate if prenatal stress impaired adult levels of both steroid hormones. Serum from female offspring of CTRL, CTRL+T, CTRL+T+SCH, DEX+T and DEX+T+SCH was analyzed by ELISA kits, specifically for corticosterone and testosterone.

A. Peripheral levels of corticosterone and testosterone increase when DEX, testosterone and SCH58261 are combined

When analyzing the peripheral levels of corticosterone, no differences were found in females (**Figure 31 A**) however, DEX+T+SCH females have a tendency for increased levels of corticosterone when compared with DEX+T.

Testosterone peripheral levels between experimental groups presented no statistical differences (**Figure 31 B**). Nevertheless, masculinized females have a tendency for an increase in serum levels of testosterone, which is reduced by SCH58261 administration. On the other hand, DEX+T females have a tendency for decreased levels of testosterone, and when SCH58261 is administered there is an increase in testosterone levels, changing the pattern of action of the antagonist.

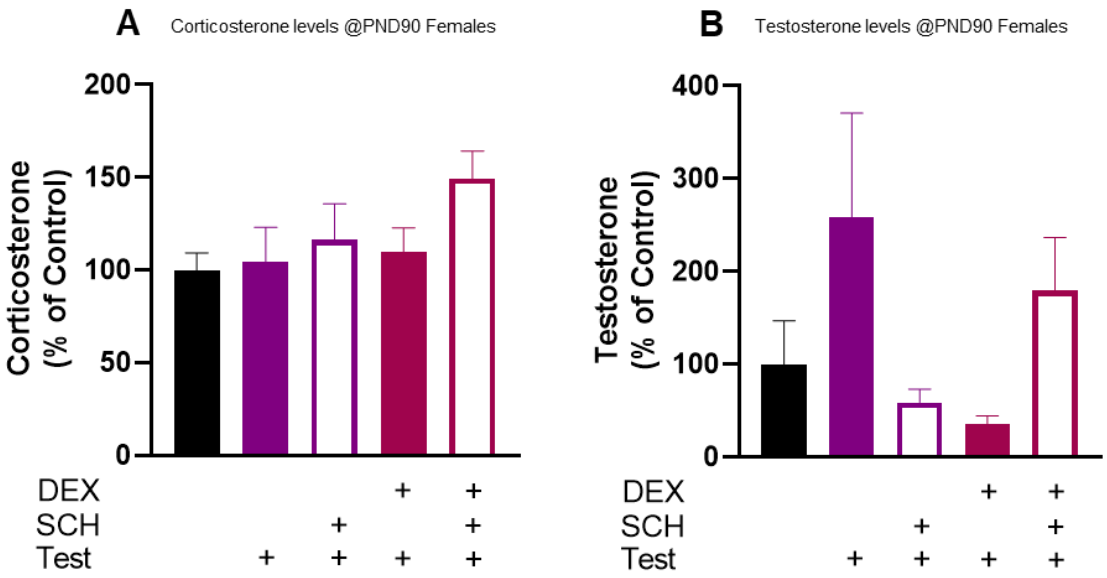

Figure 31: Combination of DEX, testosterone and SCH58261 induces an increase in corticosterone and testosterone peripheral levels in females at PND90. Peripheral concentration of corticosterone (**A**) and testosterone (**B**) from serum was assessed with an ELISA kit in females and males. Results are expressed as mean±SEM for corticosteroid and testosterone levels (n=7-11). Statistical analysis was assessed with One-way ANOVA test.

2.4 Discussion

Prenatal stress during neurodevelopment is known to cause impairments in the CNS normal function. Increased levels of DEX during critical windows are associated with anxious-like behavior in the progeny and morphologic remodeling of microglia in the PFC at adulthood (Caetano et al., 2017; Oliveira et al., 2012), which are a risk factors for neuropsychiatric disorders (Drozdowicz & Bostwick, 2014). Females are more affected by neuropsychiatric disorders, which might suggest that testosterone has a protective action in males (Celec et al., 2015). The organizational effects of testosterone can be reproduced in females by administrating androgens during sexual differentiation (Wolf et al., 2002).

In the Section 2.3.i and 2.3.ii we aimed to analyze the effect of prenatal administration of DEX in the offspring's weight and neurodevelopmental behavior (to further dissect if DEX-induced neurodevelopmental impairments could be associated with anxious behavior at adulthood) and also to assess the impact of mimicking the male surge of testosterone in females (to understand the effect of this androgen on programming brain and behavior) (Konkle & McCarthy, 2011).

In humans, weight is a risk factor for neuropsychiatric disorders development at adulthood (Willage, 2018). Data from our group, as well as other authors report a reduction of weight at PND0 in DEX offspring, an effect that is maintained until weaning day (PND21) (Bloom et al., 2001; Ferreira, 2019), but at adulthood, DEX does not have an impact on animal weight (Caetano et al., 2017). Data obtained with the present set of animals, show that DEX induced a tendency to reduce weight, an effect that was statistically different for females in particular timepoints. Furthermore, males were heavier than females in both CTRL and DEX groups, a result in part reduced by the administration of testosterone.

CNS alterations have already been reported by several authors in animals prenatally exposed to DEX (Caetano et al., 2017; Duarte et al., 2019; Oliveira et al., 2006, 2012). Hence, it becomes imperative to study neurodevelopmental behavior to assess if any alterations in this period could be used as predictive factors for psychiatric impairments at adulthood. For this analysis we made a battery of tests to assess vestibular system development, physical milestones of development, strength, discriminatory capacity and locomotion.

We observed that prenatal stress induced by DEX did not affect the vestibular system development, discriminatory capacity, auditory response and motor function during development. Conversely, exposure to DEX anticipates eye opening day and reduces strength in females. Previous data from our group, show that DEX administration, induces a delay in vestibular system development, discriminatory capacity, physical milestones, locomotion (only in males) and decreased strength, of both male and female pups, especially at PND5 and PND6 (Galvão, 2019). Additionally, Gramsbergen and Menshanov, also showed that postnatal DEX administration induced an antecipation of eye opening day, a delayed vestibular system development, increased strength and had no impact on locomotion (Gramsbergen & Mulder, 1998; Menshanov et al., 2014). Regarding locomotion and strength, our results are in agreement with the previous cited study however, different strains of animals were used and the time of exposure and dosage of DEX were also different. The delay observed in vestibular system development, discriminatory capacity and physical milestones in former analysis does not happen in this cohort of animals. The changes caused by DEX during neurodevelopment are only observed when there is anxious-like behavior at adulthood, suggesting an association between neonatal and adulthood behavior and therefore, giving a predictive value to neurodevelopmental alterations.

Regarding sex differences, in this study we observed that in physiological conditions no major differences were found besides males being heavier than females, an effect in part reduced by testosterone administration. Data not published by our group show that in physiology, females have a tendency for a delay in vestibular system development and discriminatory capacity, increased strength and an anticipation in auditory response. Nonetheless, our results are according to VanRyzin, which described no effect of sex in neurodevelopmental behavior in physiology (VanRyzin et al., 2016).

Unpublished group data demonstrates that neonatal testosterone administration does not induce a masculine behavior in CTRL females, meaning androgenized females maintain neurodevelopmental behavior similar to the females (mentioned above). Regarding testosterone exposure in this study, we observe that DEX+T females had a normalization of cliff aversion behavior, but a delay in eye opening day. Here we observe no effect of testosterone exposure in neurodevelopmental behavior, which is in line with the former data from the group.

The model of prenatal DEX exposure, used is this thesis is validated worldwide as inducing anxious-like behavior at adulthood (Caetano et al., 2017; Li et al., 2014; Oliveira et al., 2006). To assess this type of behavior we performed the gold-standard

test, the Elevated Plus Maze as showed in Section 2.3.iii. Since impairments in locomotion could induce a bias on EPM results, we assessed this parameter in the Open Field test. We analyzed mean speed and distance travelled, but also anxious-like behavior through time spent in the center of the arena. Regarding this study, we report no alterations in motor function (distance travelled and mean speed present no differences) nor in time spent in center, whether or not the anxiolytic and testosterone were administered.

Our group, shows that DEX exposure during gestation caused anxious-like behavior at adulthood in both males and females, but when these offspring are treated with an antagonist of the $A_{2A}R$, only male behavior was reverted to physiology (Caetano et al., 2017). With this work, our aim was to observe if androgenized females would be sensitive to the $A_{2A}R$ antagonist treatment and consequently revert DEX-induced anxious-like behavior. In this set of animals DEX did not induce the expected anxious-like behavior in the progeny. A possible explanation may relate to variations in compound kinetic or in dam metabolism. Additionally, we would like to highlight that in this cohort, only a small number of DEX female animals was analyzed (n=3), all of them from the same dam. Since prenatal DEX administration did not have the expected outcome, any conclusions regarding testosterone administration in DEX offspring treated with SCH58261, will have to be replicated in future studies using a new cohort of animals to confirm the results presented herein.

Here females prenatally exposed to DEX and neonatally exposed to testosterone (or in combination with SCH58261 administration) do not present an anxious-like behavior. These results may suggest a protective effect of this androgen in behavior (anxiety) of females at PND90, an anxiolytic effect similar to what was observed in other studies (Frye & Seliga, 2001; Hodosy et al., 2012). Neonatal administration of testosterone is known to change the size of brain regions as well as connectivity and density of cells (McCarthy, 2008), which can differently modulate behavior. Another possible explanation for these behavioral outcomes, is that females have a lower density of AR (Lu et al., 1998), but also a different expression of ER between brain regions (Zhang et al., 2002). Furthermore, the kinetics between endogenous and exogenous testosterone are different, which will result in different brain and behavior programming (Filová et al., 2012). Previous studies from the group show that in physiology females are more disinhibited in the EPM than males however, when testosterone is administered, females have a tendency to reduce time spent in open arms (Simões-

Henriques, 2017). Moreover, our group described that SCH58261 administration *per se* is anxiogenic, observed by a reduction in time spent in open arms (Caetano et al., 2017). With this data, testosterone administration counteracted the predicted anxiogenic outcome of SCH58261 administration in females, though there was a tendency for of CTRL+T+SCH to enter less in open arms comparing with CTRL and CTRL+T females. Overall, with these results we propose that testosterone administration, on the day of birth, has a protective effect in females prenatally exposed to DEX regarding anxious-like behavior. Taking into consideration these results, we were not able to take any conclusions regarding the permissive effect of testosterone on anxiolytic treatment with SCH58261.

Estrogens may have an impact on behavioral outcomes. For instance, a study showed that restraint stress has different effects on the activity and function of the basolateral amygdala (a region important for emotional responses), depending on the stage of the estrous cycle females were in (Blume et al., 2019). After EPM test, females were subjected to a cytology to analyze the estrous cycle stage and the possible effect of estrogens on anxious behavior. Here we observe that females were in different stages of the cycle, proving that female hormones have no impact on behavior, similarly to what was described in our previous works (Caetano et al., 2017) and in the literature (Schwarz et al., 2012).

Microglia are involved in the pathophisiology of neuropsychiatric disorders, since they can undergo morphological remodeling and consequently underlie behavioral impairments at adulthood (Hodes et al., 2015; Rial et al., 2016). Several studies have showed a sexual dimorphism in both adult behavior, microglia morphology and anxiolytic treatment (Caetano et al., 2017; McHenry et al., 2014; Schwarz et al., 2012). Microglial dynamics are controlled by A_{2A} receptors (Gyoneva et al., 2009), which are also known to be involved in anxiety and depression (Gomes et al., 2013, 2011; Gyoneva et al., 2014). To assess microglia morphology, we performed an immunohistochemistry to stain microglia, which was then tridimensionally reconstructed.

In this work we only studied microglia from the PFC of DEX+T, CTRL+T+SCH and DEX+T+SCH females, since previous studies from our group already analyzed the effect of DEX and SCH58261 administration (Caetano et al., 2017), and also the neonatal administration of testosterone to CTRL females (CTRL+T) (unpublished data). With this analysis, we aimed to study the masculinizing effect of testosterone, but also prove our initial hypothesis that testosterone was the

mediator for SCH58261 action and therefore, explain the sex-specific remodeling of microglia morphology. Results from microglia reconstructions of masculinized females are shown in Section 2.3.iv. Male and female microglia reconstructions from CTRL and DEX groups are in Caetano et al., 2017, and CTRL+T microglia are from an unpublished work.

Sex differences in microglia morphology are observed in basal conditions, where the female microglia is more complex (Schwarz et al., 2012), a reflex of the sex hormone exposure during neurodevelopment (Lenz & McCarthy, 2015). Data from the group demonstrate that at youth (PND30), males have a more complex microglia than females and neonatal administration of testosterone induces a tendency for microglia to resemble males (Simões-Henriques, 2017). However, at adulthood (PND90) females are hyper ramified when compared with males (Caetano et al., 2017) and androgenized females become partially masculinized (unpublished data). In the present work, microglia from the PFC of CTRL+T+SCH females was atrophied when compared with CTRL+T female cells (dotted line-unpublished data). This result is in conformity with previous results, where SCH58261 also induced an atrophy of both male and female microglia (Caetano et al., 2017).

Bearing in mind previous studies, we conjectured that DEX+T females would have hyper ramified microglia similar to DEX males and most importantly, that DEX+T+SCH females could revert microglia morphology to physiological conditions, similarly to what had happened to DEX+SCH males (Caetano et al., 2017). In this study our initial hypothesis concerning DEX+T females and DEX males (Caetano et al., 2017) was not confirmed, since the female cell is atrophied rather than hyper ramified. Furthermore, when comparing females from CTRL and CTRL+T groups with DEX+T we observe that there is no correlation between microglia morphology and behavior, since both groups present different microglia morphologies, but the same behavior (anxiety). These results might suggest that testosterone protects from anxious-like behavior, regardless of microglia morphology.

In previous works we observed that DEX induces an atrophy of microglia in females, which SCH58261 cannot revert. Regarding DEX+T and DEX+T+SCH female microglia, we observe that the atrophy previously reported in DEX+SCH females is maintained in these experimental groups in a more pronounced manner (Caetano et al., 2017). In this work, we also show that neonatal administration of testosterone is also not capable of reverting DEX-induced atrophy, even in the presence

of the anxiolytic. In other words testosterone was not permissive to the action of SCH58261. Still, since this model did not work properly, more studies will need to be performed to test our initial theory about microglia morphology remodeling.

Several authors have shown that testosterone administration has anxiolytic effects (Aikey et al., 2002; Celec et al., 2015; Fernández-Guasti et al., 2005). However, Hodosy and colleagues, showed that flutamide (AR antagonist) administration also had an anxiolytic effect (Hodosy et al., 2012). These studies highlight the important role of testosterone, which may have an impact on behavior and microglia (VanRyzin et al., 2020). Regarding testosterone levels, our results presented in Section 2.3.v, show that CTRL+T females have a tendency for increased levels of testosterone, an effect reduced by SCH58261 administration. Another study showed that prenatal administration of DEX is correlated with decreased levels of peripheral testosterone in males (Oliveira et al., 2011), which is concordant with the reduction in testosterone levels observed in DEX+T females. However, this result is reversed by SCH58261 administration, changing the anxiolytic pattern observed between CTRL+T and CTRL+T+SCH females. Our results suggest that $A_{2A}R$ can modulate peripheral levels of testosterone.

Caffeine intake during gestation induces an increase in PFC testosterone levels of females at GD19, but also an increase of this hormone in the hippocampus of males at PND0 (Karaismailoglu et al., 2017). This study emphasizes the interaction between the adenosine receptor antagonist, caffeine, and the production of testosterone in two brain regions, important for emotional regulation. Therefore, knowing that adenosine receptors modulate testosterone levels peripherally and centrally, we hypothesize this could happen in microglia.

Peripheral levels of corticosteroids are known to change throughout the day according to the circadian rhythm, with a peak in the onset of the active phase of the day (Chung et al., 2011). Therefore, it becomes imperative to study serum levels of this steroid hormone in this anxiety model, which results are presented in Section 2.3.v. Former data from our group, demonstrate that corticosterone levels of females from DEX and DEX+SCH groups are reduced at 8 a.m., an effect maintained at 8 p.m. for DEX+SCH females; regarding males no changes were observed between groups and time of the day (Caetano et al., 2017). In this cohort of animals, no differences were detected in corticosterone levels of females. In DEX+T females, we observe similar corticosterone levels as controls, which contrasts with the effect seen in females from previous works (Caetano et al., 2017). The administration of SCH58261 induces a tendency for an increase in corticosterone levels of DEX+T+SCH females, which is not

concordant with female corticosterone levels (Caetano et al., 2017). Muneoka et al., showed that administration of DEX between GD17 and GD19, increased corticosterone levels in male Sprague-Dawley rats, an effect has also seen in female mice subjected to unpredictable prenatal stress (Benoit et al., 2015; Muneoka et al., 1997). In summary, in past works DEX induced a reduction of corticosterone levels in females. However, our results show that corticosterone levels of androgenized females do not resemble female hormonal levels (Caetano et al., 2017). Nevertheless, further studies need to be performed to assess corticosterone levels of these groups, considering the circadian rhythm.

With the data obtained, we propose that neonatal administration of testosterone in females has a protective effect of anxious-like behavior at adulthood however, it is not capable of reverting the atrophy induced by DEX (or SCH58261) in microglia cells, nor to have a permissive effect to the action of SCH58261. Having in mind that testosterone might be modulated by the adenosinergic system, the possible $A_{2A}R$ - AR crosstalk in microglia cells will be further addressed *in vitro* in the next chapter.

3

Interaction Between Androgen and A_{2A} Receptors in Microglia

3.2 Rationale & Aims

Since males and females prenatally exposed to DEX react differently to the anxiolytic $A_{2A}R$ antagonist SCH58261, we propose a possible interaction between androgen and A_{2A} receptors in microglia, which could explain the different drug efficacy between sexes in terms of microglia morphology impacting on behavior (Caetano et al., 2017). Herein, by using a microglia cell line we aimed to analyze, not only if testosterone has a permissive effect on the A_{2A} receptor, but also to study if the A_{2A} receptor was capable of modulating androgen receptors.

Specifically, the aim of this part of the study is:

- ➔ Unveiling a possible interaction between AR and/or testosterone and $A_{2A}R$ in microglial cells, to support its putative impact on sexual dimorphism observed in microglia morphology and behavior.

3.3 Experimental Work

i. BV-2 Cell Line

A. Cells and Pharmacological Treatment

The BV-2 cell line is a murine microglial line (**Figure 32**) that is cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% Penicillin (100U/mL)-Streptomycin(μg/mL) and 2mM L-Glutamine. Cells were maintained in 75cm^2 flasks, at 37°C, 5% CO_2 in a humidified incubator. Passage of cells was made every two days. For experiments, cells were plated at a density of 1.5×10^4 cells/mL in 6 well plates for posterior western blot analysis. When plated, cells were cultured with RPMI, 2%FBS, 1% penicillin-streptomycin and 2mM L-Glutamine and maintained in conditions previously described.

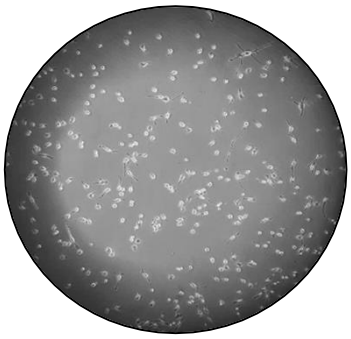

Figure 32: BV-2 microglial cells in the light microscope.

Before administering any pharmaceutical agent, cells were stabilized for 18h. Initially, BV2 cells were incubated with flutamide (1 or 10 μM) or/and testosterone (1, 10 or 100nM) for 6 or 24 hours to evaluate the possible permissive effect of testosterone on $A_{2A}R$ (**Figure 33**). When flutamide and testosterone were combined, the inhibitor was added 15 minutes prior to the agonist. Another set of cells were plated with 5.5×10^{-3}% ethanol (EtOH), which was flutamide and testosterone's vehicle.

In a second part of the study, BV-2 cells were incubated with 30nM CGS21680 Hydrochloride or/ and 50nM SCH58261 for 6 or 24 hours to evaluate if androgen receptors could be modulated by adenosine receptor signaling (**Figure 34**). Similarly to the previous assessment, cells were incubated with the antagonist SCH58261, 15 minutes prior to CGS21680 administration. Microglia was also incubated with 1.4×10^{-3}% DMSO, the vehicle of both CGS21680 and SCH58261.

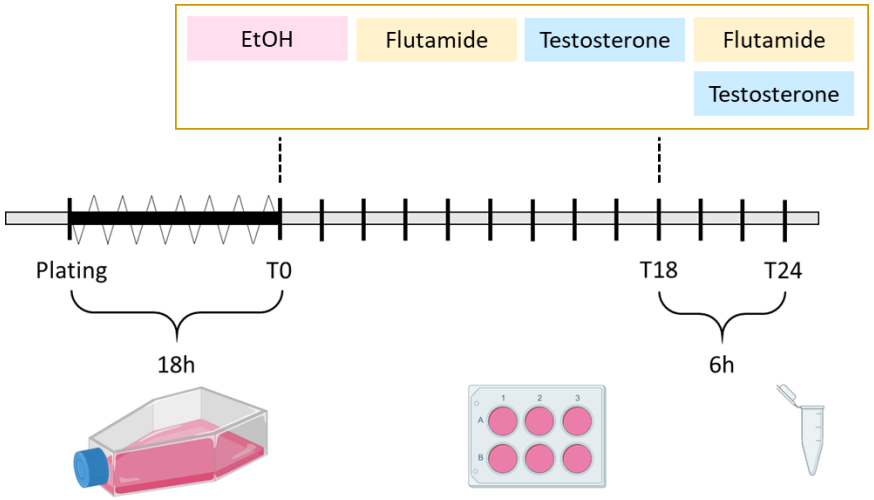

Figure 33: Timeline of BV2 pharmacological treatment. BV2 cells were incubated with testosterone and flutamide, an inhibitor of the androgen receptor, to evaluate the possible permissive effect of this sexual hormone to the action of $A_{2A}R$ signaling cascade.

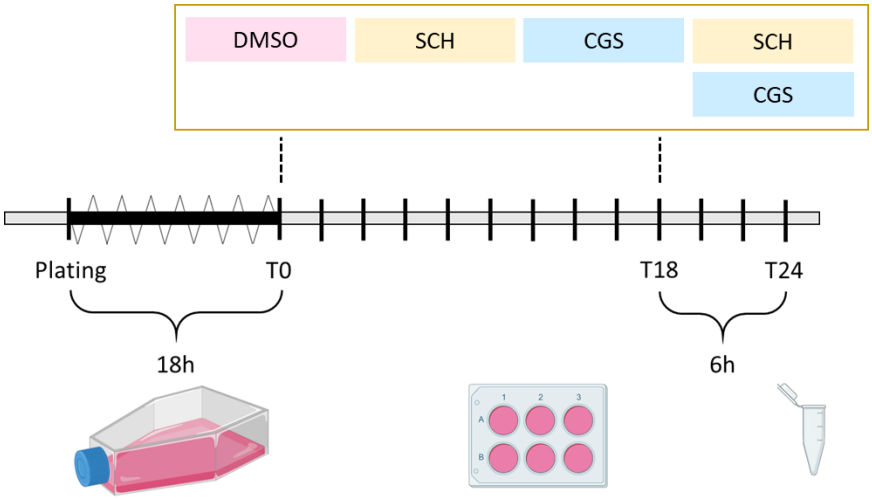

Figure 34: Timeline of BV2 pharmacological treatment. BV2 cells were incubated with the agonist (CGS21680) or antagonist (SCH58261) of the adenosine A_{2A} receptor to evaluate the effect on the androgen receptor levels.

ii. Cellular and Molecular Procedures

A. Western Blot

a. Sample Preparation

After pharmacological treatment, medium was discarded, and cells were washed 2 times with sterile PBS 1x at RT. Lysis buffer was added (RIPA supplemented with DTT and complete mini protease inhibitor) and cells were scraped while on ice. The lysed samples were sonicated in the bath for 1 minute after which they were centrifuged at 16000xg at 4°C for 10 minutes. The supernatant was collected and saved at -80°C until use.

b. SDS-PAGE

BV-2 cells protein was quantified by the bicinchoninic acid (BCA) assay as described by the manufacturer (Pierce Biotechnology, Rockford, IL, USA). After the concentration assessment, the sample was mixed with Sample Buffer 6x (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% Bromophenol Blue) and then denaturated at 95°C for 5 minutes.

For the SDS-PAGE, samples were loaded in 4% polyacrylamide gel (4% bis-acrylamide, tris-base-HCl 0.5M pH 6.8, 10% SDS, 10% APS and 1% TEMED) and left to migrate for 10 minutes at 70V. Afterwards, the protein was resolved in 6-8% polyacrylamide gels (6-8% bis-acrylamide, tris-base-HCl 1.5M pH 8.8, 10% SDS, 10% APS and 1% TEMED) during 1h at 140V. The run was made in a system filled with electrophoresis buffer (25mM tris-base, 25mM bicine, 0.1% SDS, pH 8.3). Equal amounts of protein were loaded in the gel as listed in **Table 2**. After the electrophoresis, the PVDF membrane was placed on top of the gel and the transfer was made for 1 hour and 30 minutes at 0.75A in electrotransfer buffer (10mM CAPS, 10% methanol, pH 11.0). Membranes were blocked for 1 hour at room temperature with 5% non-fat milk or 5% BSA in Tris-Buffered Saline (TBS; 10mM Tris, 150mM NaCl) with 0.1% Tween 20 (TBS-T). The primary antibodies listed in **Table 2** were prepared in blocking solution and incubated overnight at 4°C. In the following day membranes were washed 5 times for 5 minutes each with TBS-T. The membranes were incubated with secondary antibody made with 1% blocking solution in TBS-T for 2 hours at RT, followed by another set of 5 washes. Proteins were detected either by enhanced chemiluminescence (ECL) on the ImageQuant LAS 500 (GE Healthcare) or with enhanced chemifluorescence (ECF) on the Typhoon FLA 9000 (GE Healthcare). GAPDH was used as loading controls.

Table 2: List of antibodies used in western blot with BV2 cells.

Antibody	Host	Dilution	Protein (µg)	Supplier
Anti-$A_{2A}R$	Mouse	1:200	40	Santa Cruz Biotechnology
Anti-AR	Rabbit	1:200	40	Abcam
Anti-Calnexin	Goat	1:5000	-	Sicgen
Anti-GAPDH	Goat	1:5000	-	Alfagene
HRP Anti-Goat	Rabbit	1:10000	-	Life Technologies
HRP Anti-Rabbit	Goat	1:10000	-	Bio-Rad
HRP Anti-Mouse	Goat	1:10000	-	Bio-Rad
AP Anti-Goat	Rabbit	1:10000	-	Invitrogen

iii. Statistical Analysis

Western Blot quantification was made with TotalLabTL120. Statistical analysis was made with the GraphPad Prism 8 (GraphPad, USA). Between groups differences were analyzed with One-Way ANOVA. Results are represented as mean±SEM, and statistical significance was set for $p<0.05$.

iv. Reagents

All reagents and kits used to perform this thesis are listed in **Table 3**.

Table 3: List of Reagents and kits used.

Reagent	Supplier	Reference/Lot
Acrylamide/ Bis 30% Solution	Thermo Fisher Scientific,	BP1408-1
Ammonium Persulfate	Sigma-Aldrich	A3678

Pierce™ BCA protein assay Kit	Thermo Fisher Scientific	23228
Bicine	Acros Organics	172650010
Bromophenol Blue	M&B	14764
BSA	PanReac	A1391,0500
CAPS	Thermo Fisher Scientific	BP321-500
CGS21680 Hydrocloride	Tocris	1063
Clarity Western ECL substrate	Bio-Rad	170-5061
cOmplete™, Mini Protease Inhibitor	Roche	11836153001
Corticosterone ELISA Kit	Abcam	108821
DAKO Glycergel Mounting medium	Dako	C0563
Dexamethasone	Sigma-Aldrich	D-1756
DL-Dihithiothreitol (DTT)	Thermo Fisher Scientific	BP172-25
DMSO	Sigma-Aldrich	D-5879
DOC	Sigma-Aldrich	D-6750
ECF Substrate	Amersham	RPN5785
EGTA	Sigma-Aldrich	E-4378
Ethanol	Sigma-Aldrich	E/0650DF/C17
Ethylene Glycol	PanReac	121316.1211
FBS	Gibco	REF 10270-106 LOT 42F9680K
Flutamide	Sigma-Aldrich	F-9397
Glycerol	Sigma-Aldrich	G-5516
HCl	Thermo Fisher Scientific	H/1200/PB17
Isoflurane	Ecuphar	LOT 6075188
K_2HPO_4	Sigma-Aldrich	P-3786
KCl	Merk	4936
Ketamin	Dechra	LOT 111755
KH_2PO_4	Pancreac	131509

L-Glutamine	Gibco	REF 25030-081 LOT 2084102
Methanol	Termo Fisher Scientific	M/4000/17
Na_2HPO_4	Sigma	S-9763
NaCl	Termo Fisher Scientific	S/3160/65
$NaH_2PO_4H_20$	Merk	6346
Non-fat Milk	Molico	LOT 9049301B
PFA	Sigma-Aldrich	P-6148
Peanut Oil	Sigma-Aldrich	P-2144
Penicilin-Streptomicin Solution	Gibco	REF 15140-122 LOT 2145455
PVDF membrane	Millipore	IPVH00010
RPMI medium	Gibco	21875-035
SCH58261	Tocris	2270
SDS	Sigma-Aldrich	436143
Sesame Oil	Sigma-Aldrich	S-3547
Sucrose	Termo Fisher Scientific	S/8600/60
TEMED	Sigma-Aldrich	T-9281
Testosterone ELISA Kit	Abcam	108666
Testosterone Propionate	Sigma-Aldrich	86541
Tris-Base	Termo Fisher Scientific	BP152-5
TRITON X100	Merk	11869
Tween-20	Termo Fisher Scientific	BP337-500
WesternBright Sirius HRP substrate	Advansta	K-12043-D20
Xylazine	Dechra	LOT 089502

3.4 Results

In Chapter two we suggested that adenosine receptors could be modulating testosterone levels, peripherally. We hypothesized this interaction could also happen in the CNS, more specifically in microglia. If so, this communication could affect microglia morphology and function and therefore, modulate CNS development and homeostasis.

For this analysis we used the BV2 cell line, exposed with agonists and antagonists for both receptors for two periods of time after which western blot analysis was performed for both receptors.

i. Testosterone influence on A_{2A} Receptor Density in Microglia

First, we evaluated the impact of increasing concentrations of testosterone in the immunoreactivity of the $A_{2A}R$ at 6h and 24h of incubation. At 6h, results show a tendency for an increase in $A_{2A}R$ density when 10nM of testosterone is administered to the cells (**Figure 35 A**). At 24h, increasing concentrations of the sexual hormone caused no differences in the immunoreactivity of the $A_{2A}R$ (**Figure 35 B**). When combining results from both time points we observe an effect of testosterone in $A_{2A}R$ density over time, since there is a tendency for this receptor to reduce its levels at 24 hours of exposure comparing to 6h, especially with 10nM of testosterone (**Figure 35 C**).

To analyze if the impact on the receptor immunoreactivity was in fact due to testosterone, we blocked the AR with the specific antagonist flutamide. Results show that the combination of testosterone and flutamide maintained $A_{2A}R$ immunoreactivity similar to control, at 6 hours (**Figure 36 A**) and 24 hours (**Figure 36 B**) of exposure. The combination of results shows that a longer exposure to flutamide induces a tendency for decreased density of the $A_{2A}R$ (**Figure 36 C**).

i. $A_{2A}R$ Agonist and Antagonist effect on Androgen Receptor immunoreactivity in Microglial cells

In order to analyze if adenosine receptors were modulating AR levels, a second study was performed. For that purpose, cells were incubated for 6 or 24 hours with either CGS21680 ($A_{2A}R$ agonist), SCH58261 or both after which AR protein levels were analyzed. The administration of both agonist and antagonist of the $A_{2A}R$ have no impact on AR density at 6 hours (**Figure 37 A**) nor at 24 hours of exposure (**Figure 37 B**). In

Figure 37 C, we present joined results, which further show that neither CGS21680 nor SCH58261 influence AR expression and therefore, we suggest that testosterone modulates $A_{2A}R$ density, but adenosine receptors do not modulate AR.

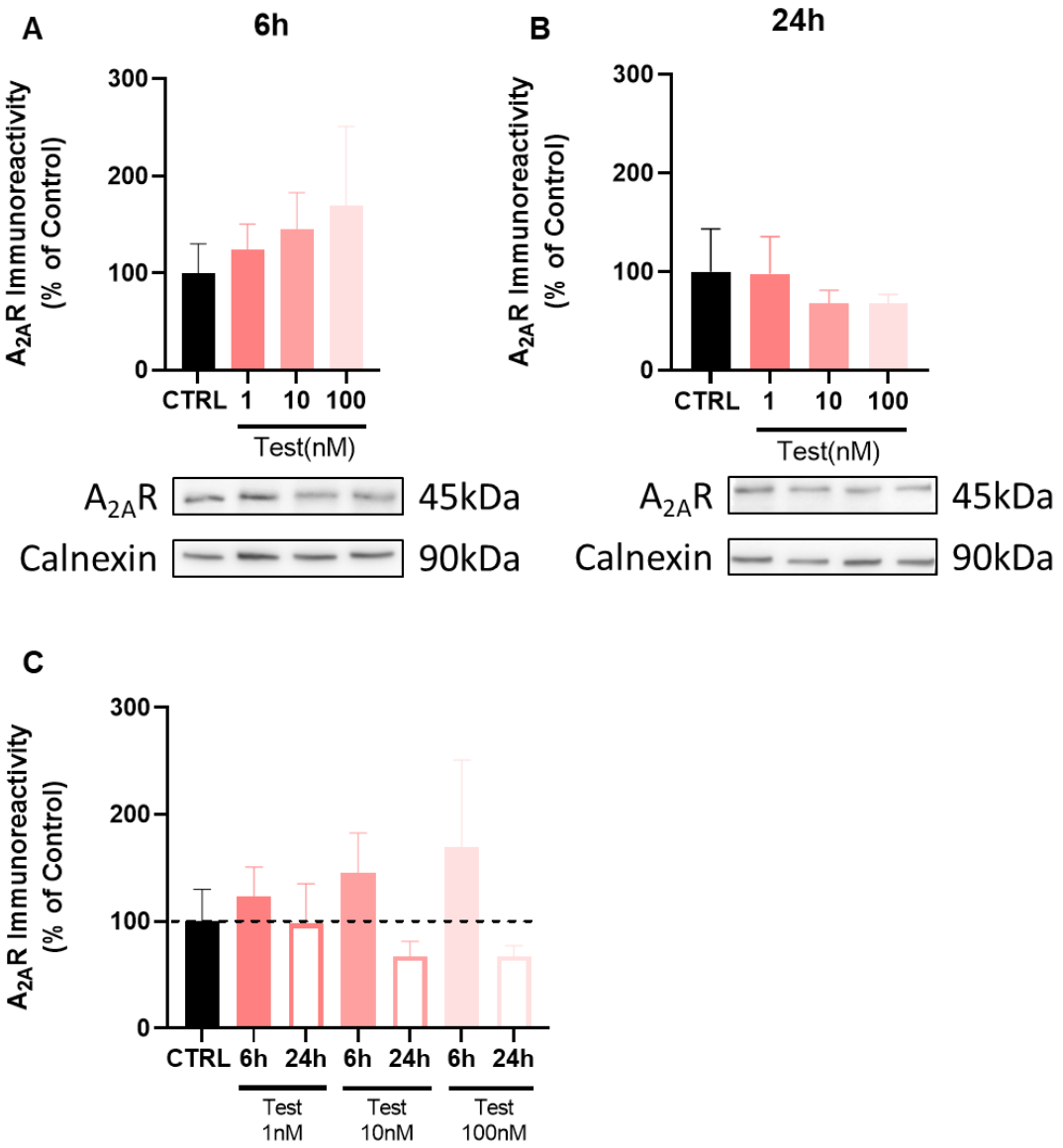

Figure 35: Testosterone has an impact on $A_{2A}R$ density. The effect of testosterone on $A_{2A}R$ immunoreactivity at 6h (**A**) and 24h (**B**) was assessed in the BV-2 cell line. For better understanding of this results, data from both time points was joined in graph **C**. Results are expressed as mean±SEM (n=3-4). Statistical analysis was assessed with One-way ANOVA with Tukey's multiple comparison test.

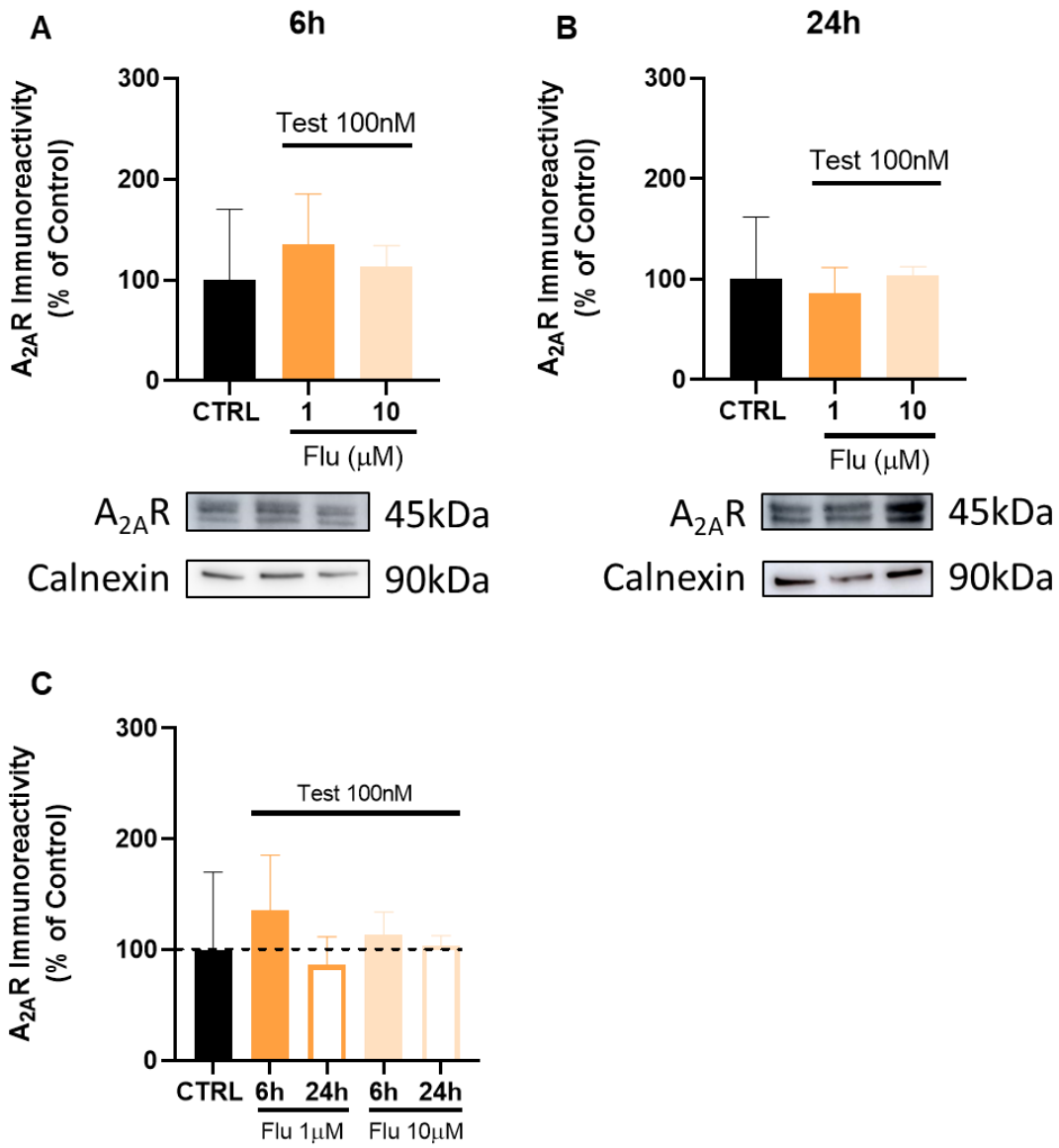

Figure 36: Effect of Flutamide and Testosterone on $A_{2A}R$ density in microglial cells. The inhibitor of AR was used with 100 nM of testosterone, and $A_{2A}R$ density was assessed in the BV-2 cell line at 6h (**A**) and 24h (**B**) of exposure. Data from both time points was combined in graph **C**. Results are expressed as mean±SEM (n=2-4). Statistical analysis was assessed with One-way ANOVA with Tukey's multiple comparison test.

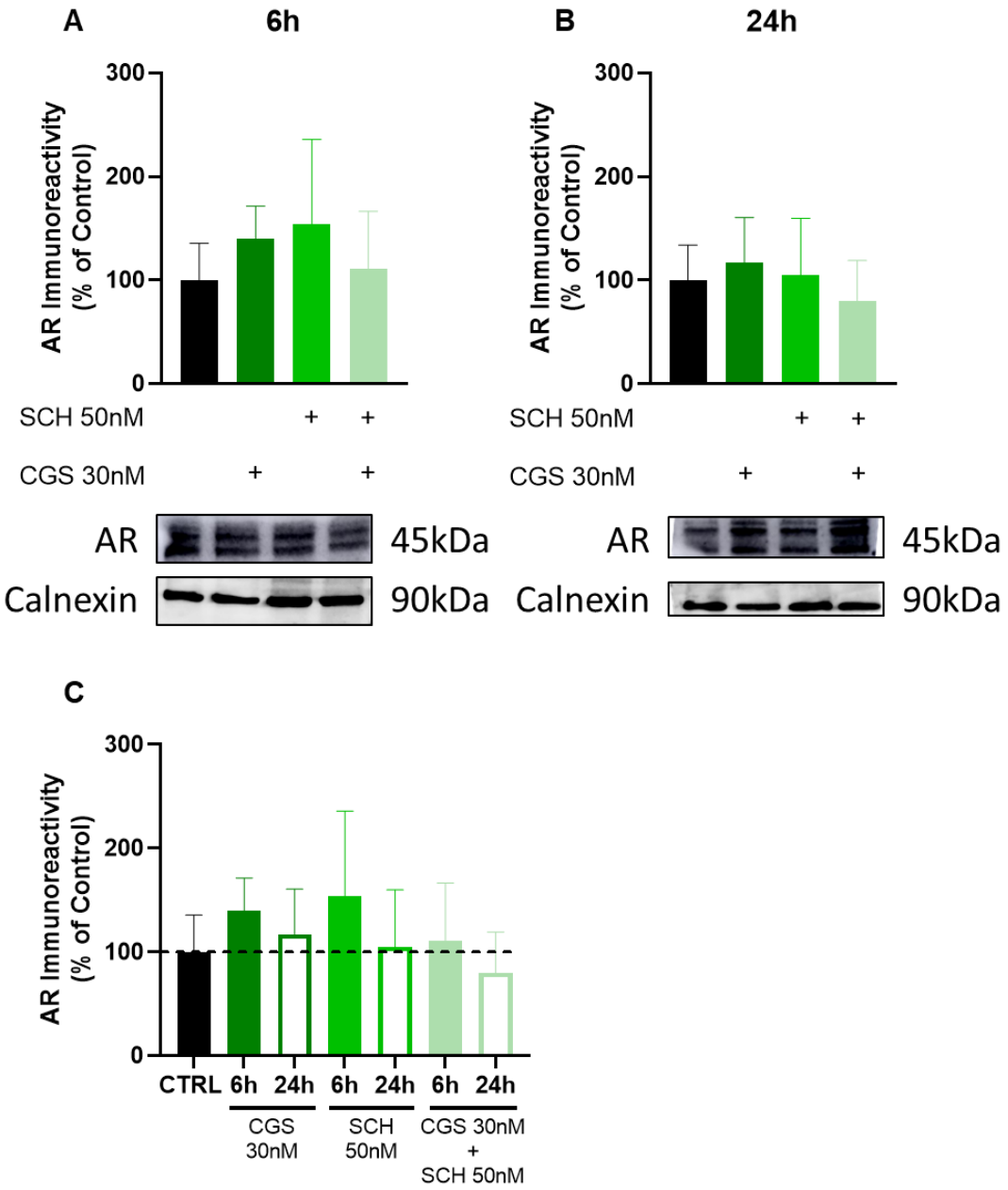

Figure 37: AR content in microglia when $A_{2A}R$ agonist, CGS21680, and the antagonist, SCH58261 are administered. To demonstrate a possible interaction between AR and $A_{2A}R$, CGS21680 and SCH58261 were administrated to the cells and AR reactivity was assessed through western blot analysis at 6h (**A**) and 24h (**B**). In graph **C**, results were combined for better understanding the effect of both drugs. Results are expressed as mean±SEM (n=4-5). Statistical analysis was assessed with One-way ANOVA with Tukey's multiple comparison test.

3.5 Discussion

Regarding the data from Chapter 2, we demonstrated that testosterone peripheral levels are modulated by the administration of SCH58261 in both CTRL or DEX groups, which leads us to hypothesize this interaction could also be happening in the CNS. Furthermore, GR could also play a role on this interaction since the pattern of response to SCH58261 changed whether DEX was or not administered. The interaction between GR and $A_{2A}R$ was already observed *in vitro* by our group, with N9 microglial cells. In this former study it was observed an increase in $A_{2A}R$ density after 24 and 48 hours of exposure to 1μM of DEX (Caetano, 2014). Moreover, adenosine played a role in regulating GR levels in the cytoplasm and nucleus, since the translocation of the receptor was dependent on the activation of $A_{2A}R$ by adenosine, in BV-2 cells (Duarte, 2016). In this study, we focused on the possible interaction between AR and $A_{2A}R$, which was analyzed by activating or inhibiting both receptors in BV-2 cells.

Androgens and adenosine can influence microglia morphology (Orr et al., 2009; Schwarz et al., 2012) through their receptors AR (García-Ovejero et al., 2002) and $A_{2A}R$ (Fredholm et al., 2011). Our results show an effect of testosterone dependent of time and concentration, since increasing concentrations of testosterone at 24 hours of exposure induce a tendency for a reduction in $A_{2A}R$ density, an effect reverted with 24 hours of exposure to flutamide. Cordycepin, an analogous of adenosine, was shown to increase testosterone levels in serum and in mouse Leydig cells, in a dose and time-dependent manner. Furthermore, cordycepin, regulates all 4 adenosine receptors, and their activation was responsible for testosterone production, via Protein Kinase A (PKA)- cyclic adenosine monophosphate (cAMP) signaling pathway (responsible for the regulation of enzyme activation and gene expression) (Leu et al., 2011). However, in this study when CGS21680 and/or SCH58261 were administered to the cells we observe no differences in AR density.

With these results we show that testosterone induces an effect on $A_{2A}R$ density, but $A_{2A}R$ does not modify AR levels, which could mean testosterone levels did not change. Therefore, it would be relevant to study testosterone levels in microglia to understand the effect of $A_{2A}R$ agonists directly. We propose that testosterone modulates $A_{2A}R$ density, which might be responsible for the remodeling of microglia morphology during development in males and therefore, account for the sex differences seen at adulthood in the response to SCH58261 in this model of anxiety.

4

Main Conclusions & Future Perspectives

4.1 Main Conclusions

In this work we observed no effect of prenatal DEX administration on neurodevelopmental behavior nor in anxious-like behavior. Furthermore, no sex differences were observed in CTRL nor DEX groups except for decreased strength in DEX females and delayed eye opening day in CTRL females. However, neonatal testosterone administration, normalized strength and eye opening day, but in general it was not capable of masculinizing behavior. Data not published from our group has detected changes in neurodevelopmental behavior when DEX is administered, as well as anxious-like behavior at adulthood. Therefore, we propose a possible correlation between behaviors at the two time points and suggest that the alterations observed on neurodevelopmental tests may be possible predictive factors for the development of neuropsychiatric disorders later in life.

In this thesis we could not validate the anxiety model, meaning further studies will be needed to assess the original aims of this work. Nonetheless, neonatal administration of testosterone may have a protective effect in androgenized females exposed to DEX. Furthermore, testosterone did not revert DEX-induced atrophy in female microglia and did not have a permissive effect for SCH58261 action. In summary, testosterone influenced behavior, but not microglia morphology, meaning this androgen has a mechanism of modulating behavior independent of these cell's morphology.

Peripheral testosterone levels suggest that blockage of $A_{2A}R$ modulates testosterone levels in the periphery. *In vitro* studies made to analyze this effect centrally, specifically in microglia cells, show a dose and time dependent response of testosterone on $A_{2A}R$ density. However, when activating or blocking the $A_{2A}R$ no differences were detected in AR density, which might be correlated with no changes in testosterone levels. These results lead us to suggest that testosterone might have an impact on microglia morphology during development by modulating $A_{2A}R$. This effect could account for the sex dimorphism observed in microglia morphology and also in anxiety prevalence and treatment.

4.2 Future Perspectives

Taking into account the results reported in the present study, further analysis needs to be performed, namely, to replicate the study in a new cohort of animals to confirm obtained results and to respond to the initial hypothesis of this thesis.

Furthermore, to prove that the sex-specific effects observed on behavior and microglia morphology are due to the action of testosterone, we could use an antagonist of the AR and an inhibitor of aromatase combined or alternatively inject estrogens in males. Lastly, to infer on the impact of DEX, sex and anxiolytic treatment on behavior, the mechanism behind the tripartite communication (GR-A_{2A}R and AR) should be unveiled.

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Supplementary Data

Table S1: Offspring weight from PND0 to PND90. Results described in Chapter 2, Section 2.3.i.

	CTRL ♀			DEX ♀			CTRL + T ♀			DEX + T ♀			CTRL ♂			DEX ♂		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
P0	5.91	0.07	7	5.03	0.09	12	6.06	0.19	16	5.65	0.16	22	6.46	0.11	36	5.58	0.19	31
P7	16.29	0.18	7	13.00	0.51	12	15.00	0.26	16	14.45	0.26	22	16.33	0.17	36	14.70	0.29	33
P14	30.71	0.36	7	22.67	1.21	12	27.88	0.48	16	27.86	0.58	22	30.17	0.31	36	27.48	0.64	33
P21	47.43	0.81	7	36.92	1.62	12	45.38	0.64	16	42.23	0.88	22	45.58	1.09	12	38.67	2.14	12
P28	82.57	0.90	7	67.50	2.09	12	81.50	1.27	16	73.64	1.71	22	81.67	1.79	12	71.50	4.52	12
P35	120.43	1.49	7	105.17	3.35	12	120.81	1.85	16	111.77	2.62	22	131.17	2.56	12	115.92	8.44	12
P42	146.00	2.57	7	133.08	3.41	12	147.94	2.63	16	139.64	3.19	22	177.92	2.91	12	166.64	7.05	11
P49	164.29	2.67	7	153.42	3.44	12	169.25	3.07	16	158.68	3.56	22	219.42	3.99	12	205.64	8.16	11
P56	185.00	2.08	7	174.33	3.25	12	192.75	3.46	16	180.14	4.24	22	258.25	4.55	12	246.64	8.99	11
P63	196.57	1.99	7	187.00	3.51	12	206.06	3.87	16	195.41	5.35	22	282.00	7.15	6	276.36	9.10	11
P70	209.14	3.03	7	198.75	3.06	12	218.81	4.41	16	213.25	3.17	20	300.00	7.72	6	296.45	9.17	11
P77	213.29	3.80	7	206.17	3.46	12	229.31	4.50	16	221.95	3.16	20	321.00	9.70	6	316.45	10.58	11
P84	222.14	3.91	7	215.58	3.95	12	238.00	4.50	16	231.70	3.30	20	340.67	9.69	6	332.55	10.99	11
P90	231.43	4.69	7	218.50	4.37	12	243.69	4.80	16	234.65	4.67	20	349.50	9.54	6	342.91	11.57	11

Table S2: Locomotion raw data from PND5 until PND14. Results described in Chapter 2, Section 2.3.ii.

	CTRL ♀			DEX ♀			CTRL + T ♀			DEX + T ♀			CTRL ♂			DEX ♂		
	Mean	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**
P5	30.00	0.00	7.00	30.00	0.00	12.00	29.63	0.38	16.00	28.36	1.00	22.00	29.89	0.11	36.00	30.00	0.00	33.00
P6	29.57	0.30	7.00	28.58	1.42	12.00	30.00	0.00	16.00	28.23	1.26	22.00	29.57	0.43	23.00	28.32	1.18	22.00
P7	25.57	3.29	7.00	29.50	0.50	12.00	28.00	1.22	16.00	28.82	0.65	22.00	28.39	0.90	23.00	28.59	0.64	22.00
P8	23.43	3.12	7.00	28.50	0.95	12.00	29.25	0.54	16.00	29.00	0.70	22.00	27.39	0.93	23.00	27.77	0.97	22.00
P9	25.00	2.57	7.00	25.83	2.36	12.00	26.63	1.47	16.00	28.86	0.80	22.00	25.09	1.45	23.00	28.32	0.88	22.00
P10	23.86	3.83	7.00	26.17	1.69	12.00	26.69	1.26	16.00	24.09	1.72	22.00	22.13	1.69	23.00	25.86	1.28	22.00
P11	18.57	3.29	7.00	21.08	2.86	12.00	19.88	2.37	16.00	22.77	1.72	22.00	18.09	1.64	23.00	20.18	1.91	22.00
P12	13.71	2.18	7.00	17.58	2.61	12.00	17.13	1.14	16.00	20.00	1.94	22.00	11.70	1.30	23.00	12.59	2.06	22.00
P13	10.43	2.03	7.00	15.00	2.47	12.00	15.19	2.02	16.00	11.73	1.99	22.00	9.52	1.56	23.00	13.55	1.74	22.00
P14	8.57	2.63	7.00	10.67	2.02	12.00	9.81	1.22	16.00	13.14	1.75	22.00	10.87	1.52	23.00	10.91	1.57	22.00

Table S3: Surface Righting Reflex raw data from PND5 until PND10. Results described in Chapter 2, Section 2.3.ii.

	CTRL ♀			DEX ♀			CTRL + T ♀			DEX + T ♀			CTRL ♂			DEX ♂		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
P5	1,21	0,15	7	1,71	0,17	12	1,38	0,11	16	1,68	0,20	22	1,36	0,07	36	1,41	0,08	33
P6	1,21	0,10	7	1,38	0,11	12	1,31	0,09	16	1,64	0,14	22	1,35	0,05	23	1,30	0,06	22
P7	1,29	0,10	7	1,50	0,06	12	1,47	0,06	16	1,48	0,06	22	1,37	0,05	23	1,39	0,07	22
P8	1,29	0,10	7	1,50	0,06	12	1,38	0,09	16	1,43	0,04	22	1,30	0,05	23	1,39	0,06	22
P9	1,43	0,07	7	1,50	0,00	12	1,47	0,06	16	1,48	0,02	22	1,37	0,05	23	1,36	0,05	22
P10	1,36	0,09	7	1,33	0,07	12	1,34	0,08	16	1,36	0,05	22	1,20	0,05	23	1,36	0,05	22

Table S4: Cliff Aversion raw data from PND5 to PND10. R Results described in Chapter 2, Section 2.3.ii.

	CTRL ♀			DEX ♀			CTRL + T ♀			DEX + T ♀			CTRL ♂			DEX ♂		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
P5	20.43	4.67	7.00	19.75	3.69	12.00	21.25	2.96	16.00	21.77	2.30	22.00	22.19	1.94	36.00	20.06	2.01	32.00
P6	7.43	3.77	7.00	6.17	2.25	12.00	22.81	2.83	16.00	7.82	1.96	22.00	14.87	2.76	23.00	9.14	2.18	22.00
P7	8.29	3.77	7.00	8.75	2.92	12.00	10.94	2.55	16.00	8.64	1.16	22.00	9.09	1.85	23.00	7.00	1.43	21.00
P8	3.43	0.48	7.00	9.17	2.19	12.00	12.06	2.13	16.00	8.50	1.68	22.00	9.04	1.82	23.00	7.68	1.68	22.00
P9	7.86	1.93	7.00	5.83	0.67	12.00	6.31	0.76	16.00	6.68	1.00	22.00	6.74	0.69	23.00	5.52	0.67	21.00
P10	6.86	0.80	7.00	7.75	1.24	12.00	5.94	0.64	16.00	6.09	0.72	22.00	7.00	0.63	23.00	7.18	0.82	22.00

Table S5: Negative Geotaxis Reaction test raw data from PND5 to PND14. Results described in Chapter 2, Section 2.3.ii.

	CTRL ♀			DEX ♀			CTRL + T ♀			DEX + T ♀			CTRL ♂			DEX ♂		
	Mean	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**
P5	23.43	3.29	7.00	27.58	1.32	12.00	27.88	1.27	16.00	25.73	1.41	22.00	25.86	1.18	36.00	23.13	1.41	32.00
P6	21.29	3.27	7.00	17.75	2.87	12.00	20.63	1.99	16.00	20.41	1.63	22.00	19.00	1.82	23.00	16.23	2.05	22.00
P7	13.14	3.34	7.00	15.67	1.92	12.00	19.31	1.92	16.00	18.27	1.63	22.00	14.91	1.55	23.00	16.36	1.59	22.00
P8	11.57	1.93	7.00	13.33	1.34	12.00	12.81	1.65	16.00	15.41	1.68	22.00	11.57	0.97	23.00	11.45	1.07	22.00
P9	9.43	1.48	7.00	9.83	1.31	12.00	10.88	0.88	16.00	14.00	1.45	22.00	9.74	1.20	23.00	9.86	1.12	22.00
P10	10.00	2.55	7.00	11.25	2.23	12.00	8.44	1.06	16.00	12.09	1.18	22.00	10.04	0.94	23.00	9.73	0.83	22.00
P11	10.00	2.29	7.00	7.75	1.33	12.00	8.88	1.37	16.00	9.18	0.69	22.00	7.48	0.84	23.00	7.45	0.92	22.00
P12	7.43	1.82	7.00	7.92	1.03	12.00	7.31	0.96	16.00	6.73	0.69	22.00	7.04	0.67	23.00	7.91	1.25	22.00
P13	6.14	1.10	7.00	7.50	0.65	12.00	5.44	0.68	16.00	6.55	0.86	22.00	5.00	0.51	23.00	5.64	0.66	22.00
P14	6.29	0.52	7.00	6.00	0.92	12.00	5.50	0.40	16.00	5.14	0.37	22.00	4.52	0.27	23.00	6.86	0.57	22.00

Table S6: Nest Seeking raw data from PND5 until PND15. Results described in Chapter 2, Section 2.3.ii.

	CTRL ♀			DEX ♀			CTRL + T ♀			DEX + T ♀			CTRL ♂			DEX ♂		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
P5	29.93	8.25	7.00	58.58	5.26	12.00	73.78	10.22	16.00	52.66	8.19	22.00	55.11	6.65	35.00	48.17	5.71	33.00
P6	73.29	18.63	7.00	85.13	10.92	12.00	38.56	8.41	16.00	69.50	9.91	22.00	46.89	6.69	22.00	64.18	8.54	22.00
P7	57.64	14.01	7.00	35.25	8.30	12.00	38.91	7.02	16.00	35.61	6.90	22.00	69.18	6.23	22.00	44.66	9.23	22.00
P8	39.57	16.81	7.00	55.29	13.28	12.00	35.47	8.79	16.00	37.77	8.63	22.00	45.09	7.85	22.00	41.05	6.92	22.00
P9	23.64	10.15	7.00	43.46	8.54	12.00	49.34	8.19	16.00	24.84	5.61	22.00	38.95	8.12	22.00	54.82	8.69	22.00
P10	63.50	11.36	7.00	17.13	6.34	12.00	35.22	10.38	16.00	34.34	8.16	22.00	52.16	9.10	22.00	42.64	6.87	22.00
P11	47.93	17.47	7.00	33.29	8.62	12.00	33.09	10.61	16.00	33.20	9.11	22.00	46.09	9.48	22.00	33.48	6.52	22.00
P12	36.29	17.45	7.00	38.67	8.18	12.00	43.66	10.43	16.00	28.68	5.94	22.00	33.70	7.35	22.00	35.48	7.47	22.00
P13	36.50	12.20	7.00	23.25	10.96	12.00	24.84	7.25	16.00	24.41	8.23	22.00	31.93	8.40	22.00	8.55	3.69	22.00
P14	52.93	15.19	7.00	17.63	7.61	12.00	21.16	7.01	16.00	11.82	4.36	22.00	16.32	5.34	22.00	22.55	5.83	22.00
P15	36.57	17.32	7.00	12.71	6.55	12.00	28.22	7.47	16.00	13.18	6.28	22.00	8.18	3.67	22.00	10.48	5.85	22.00

Table S7: Wire Suspension raw data from PND10 until PND14. Results described in Chapter 2, Section 2.3.ii.

	CTRL ♀			DEX ♀			CTRL + T ♀			DEX + T ♀			CTRL ♂			DEX ♂		
	Mean	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**
P10	8.57	0.00	7.00	6.00	0.00	12.00	9.19	0.00	16.00	9.55	0.00	22.00	9.30	0.00	23.00	8.73	0.00	22.00
P11	10.00	0.00	7.00	8.25	0.00	12.00	9.63	0.00	16.00	8.91	0.00	22.00	9.26	0.00	23.00	9.05	0.00	22.00
P12	10.00	0.00	7.00	8.08	0.60	12.00	10.00	0.00	16.00	9.77	0.23	22.00	9.70	0.23	23.00	9.41	0.28	22.00
P13	9.86	0.14	7.00	8.58	0.62	12.00	9.81	0.19	16.00	9.82	0.14	22.00	9.83	0.14	23.00	8.86	0.39	22.00
P14	10.00	0.00	7.00	9.17	0.56	12.00	9.81	0.19	16.00	9.91	0.09	22.00	10.00	0.00	23.00	9.27	0.27	22.00

Table S8: Auditory Startle raw data. Results described in Chapter 2, Section 2.3.ii.

	CTRL ♀			DEX ♀			CTRL + T ♀			DEX + T ♀			CTRL ♂			DEX ♂		
	Mean	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**
P11	0.00	0.00	7.00	0.00	0.00	12.00	0.00	0.00	16.00	0.00	0.00	22.00	0.00	0.00	23.00	0.00	0.00	22.00
P12	0.00	0.00	7.00	8.33	8.33	12.00	0.00	0.00	16.00	0.00	0.00	22.00	4.35	4.35	23.00	0.00	0.00	22.00
P13	100.00	0.00	7.00	91.67	8.33	12.00	93.75	6.25	16.00	90.91	6.27	22.00	95.65	4.35	23.00	90.91	6.27	22.00
P14	100.00	0.00	7.00	100.00	0.00	12.00	100.00	0.00	16.00	100.00	0.00	22.00	100.00	0.00	23.00	100.00	0.00	22.00

Table S9: Eye opening raw data. Results described in Chapter 2, Section 2.3.ii.

	CTRL ♀			DEX ♀			CTRL + T ♀			DEX + T ♀			CTRL ♂			DEX ♂		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
P12	0.00	0.00	7.00	0.00	0.00	12.00	0.00	0.00	16.00	0.00	0.00	22.00	0.00	0.00	23.00	0.00	0.00	22.00
P13	0.00	0.00	7.00	25.00	13.06	12.00	12.50	8.54	16.00	0.00	0.00	22.00	8.70	6.01	23.00	18.18	8.42	22.00
P14	28.57	18.44	7.00	50.00	15.08	12.00	37.50	12.50	16.00	54.55	10.87	22.00	30.43	9.81	23.00	68.18	10.16	22.00
P15	100.00	0.00	7.00	91.67	8.33	12.00	93.75	6.25	16.00	95.45	4.55	22.00	100.00	0.00	23.00	86.36	7.49	22.00
P16	100.00	0.00	7.00	100.00	0.00	12.00	100.00	0.00	16.00	100.00	0.00	22.00	100.00	0.00	23.00	100.00	0.00	22.00
P17	100.00	0.00	7.00	100.00	0.00	12.00	100.00	0.00	16.00	100.00	0.00	22.00	100.00	0.00	23.00	100.00	0.00	22.00

Table S10: Open Field Test raw data of females. Results described in Chapter 2, Section 2.3.iii.

	CTRL ♀			DEX ♀			DEX+SCH ♀			CTRL+T ♀			CTRL+T+SCH ♀			DEX+T ♀			DEX+T+SCH ♀		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Distance Travelled (m)	16.35	0.62	7.00	15.76	0.77	4.00	12.07	0.82	8.00	17.62	1.16	5.00	16.14	0.87	11.00	13.55	1.01	10.00	15.07	0.76	10.00
Mean Speed (m/s)	0.05	0.00	7.00	0.05	0.00	4.00	0.04	0.00	8.00	0.06	0.00	5.00	0.05	0.00	11.00	0.05	0.00	10.00	0.05	0.00	10.00
Time in Center (s)	17.36	4.95	7.00	27.35	3.58	4.00	13.76	3.07	8.00	20.30	2.29	5.00	17.89	3.78	11.00	22.62	3.33	10.00	18.51	3.79	10.00

Table S11: Elevated Plus Maze raw data of females. Results described in Chapter 2, Section 2.3.iii.

	CTRL ♀			DEX ♀			DEX+SCH ♀			CTRL+T ♀			CTRL+T+SCH ♀			DEX+T ♀			DEX+T+SCH ♀		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Time in OA (s)	0.43	0.06	7.00	0.54	0.02	4.00	0.31	0.07	8.00	0.45	0.05	5.00	0.49	0.07	10.00	0.43	0.05	10.00	0.51	0.02	10.00
OA Entries	9.29	1.13	7.00	12.50	1.76	4.00	7.63	1.28	8.00	11.60	1.29	5.00	7.73	1.25	11.00	10.50	1.17	10.0	9.80	0.93	10.00