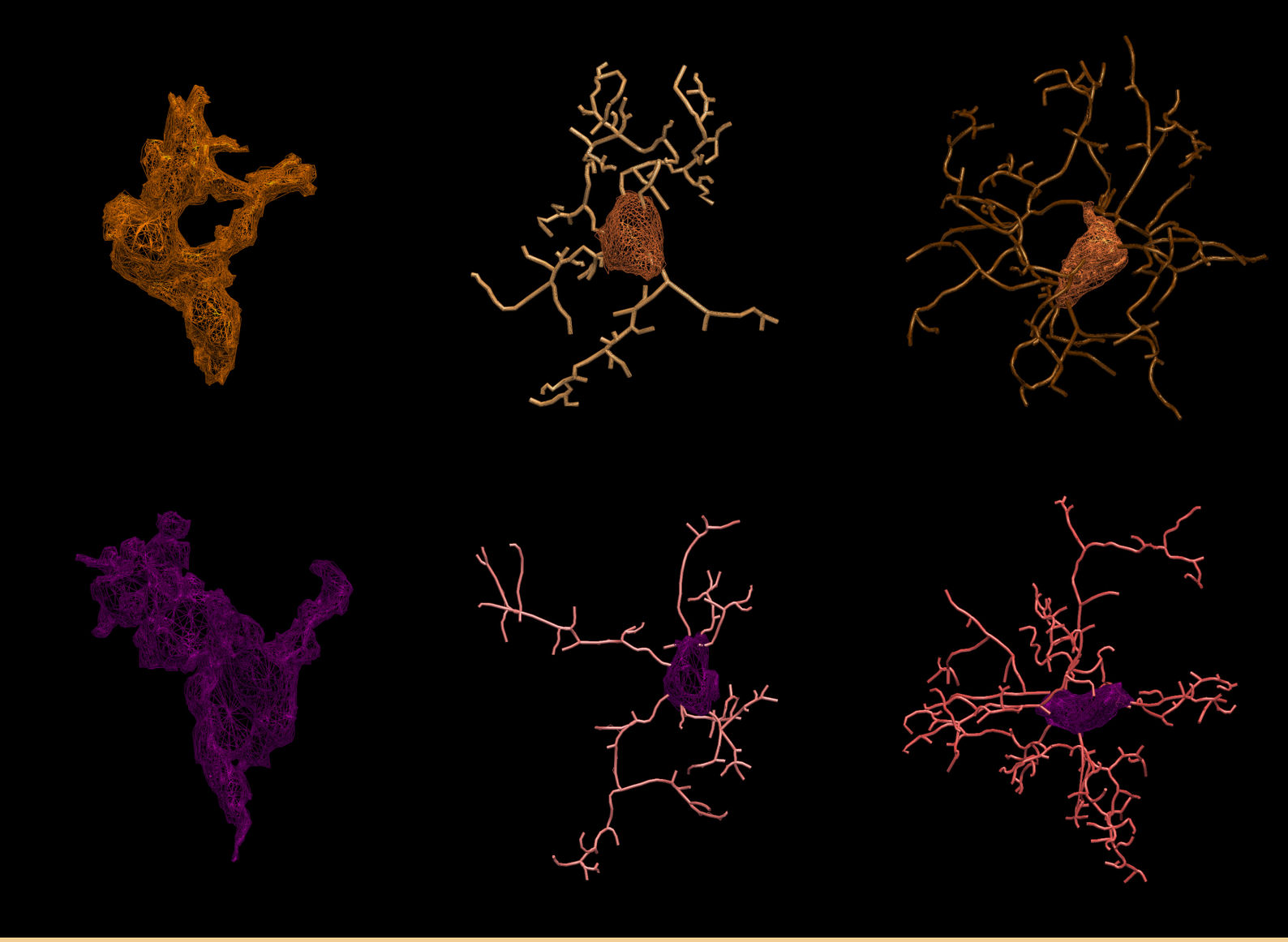


Carla Filipa Simões Henriques

GENDER DIMORPHISM OF MICROGLIA MORPHOLOGY

ADENOSINE AND TESTOSTERONE AS PUTATIVE MODULATORS IN TRANSGENDER MICROGLIA EXPERIMENTS

Dissertation to obtain the Master degree in Biomedical Research, performed under the scientific supervision of Doctor Catarina Alexandra Reis Vale Gomes and co-supervision of Doctor António Francisco Rosa Gomes Ambrósio and presented to the Faculty of Medicine of the University of Coimbra

July 2017

· U C ·

Universidade de Coimbra

On the front of the page: Microglia cells from the prefrontal cortex of male (top) and female (buttom) Wistar rats at PND0 (left), PND7 (center) and PND33 (right). Brain slices were stained for Iba-1 and microglia cells were tridimensionally reconstructed with Neurolucida software.

GENDER DIMORPHISM OF MICROGLIA MORPHOLOGY

ADENOSINE AND TESTOSTERONE AS PUTATIVE MODULATORS IN TRANSGENDER MICROGLIA EXPERIMENTS

Carla Filipa Simões Henriques

Dissertation presented to the Faculty of Medicine of the University of Coimbra. The work was performed in the Retinal Dysfunction and Neuroinflammation Lab of the Institute for Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, under the scientific supervision of Doctor Catarina Alexandra Reis Vale Gomes and co-supervision of Doctor António Francisco Rosa Gomes Ambrósio.

University of Coimbra

2017

The experimental work described in the present thesis was performed in the Retinal Dysfunction and Neuroinflammation Lab, at the Institute for Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, Portugal.

Financial support was granted by the Research Support Office (GAI, Faculty of Medicine, University of Coimbra, Portugal); the Foundation for Science and Technology, Portugal (PEst UID/NEU/04539/2013); COMPETE-FEDER (POCI-01-0145-FEDER-007440); Centro 2020 Regional Operational Programme (CENTRO-01-0145-FEDER-000008: BrainHealth 2020); and Santander Totta.

À minha família

“Considere a sua origem. Não foste formado para viver como os brutos, mas para seguir a virtude e o conhecimento.”

A Divina Comédia, de Dante Alighieri

ACKNOWLEDGEMENTS

Nesta secção empenho-me em homenagear aqueles com os quais me cruzo e que deixam marcas, porque ninguém é completo sozinho.

Agradeço, em primeiro lugar, à minha orientadora, Professora Catarina Gomes, por ter despertado a minha paixão pelo tema sobre o qual me debrucei durante este ano. Encontrei em si um exemplo de trabalho e dedicação exaustivos, alguém possuidor de uma perspicácia e convicção absolutamente cativantes, incapaz de não se questionar perante os factos. São muitos os ensinamentos que levo comigo, mas aquele no qual reflito a cada dia de trabalho vem das suas palavras “O trabalho é (quase) sempre compensado!”. Assim, mais importante do que a recompensa, é a certeza de que fizemos o nosso melhor. É um orgulho ser sua aluna!

Ao Professor Francisco Ambrósio, por me ter acolhido no seu grupo, enquanto aluna de mestrado e, proporcionado as condições necessárias à elaboração desta tese.

Ao Professor Henrique Girão, por me ter permitido fazer parte desta família que é o MIB, pela energia e alegria que deposita na ciência que faz. É um exemplo!

Agradeço também à Filipa Baptista, à Inês Almeida, ao Miguel Pinheiro e à Rita Gaspar, aqueles que acompanharam a minha jornada mais de perto. Foram essenciais os momentos de partilha, discussão e argumentação, que tanto me fizeram crescer enquanto cientista.

À Helena e à Joana dirijo um agradecimento especialmente doce por me terem acompanhado durante os primeiros passos deste percurso. Muitas vezes sorrio ao pensar que eram as minhas irmãs na ciência. Do mesmo modo que os irmãos mais novos almejam alcançar os mais velhos, eu persegui o vosso exemplo de dedicação e proatividade.

À Catarina Neves, à Joana Martins e à Raquel Boia, agradeço pela companhia, pela exigência, por nunca permitirem que desejasse apenas o alcançável. Apesar de uma convivência que se iniciou tímida, tornaram-se três das pessoas que mais me fazem sorrir, três colegas e amigas.

Ao Rafael, que mais do que um colega é um amigo, agradeço a amizade e o companheirismo. Aos restantes membros do grupo que fizeram parte desta jornada, ao António, à Elisa Campos, à Inês Aires, ao João Martins, à Maria, à Raquel Santiago e ao Samuel, bem-haja! As horas infindáveis de trabalho são tão mais fáceis em boa companhia! À Susana Bacelar, pela amabilidade e serenidade que sempre demonstrou.

Um obrigada muito especial aos grandes amigos que Coimbra me trouxe, aqueles que nunca saem do coração. Levo-vos sempre comigo pois não consigo separar-me de vós!

À Maria Inês, à Ana Sofia, ao Fábio, ao André, ao Miguel, ao Eurico, à Tomás, à Café, e à Juliana. Quero ver-vos brilhar!

À Cátia Antunes e ao Zé Miguel Branco, aqueles que nem a distância consegue afastar. É um orgulho caminhar ao vosso lado por tantos anos.

Ao Carlos, agradeço a sua exigência que me faz ser melhor, a serenidade que compensa a minha euforia, as palavras parcas que dizem tudo. Completas-me.

À minha família, aquele amor incondicional que me proporcionou tudo. Aos meus avós, Alice e António, que sempre me incentivaram sempre a seguir os meus sonhos. Ao tio António por nunca esconder orgulho a cada pequeno passo meu. Aos meus pais agradeço o seu empenho na minha educação e a sensatez que me incutiram. Pelo equilíbrio que representam, pelo suporte indispensável que são e pela exigência que demonstraram em todas as etapas do meu percurso académico. Nunca foi demais seguir o vosso exemplo! À minha irmã Catarina, que caminhou sempre a meu lado, respeitando as minhas decisões como se estas fossem as mais acertadas. Agradeço exemplo sóbrio e o gesto meigo que te caracterizam, e a tua presença em todas as minhas conquistas, que tomavas como certas antes de se concretizarem. Ao Bruno e à Débora, que entraram na família e a completaram. Obrigada família, porque só me ensinaram a sorrir.

TABLE OF CONTENTS

TABLE OF CONTENTS

FIGURES AND TABLES LIST

FIGURES LIST

TABLES LIST

PUBLICATIONS

Publications

M. Pinheiro, **C. Henriques**, R. Gaspar, H. Pinheiro, J. M. Duarte, A. F. Ambrósio, C. A. Ribeiro, R. A. Cunha, C. A. Gomes. ***Microglia morphology across the brain in A_{2A}R KO mice: gender specificities.*** (manuscript in preparation)

Poster Presentations

C. Henriques; J. Duarte; H. Pinheiro; R. Gaspar; I. Almeida; P. Patrício; A. Mateus-Pinheiro; N. Alves; B. Coimbra; S. Henriques; C. Cunha; C. A. Ribeiro; N. Sousa; R. A. Cunha; A. J. Rodrigues; L. Pinto; A. F. Ambrósio; C. A. Gomes. ***Chronic blockade of adenosine A_{2A} receptors: gender-specific reprogramming of microglia morphology in the PFC.*** *XV Portuguese Society for Neuroscience meeting.* Poster presentation. 25-26 May 2017, Braga, Portugal.

M. Pinheiro, R. Gaspar, H. Pinheiro, **C. Henriques**, J. M. Duarte, R. A. Cunha, A. F. Ambrósio, C. A. Gomes. ***Portraying gender differences in microglia morphology across brain regions.*** *XV Portuguese Society for Neuroscience meeting.* Poster presentation. 25-26 May 2017, Braga, Portugal.

Oral Communications

C. Henriques, J. Duarte, H. Pinheiro, R. Gaspar, A. F. Ambrósio, Catarina A. Gomes. ***Gender determination of microglia morphology in the prefrontal cortex: born similar, age differently.*** *XLVII Meeting of the Portuguese Society for Pharmacology*. Oral communication. 2-4 February 2017, Coimbra, Portugal.

C. Henriques; J. Duarte; H. Pinheiro; R. Gaspar; I. Almeida; P. Patrício; A. Mateus-Pinheiro; N. Alves; B. Coimbra; S. Henriques; C. Cunha; C. A. Ribeiro; N. Sousa; R. A. Cunha; A. J. Rodrigues; L. Pinto; A. F. Ambrósio; C. A. Gomes. ***Chronic blockade of adenosine A_{2A} receptors: gender-specific reprogramming of microglia morphology in the PFC.*** *XV Portuguese Society for Neuroscience meeting.* Oral communication. 25-26 May 2017, Braga, Portugal.

ABBREVIATIONS LIST

A

$A_{2A}R$ Adenosine A_{2A} receptor
Amy Amygdala

B

BDNF Brain-derived neurotrophic factor
BSA Bovine serum albumin

C

CNS Central nervous system

D

DAPI 4',6-diamidino-2-phenylindole
DEX Dexamethasone

E

E# Embryonic day #
EPM Elevated plus maze
ER Estrogen receptors

G

CG Glucocorticoids
GW# Gestational week #
GNX Gonadectomy

H

H Hours
HBSS Hank's balanced salt solution
Hip Hippocampus
HPA Hypothalamic-pituitary-adrenal axis

I

Iba1 Ionized calcium-binding adaptor molecule 1
Ip Intraperitoneal

K

KO Knockout

L

LPS Lipopolysaccharide

N

NOR Novel object recognition
NT Non-treated

O

OB Olfactory bulb
OF Open field
OVX Ovariectomy

P

PBS Phosphate buffered saline
PFA Paraformaldehyde
PFC Prefrontal cortex
PND# Postnatal day #

R

RT Room temperature

S

SEM Standard error of mean
Str Striatum

T

Tfm Testicular feminization
TP Testosterone propionate

V

VH Vehicle

W

WT Wild-type

Y

YS Yolk sac

3D Tridimensional

ABSTRACT

Stressful events in the prenatal and in the early life periods have been associated with the genesis of neuropsychiatric disorders. Importantly, personalised therapeutics are essential in these disorders, since some of them possess gender specificities in the genesis, clinical presentation and therapeutic efficacy. Microglia, the resident immune cells of the central nervous system, have been implicated in the pathophysiology of these disorders, namely anxiety. For instance, in a previous study from our group, we reported a gender-specific morphological rearrangement of microglia cells in a model of chronic anxiety with developmental genesis (prenatal exposure to glucocorticoids). Moreover, these cells present a gender-specific morphology at adulthood, under physiological conditions.

Adenosine A_{2A} receptors ($A_{2A}R$) are well described modulators of microglia, proposed as therapeutic targets in neuropsychiatric disorders. The modulation of $A_{2A}R$ promotes morphologic alterations in these cells, which are widely associated with their function. In our model of chronic anxiety with developmental genesis, the chronic blockade of these receptors normalized microglia morphology and ameliorated anxious-like behaviour in males, but not in females. Moreover, this modulation of $A_{2A}R$ at adulthood *per se* promoted a gender-biased effect: an atrophy in microglia morphology and an increase in anxious-like behaviour in females, without affecting males. These gender specificities in the modulation of microglia morphology and in the presentation of anxious-like behaviour in this animal model led us to hypothesize an hormonal influence underlying this process. A protective role was suggested for testosterone since, in males, an ontogenic surge of this hormone at the day of birth is responsible for the masculinisation of brain and behaviour.

The present thesis pursues three main objectives: to further clarify the gender-biased modulation of microglia morphology by $A_{2A}R$, to screen the time window of appearance of gender differences in microglia morphology in the prefrontal cortex (PFC), and to disclose the role of testosterone in the presentation of anxious-like behaviour and associated morphological reassignment of microglia in the PFC.

In Section 3.1., we analysed microglia morphology in the PFC from $A_{2A}R$ knockout mice, in order to assess whether the absence of expression of this receptor early in development promotes a gender-specific morphological rearrangement of these cells. The lack of expression of $A_{2A}R$ in these animals promoted an hypertrophy of microglia morphology in the PFC of females, but had no effect in males. Thus, we can report the same gender-effect previously described by blocking $A_{2A}R$ at adulthood. However, in

females, the morphological rearrangement of microglia occurs in opposite fashions, according to the developmental stage of interference.

In Section 3.2., we analysed microglia morphology in the PFC from Wistar rats, screening for the surge of gender differences in these cells, from the early neurodevelopment (PND0 and PND7) until youth (PND33). We did not observe differences in microglia morphology in the PFC between genders until PND33. However, when we analysed the performance of these animals in the elevated plus maze test, a test that measures anxious-like behaviour, we were able to report a higher “disinhibition” of females at that age. In this section, we determined that the differences previously described in microglia morphology in the PFC may arise between PND33 and PND90. Moreover, we concluded that behavioural gender differences surge before the differences in microglia morphology are observable.

In Section 3.3., we mimicked the neonatal peak of testosterone that occurs in males by injecting testosterone in females, and analysed microglia morphology in the PFC and anxious-like behaviour at PND30. We observed that the neonatal androgenisation did not alter microglia morphology in the PFC of females at PND33, however this was able to partially masculinise their behaviour: we reported an intermediate phenotype between males and females in anxious-like behaviour. These results led us to conclude that the neonatal peak of testosterone in males is necessary, but not sufficient to fully masculinise anxious-like behaviour.

This work highlights the imperative role of gender in the neuroimmune development. It would be valuable to consider gender in pharmacological research, since differences in anxious-like behaviour are already observable at youth and can be partially reversed by male hormones. In the future, gender-directed therapeutics may increase the effectiveness of drugs, reducing these public health problems and improving lifestyle.

RESUMO

Eventos indutores de *stress* que ocorrem cedo no desenvolvimento têm sido associados à génese de doenças neuropsiquiátricas. Terapêuticas personalizadas são essenciais nestas doenças, atendendo a especificidades de género que algumas destas apresentam na sua génese, apresentação clínica e resposta à terapêutica. As células da microglia, células imunes do sistema nervoso central, têm sido implicadas na fisiopatologia destas doenças, nomeadamente na ansiedade. Por exemplo, num estudo anterior do nosso grupo, observámos alterações morfológicas destas células, que são dependentes do género, num modelo de ansiedade crónica com génese no desenvolvimento (exposição pré-natal a glucocorticóides). Além disso, estas células apresentam um dimorfismo morfológico dependente do género na idade adulta, em condições fisiológicas.

Os recetores A_{2A} da adenosina ($A_{2A}R$) são moduladores bem descritos das células da microglia, que têm sido propostos como alvos terapêuticos em doenças neuropsiquiátricas. A modulação destes recetores promove alterações na morfologia da microglia, que se associam à sua função. No modelo de ansiedade crónica com génese no desenvolvimento, o bloqueio crónico dos $A_{2A}R$ reverte as alterações morfológicas e o comportamento de tipo ansioso em machos, mas não em fêmeas. Esta modulação dos $A_{2A}R$ na idade adulta, por si só, promove um efeito dependente do género: uma atrofia na morfologia da microglia e um aumento do comportamento de tipo ansioso em fêmeas, sem afetar nenhum destes parâmetros em machos. Estas especificidades de género na modulação da morfologia da microglia e na apresentação do comportamento de tipo ansioso neste modelo animal levaram-nos a colocar como hipótese uma influência hormonal neste processo. Foi, assim, sugerido um papel protetor da testosterona nos machos, atendendo à ocorrência de um pico ontogénico desta hormona no dia do nascimento, que é responsável pela masculinização do cérebro e do comportamento.

Os principais objetivos da presente tese são: ajudar a clarificar a modulação específica de género que ocorre na morfologia da microglia pelos $A_{2A}R$, clarificar a janela temporal de surgimento das diferenças de género na morfologia da microglia no córtex pré-frontal (CPF), e avaliar o papel da testosterona na apresentação do comportamento de tipo ansioso e do rearranjo morfológico da microglia que lhe está associado no CPF.

Na Secção 3.1., analisámos a morfologia da microglia no CPF de ratinhos que não expressam o $A_{2A}R$, a fim de verificar se a ausência da expressão do mesmo cedo no desenvolvimento promove um rearranjo morfológico destas células de um modo dependente do género. A ausência da expressão do $A_{2A}R$ promoveu uma hipertrofia da

morfologia da microglia no CPF de fêmeas, mas não teve efeito nos machos. Deste modo, observámos o mesmo efeito do género previamente descrito aquando do boqueio farmacológico do recetor na idade adulta. Contudo, nas fêmeas, o rearranjo morfológico da microglia ocorreu em sentidos opostos, em função do estado de desenvolvimento aquando da intervenção.

Na Secção 3.2., analisámos a morfologia da microglia no CPF de ratos *Wistar*, em várias fases da vida dos animais, procurando identificar o momento em que surgem as diferenças de género nestas células, desde cedo no desenvolvimento (dia pós-natal, DPN0 e DPN7) até à juventude (DPN33). Não observámos diferenças de género na morfologia da microglia no CPF até ao DPN33. Contudo, quando analisámos o desempenho destes animais num teste que avalia comportamento de tipo ansioso, observámos uma maior "desinibição" das fêmeas naquela idade. Assim, concluímos que as diferenças de género na morfologia da microglia previamente descritas no CPF surgirão entre os DPN33 e DPN90. Concluímos também que as diferenças de género no comportamento surgem antes que as diferenças na morfologia da microglia sejam observáveis.

Na Secção 3.3., mimetizámos o pico neonatal de testosterona que ocorre nos machos através da injeção desta hormona em fêmeas, e analisámos a morfologia da microglia no CPF e o comportamento de tipo ansioso ao DPN30. Observámos que a androgenização neonatal não modificou a morfologia da microglia no CPF das fêmeas ao DPN33; contudo, masculinizou parcialmente o seu comportamento: observámos um fenótipo intermédio entre o comportamento de tipo ansioso de machos e de fêmeas. Concluímos que o surgimento neonatal de testosterona que ocorre nos machos é necessário, mas não suficiente para masculinizar completamente o comportamento tipo ansioso.

Este trabalho evidencia o papel imperativo do género no desenvolvimento neuroimune. Ter em consideração o género seria uma mais valia para a investigação em farmacologia, tendo em conta que as diferenças no comportamento de tipo ansioso são já visíveis na juventude e que podem ser parcialmente revertidas por hormonas masculinas. No futuro, terapêuticas direcionadas para o género poderão aumentar a eficácia dos fármacos, minimizando problemas de saúde pública.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Microglia: sculpting the developing brain in a gender-specific manner

1.1.1. Historic overview

In 1856, Rudolf Virchow coined the term “neuroglia” as the non neuronal elements, an amorphous structure embedding the neuronal elements (reviewed in Tremblay et al. 2015). Michael von Lenhossek (1895) created the term “astrocyte” to name the cellular element of the neuroglia, intending to emphasize that glia were non neuronal brain cells (reviewed in Tremblay et al. 2015). Two years later, Ramón y Cajal recognized the existence of other cell type that is neither a neuron nor an astrocyte (Ramón y Cajal, 1897). In 1899, Franz Nissl was the first to describe *Stäbchenzellen*, rod cells now known as a form of activated microglia. Nissl characterized these cells as reactive neuroglia and proposed them to be able to migrate and phagocytose (Nissl, 1899). In 1913, Cajal observed poorly stained non-astrocyte glial cells that he named the “third element” of the nervous system, by performing the gold chloride sublimate method developed by himself. These cells were morphologically different from the “first element” (neurons) or the “second element” (astrocytes) (Ramón y Cajal, 1913). Pío Del Río-Hortega, a disciple of Cajal, created new staining techniques, as the ammoniacal silver carbonate method, which allowed him to separate the “third element” described by Cajal in two different cell types: microglia (or mesoglia) and oligodendrocytes (first described as interfascicular glia) (reviewed in (Tremblay *et al*, 2015)).

1.1.2. Microglia origin and colonization of the developing brain

Microglia colonize the central nervous system (CNS) early in neurodevelopment. The most recent hypothesis for the origin of these cells support that they are the only resident macrophages entirely derived from primitive macrophages originated in the extra-embryonic yolk sac (YS) (Figure 1) (Ginhoux *et al*, 2010; Schulz *et al*, 2012; Kierdorf *et al*, 2013; Gomez Perdiguero *et al*, 2014; Hoeffel *et al*, 2015; Sheng *et al*, 2015).

The circulatory system plays an important role in the CNS colonization by YS macrophages (Ginhoux *et al*, 2010). In the mouse embryo, haematopoietic and endothelial precursor cells expressing vascular endothelial growth factor receptors migrate from the primitive streak to the proximal YS to form the blood islands around embryonic day (E) 7 (Jones, 2011). The primitive hematopoiesis takes place in the YS also around E7, which corresponds to the first trimester of pregnancy in humans,

contributing to the production of macrophages and erythrocytes (Moore & Metcalf, 1970; Palis *et al*, 1999; Bertrand *et al*, 2005).

Ginhoux and colleagues showed that the specification of YS microglia in mice occurs between E7.0 and E7.5, in a fate mapping study performed between E6.5 and E10.5. Briefly, the authors followed haematopoietic precursors expressing the enhanced yellow fluorescent protein reporter gene: mice expressing the tamoxifen-inducible *MER-Cre-MER* recombinase gene under the control of the endogenous promotors of runt-related transcription factor 1 (*Runx1*) were crossed with a mouse strain that expresses the Cre-reporter under the promotor Rosa26, being the recombination induced by the injection of 4-hydroxytamoxifen in pregnant females (Ginhoux *et al*, 2010). Around E9.5 microglia pass through the leptomeninges and lateral ventricles, penetrate the rudimentary brain and distribute along the cortical wall in both directions at dissimilar velocities and rates of proliferation and maturation, depending on the region and on the developmental stage (Ginhoux *et al*, 2010; Arnoux *et al*, 2013; Swinnen *et al*, 2013). YS precursors undergo a process of maturation in the blood islands and cephalic mesenchyme, becoming mature macrophages in the neuroepithelium at E10.5 (Roumier *et al*, 2008; Mizutani *et al*, 2012).

The primitive haematopoiesis decreases until E10.5, when it starts being gradually replaced by the definitive haematopoiesis which occurs in the aorta, gonads and mesonephros regions (Ginhoux *et al*, 2013). Progenitors resulting from the definitive haematopoiesis transiently establish themselves in the fetal liver between E11.5 and the day of birth, when they start being produced by the bone marrow (Hoeffel & Ginhoux, 2015). Controversially, Askew and colleagues recently suggested that fetal liver-derived monocytes that infiltrate the brain, that peak at postnatal day (PND) 3, are rapidly depleted by apoptosis, not contributing to the final microglia core (Askew *et al*, 2017). There are no available data concerning these developmental events in rats.

Regarding the number of microglia cells, in mice, in the first two postnatal weeks it increases and gradually decline (50%) from the third to the sixth weeks, stabilizing their density after that (Nikodemova *et al*, 2015). The maintenance of microglia numbers throughout life is dependent on a process of self-renewal (Hashimoto *et al*, 2013).

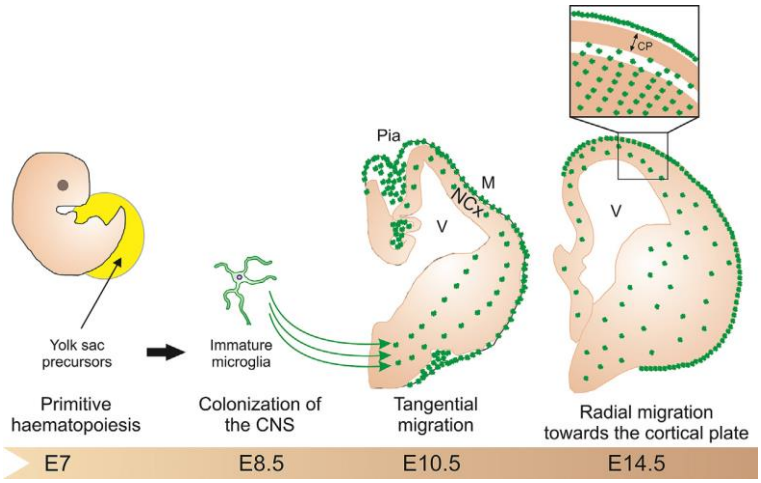


Figure 1 Colonization of the developing CNS by microglia. CP: cortical plate, M: meninges, NCx: neocortex, V: ventricle. Adapted from (Mosser *et al*, 2017).

Casano and colleagues (using a zebrafish model) reported neuronal apoptosis to drive the entrance and positioning of microglial precursor cells into the developing brain, since enhanced brain colonization by microglia was observed when neuronal cell death is increased and the opposite was observed when it is decreased. Moreover, these cells are attracted to the brain through a nucleotide-mediated chemotaxis (Casano *et al*, 2016), the same ‘‘find me’’ signal emitted by apoptotic cells, adenosine triphosphate (ATP).

Schwarz and co-workers reported that male Sprague-Dawley rats possess higher number of microglia cells than females during the early postnatal period (PND4). However, from the late development (PND30) onwards, the opposite was observed (Schwarz *et al*, 2012).

Doorn and colleagues quantified microglia in different brain regions from adult male Wistar rats, by performing fluorescence-activated cell sorting for microglia cells, and measured the levels of expression of established microglial markers. In a decreasing order, the authors counted microglia cells in the olfactory bulb (OB), the substantia nigra, the hippocampus (Hip), the striatum (Str) and the amygdala (Amy). Regarding gene expression, no regional differences were observed in the levels of mRNA of common microglia markers, as ionized calcium-binding adaptor molecule 1 (Iba1) or CD11b. However, for instance, CD68, a marker for microglial activation, presented a higher expression in the OB comparing to the Str, Hip and Amy and in Str comparing with Amy, and the pro-inflammatory cytokine interleukin-1β (IL-1β) presented a higher expression in the OB comparing to Str (Doorn *et al*, 2015).

In the adult mouse brain, microglia are not uniformly distributed, being the most populated areas the Hip, olfactory telencephalon, basal ganglia and substantia nigra, the average populated areas the cerebral cortex, thalamus and hypothalamus, and the less populated ones the cerebellum and much of the brainstem. In the cortex, the number of microglia cells was similar to that in the whole brain. When dissecting this brain region, the authors counted a similar number of microglia cells in frontal, cingulate, parietal, occipital and sensorimotor cortex, and a lower number in the occipital cortex (Lawson *et al*, 1990).

In humans, the developing brain cortex is colonized by amoeboid microglia, which are thought to come from the YS, passing through the pial surface, ventricles and choroid plexus at gestational week (GW) 4.5, and cross the blood vessels at GW10. A limited number of amoeboid microglia is observed at GW4.5 (Andjelkovic *et al*, 1998). Fetal microglia appears in clusters located in highly vascularized regions (Rezaie *et al*, 2005), whose size and number increase from GW9 to GW14. A second entrance via the vasculature occurs at GW12-13 only in the white matter (Monier *et al*, 2007; Verney *et al*, 2010). At GW7, microglia progressively colonize the cerebral wall of the telencephalon from the outside, beginning at the ventricular zone and moving to the deep cortical plate, in parallel with the white fiber tract development (Monier *et al*, 2006; Rezaie *et al*, 2005; Verney *et al*, 2012). During microglia progression towards the cortical plate, there is a decrease in their density and these cells acquire a ramified morphology, similar to that observed at adulthood (Pogledic *et al*, 2014). Microglia morphology will be discussed bellow. Mittelbronn and co-workers described regional differences in the number of ramified microglia cells in the brain in a cohort of patients aged between 21 and 92 years old. In a comparison between the grey and the white matter, the authors examined different brain areas and reported a higher number of microglia cells in the white matter (Mittelbronn *et al*, 2001).

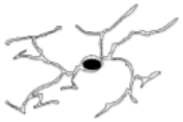
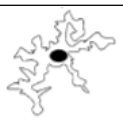
Concluding, besides gender differences in the overall number of microglia cells in the developing brain (Schwarz *et al*, 2012), these cells also colonize the brain in a region-specific manner (Ginhoux *et al*, 2010; Arnoux *et al*, 2013; Swinnen *et al*, 2013; Nikodemova *et al*, 2015). In fact, studies in mice, rats and humans show regional differences concerning microglia morphology, number and gene expression (Lawson *et al*, 1990; Mittelbronn *et al*, 2001; Doorn *et al*, 2015).

1.1.3. Microglia morphology throughout life

While colonizing the CNS, microglia undergo a process of differentiation. Dalmau and colleagues were the first to characterize microglia morphology in the developing rat

Hip (Table 1). The authors described four different morphological presentations of microglia: type 1 to type 3 amoeboid microglia and primitive ramified microglia, in the prenatal rat Hip (Dalmau *et al*, 1997). Then, in the postnatal rat Hip, they described five different morphological presentations of these cells, types 2 and 3 amoeboid microglia, primitive ramified microglia, resting microglia and reactive-like microglia (Dalmau *et al*, 1998).

Table 1 Classification of microglia morphology througout neurodevelopment in the rat hippocampus (adapted from (Dalmau *et al*, 1997, 1998)**).**

Classification	Shape	Cell processes	Measuring (µm)	Appearance	Morphology
Amoeboid microglia type 1	Roundish or lobular	None	15-40	From Ed14	
Amoeboid microglia type 2	Round	None, occasional filopodia	15-20	From Ed19 to PND9 Scarcely @ PND12	
Amoeboid microglia type 3	Pleomorphic	Filopodia and/or pseudopodia	20-50	From Ed14 to PND9 Some @ PND15	
Primitive ramified microglia	Elongated	Scantly developed processes showing a beaded shape	50-110	From Ed19 to PND12 Some @ PND15 and rarely @ PND18	
Resting microglia	Oval to roundish	Fully developed processes	85-100	Some @ PND12 From PND15 to PND18	
Reactive-like microglia	Large, plump, round to oval	Retracted, coarse processes	40/50-80	From PND9 to PND18	

Following these studies, only comparative studies regarding gender or regional differences in microglia morphology were performed, using a dichotomic characterization, in accordance with the presence or absence of microglial processes. Recently, we described morphometric characteristics of microglia in the prefrontal cortex (PFC) from Wistar rats (Caetano *et al*, 2016), detailed in Section 1.1.3.1.

In humans, microglia already colonized the brain parenchyma by GW16 and the characteristic ramified morphology is already observable at GW18 (Monier *et al*, 2006). This morphological maturation comprises the same stages as in rodents, with a parallel decrease in amoeboid and increase in ramified microglia, which display thin long processes (Harry, 2013).

1.1.3.1. Gender differences in microglia morphology and number

Despite the similarities in microglia between genders and brain regions concerning gross microglia morphology and intervention in inflammatory processes, microglia colonization and maturation are gender- and region-specific (Figure 2).

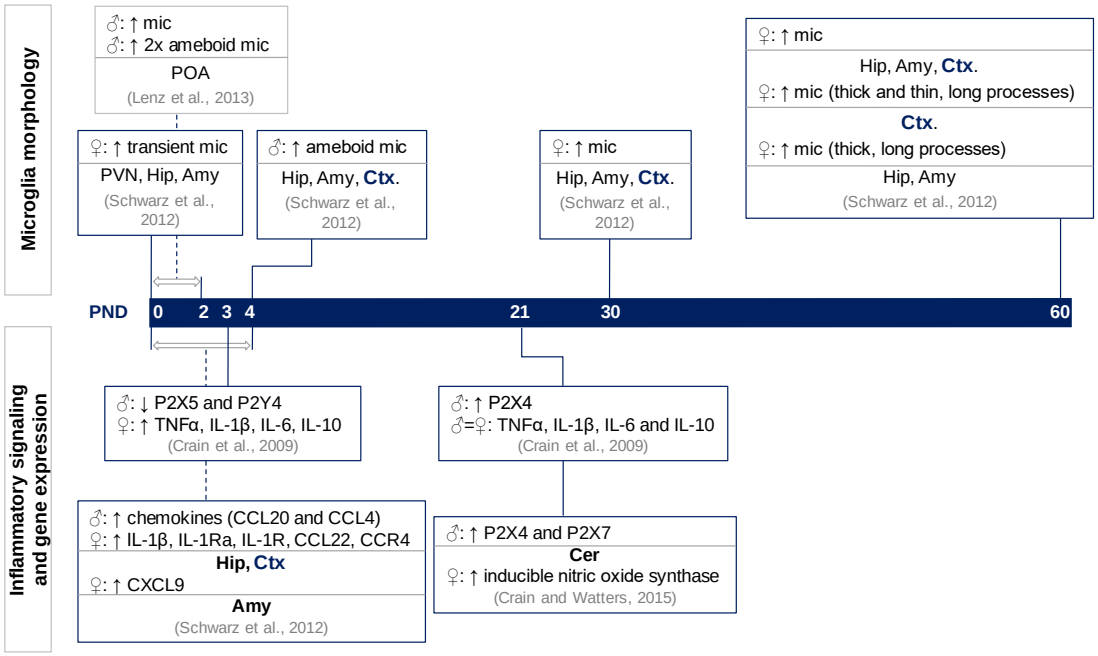


Figure 2 Physiological gender differences in microglia morphology, inflammatory signalling and gene expression between brain regions throughout neurodevelopment: a timeline. The abbreviations on scheme mean: amygdala (Amy), C-C chemokine receptor type 4 (CCR4), cerebellum (Cer), chemokine (C-C motif) ligand # (CCL#), chemokine (C-X-C motif) ligand 9 (CxCL9), cortex (Ctx), hippocampus (Hip), hypotalamus (Hyp), interleukin # (IL- #), interleukin-1 receptor antagonist (IL-1Ra), microglia (mic), number (#), P2X purinoceptor # (P2X#), P2Y purinoceptor 4 (P2Y4), paraventricular nucleus (PVN) of the Hyp, preoptic area (POA), similar to opposite gender (=), tumor necrosis factor alpha (TNFα). The arrows represent higher (↑) and lower (↓) number or expression than in the opposite gender, males (♂) and females (♀). Based on (Crain *et al*, 2009; Schwarz *et al*, 2012; Lenz *et al*, 2013; Crain & Watters, 2015).

Concerning gender differences in microglia morphology, our group performed, for the first time, a morphometric analysis of microglia cells from the PFC of Wistar rats at PND90. We observed that females possess a higher number of processes and longer processes *per* order, compared to males (Caetano *et al*, 2016).

1.1.4. Microglia functions throughout neurodevelopment

In a traditional view, microglia cells were largely associated with their functions in the inflammatory processes underlying diseases (Wolf *et al*, 2017). When exposed to an

injury, microglia release pro- and anti-inflammatory cytokines and neurotrophic factors and engulf cellular debris (Chen & Trapp, 2016), taking both classical pro-inflammatory activation (called M1) and alternative anti-inflammatory activation (called M2) which function in concert (Kigerl *et al*, 2009). After an inflammatory stimulus, these cells can display opposite functions (e.g. displace synaptic terminals (Chen *et al*, 2014) or prompt synapse formation (Cristovao *et al*, 2014)). These functions of microglia cells can be driven not only by chemotaxis, but also through the direct contact with neuronal processes. Wake and colleagues showed that the brief contact between microglia and neurons leads microglial processes to enlarge and promote the elimination of the synaptic structure (Wake *et al*, 2009).

From 2005, microglia have been more consistently associated with the sculpture and maintenance of the healthy brain, since two studies described microglia processes to be highly motile and sensitive to fine changes in the surrounding environment (Nimmerjahn *et al*, 2005; Davalos *et al*, 2005). Nimmerjahn and colleagues observed that microglial processes are highly dynamic under physiological conditions at adulthood, using two-photon microscopy. The permanent extension and retraction of microglial processes allows microglia to survey the whole brain parenchyma every few hours (h) (Nimmerjahn *et al*, 2005), to detect and answer to subtle variations in the surrounding environment in a manner dependent on purinergic receptors (Davalos *et al*, 2005). Moreover, other molecules were reported to be involved in this process, such as complement protein receptors, cell adhesion molecules and inflammatory cytokines and chemokines (reviewed in (Rock *et al*, 2004)), as well as the fractalkine receptor, C-X3-C motif chemokine receptor 1 (CX3CR1), whose ligand is fractalkine, produced and released by neurons (Pagani *et al*, 2015; Sheridan & Murphy, 2013). In fact, microglia express receptors for all neuronal neurotransmitters and neuromodulators, reinforcing their monitoring role (Kettenmann *et al*, 2013).

Regarding the interaction of microglia with other cell types, it does not occur exclusively in the injured brain. Tremblay and colleagues also described microglia to briefly contact with synaptic structures in the adolescent mouse visual cortex, under physiological conditions, by *in vivo* imaging (Tremblay *et al*, 2010).

In what concerns to neurons, in the early development, microglia regulate the number of neuronal precursor cells through phagocytosis (Cunningham *et al*, 2013). Moreover, these cells induce neuronal cell death during the programmed neuronal apoptosis (Wakselman *et al*, 2008), digest dying neurons to avoid the dispersion of degradation products (Peri & Nüsslein-Volhard, 2008) and prune synapses and actively phagocytose them (Paolicelli *et al*, 2011). The selection of synapses to be eliminated is

poorly understood, but one hypothesis is that the weaker and less efficient ones are eliminated, perhaps to “protect” the stronger ones (Schafer *et al*, 2012b). The tagging of weaker synapses for removal was also hypothesized (Stephan *et al*, 2012). During brain development, the C1q protein, an initiator of the complement cascade, is up-regulated and localized at synapses. Mice lacking C1q or the downstream chemokine C3 have deficiencies in synapse elimination (Stevens *et al*, 2007) and mice lacking the receptor for C3 (CR3) in microglial cells possess higher number of spines, suggesting that synaptic pruning may be impaired (Schafer *et al*, 2012b). Thus, the hypothesis of tagging synapses for elimination was reinforced and proteins involved in the complement cascade, namely C1q and C3, are important for the engulfment of pre-synaptic terminals. Furthermore, that engulfment of pre-synaptic terminals is related with lower neuronal activity (Schafer *et al*, 2012a; Li *et al*, 2012).

1.1.5. Microglia dysfunction: triggering the genesis of neuropsychiatric disorders

In microglia, morphology and function are strongly associated (Hinwood *et al*, 2013; Kreisel *et al*, 2014), since their highly dynamic processes are responsible for surveying functions and the pruning of synapses (Paolicelli *et al*, 2011). An harmonious conversation between microglia and neurons is essential for a healthy brain, as previously discussed. Thus, deficiencies in that crosstalk lead to weak synaptic transmission, decreased functional brain connectivity and behaviour abnormalities (Zhan *et al*, 2014). The referred permanent deficiencies in neuronal circuits are associated with the genesis of neuropsychiatric disorders, such as autism spectrum disorders (D’Mello & Stoodley, 2015), schizophrenia (Ferrarelli *et al*, 2015), depression (Yirmiya *et al*, 2015; Rial *et al*, 2016) and anxiety disorders (Caetano *et al*, 2016).

Prenatal and early-life stressful events may amplify the susceptibility to develop these neuropsychiatric disorders (Sousa, 2016). This amplification occurs due to an increase of the levels of steroid hormones, namely glucocorticoids (GC) (Smith & Vale, 2006), whose premature rise during development activates the fetal hypothalamic-pituitary-adrenal (HPA) axis, which promotes earlier tissue differentiation resulting in a malformed organism (Fowden *et al*, 1998).

Regarding endogenous GC, cortisol in humans and corticosterone in rodents, they are released by the adrenal glands in response to the circadian rythm or under stressful conditions (Smith & Vale, 2006; Chung *et al*, 2011). The fetus possesses lower levels of GC comparing to the circulating levels in the mother, what can be explained by the

conversion of GC in inactive metabolites by the placental enzyme 11β-hydroxylase 2, whose expression decreases at the end of gestation (Mesquita *et al*, 2009). In the late pregnancy, the intrauterine levels of GC raise and induce fetal maturation (Thorburn *et al*, 1977). The endogenous levels of GC are strongly regulated by the HPA axis: the hypothalamus produces and releases corticotropin-releasing hormone that stimulates the pituitary gland to release adenocorticotropic hormone into the circulation, inducing the synthesis and release of GC by the adrenal glands. A negative feedback regulates the HPA axis: when there are elevated levels of GC in circulation, they bind to receptors in the brain to normalize their own production (Waffarn & Davis, 2012).

Concerning the required levels of GC for a normal development, women at risk of preterm delivery are treated with synthetic GC, such as dexamethasone (DEX). However, this treatment has long-term effects on HPA axis regulation of the fetus, as observed in both humans and animal models (Nagano *et al*, 2008)

1.1.5.1. The specific case of anxiety

Stressful stimuli in different stages of neurodevelopment may trigger the genesis of neuropsychiatric disorders, namely anxiety, with measurable effects in microglia. Some of these studies used well described animal models related to anxious-like behaviour measures, but others manipulated directly microglia cells trying to correlate their morphology and function with behavioural outcomes.

For instance, in a model of chronic anxiety with developmental genesis (*in utero* exposure to DEX at E18 and E19), our group correlated alterations in anxious-like behaviour with a gender-specific morphological remodelling of microglia in Wistar rats. We described long-term effects, observable at PND90, of the prenatal stressful stimulus in increasing the anxious-like behaviour in both genders and in microglia morphology in the PFC in a gender-specific manner: an hypertrophy in males and an atrophy in females (Caetano *et al*, 2016).

Also in the neonatal period, an inflammatory stimulus can influence long-term anxious-like behaviour. Claypoole and co-workers (2017) studied that hypothesis through neonatal injection of lipopolysaccharide (LPS), an inflammatory trigger present in the membrane of the Gram-negative bacteria, in two different bred strains of rats that display low or high anxious-like behaviour. At PND7, under physiological conditions, males presented a higher density of microglia than females in the Hip, but when exposed to LPS, females experienced an increase in that density. Regarding anxious-like behaviour, high line animals express significantly more ultrasonic vocalizations comparing to low line

animals, independently of LPS exposure. At adulthood (PND70 to PND100), neonatal exposure to LPS increased anxious-like behaviour in low line rats, but decreased in high line rats. Yet, low line animals showed lower anxious-like behaviour than high line ones and animals exposed to LPS display lower anxious-like behaviour than control ones. In parallel, another group of animals was exposed to 3 days of a stress paradigm, starting at PND180, and the authors described that high line animals presented higher anxious-like behaviour than low line ones and males presented higher anxious-like behaviour than females. Concluding, neonatal inflammatory stimuli triggered anxious-like behaviour in a line and gender dependent manner (Claypoole *et al*, 2017).

To further explore the role of microglia in the early post-natal period, Nelson and Lenz injected clodronate liposomes in rats at PND2 and PND4, leading to microglia depletion for two weeks. This promoted a decrease in anxious-like behaviour measures in the elevated plus maze (EPM) test in males and females at youth, but only in females at adulthood (Nelson & Lenz, 2017a).

At adulthood, stressful stimuli are also able to modify both anxious-like behaviour and microglia morphology. Wohleb and colleagues exposed male C57BL/6 mice (6 to 8 weeks old) to a social disruption stress protocol (Avitsur *et al*, 2001) and evaluated microglia morphology and anxious-like behaviour. After the social disruption stress protocol, these animals presented increased anxious-like behaviour, measured through light/dark preference test (Kinsey *et al*, 2007), as well as alterations in microglia morphology that became hypertrophic, but with shorter and thicker processes in the medial Amy, PFC, and Hip (Wohleb *et al*, 2011).

Altogether, these data lead us to speculate about the involvement of microglia in the genesis and pathophysiology of anxiety. Despite the dimorphic brain among genders, it is intriguing how *in utero* exposure to synthetic GC promotes opposite gender-specific morphological fine-tuning. Moreover, this contradictory morphological remodelling of microglia parallels an anxiogenic effect similar among genders. If the morphological rearrangement of microglia is a cause or a consequence of anxiety surge still unclear. When the anxiogenic stimuli occur in the postnatal period, it also changes microglia morphology and anxious-like behaviour. An interesting question for discussion is the interaction between the developmental stage in which the animal is exposed to stressful stimuli, its gender and genetic background, as well as other environmental factors that can trigger the genesis of anxiety. More studies are needed to reveal the molecular mechanisms behind these queries.

1.2. Adenosine A_{2A} receptor: modulating microglia and behaviour

1.2.1. Overview of adenosine and its receptors

Adenosine is a neuromodulator and homeostatic regulator in the brain that belongs to the family of purines and is produced by all cell types, including microglia (Ribeiro *et al*, 2002; Gomes *et al*, 2009, 2013; Cristovao *et al*, 2014; George *et al*, 2015; Caetano *et al*, 2016). Adenosine has four metabotropic (G-protein coupled) receptors: A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm *et al*, 2001). The expression of these receptors differs between brain regions (Ribeiro *et al*, 2002) and their distribution varies during development, stabilizing at adulthood (Shaw *et al*, 1986). Microglia express all adenosine receptor subtypes (Daré *et al*, 2007).

1.2.2. Adenosine A_{2A} receptor in the developing brain

Adenosise A_{2A} receptor ($A_{2A}R$) is distributed in the brain (Fredholm *et al*, 2005) and mostly located in synapses (Rebola *et al*, 2005a). During pregnancy and early postnatal life, the expression of this receptor varies, indicating important roles during neurodevelopment (Ådén *et al*, 2000). In rats, the expression of $A_{2A}R$ mRNA in the brain was first found at E14, raises during development, reaches a maximum at PND7 and decreases from PND21 until adulthood, with exception of the Str, where this expression persists in high levels (Weaver, 1993; Ådén *et al*, 2000; Johansson *et al*, 1997).

Besides the important roles of $A_{2A}R$ during brain development, as the control in the migration speed of some GABA neurons that is delayed by prenatal $A_{2A}R$ blockade (Silva *et al*, 2013), its activation in the adult brain was described to modulate processes such as synaptic transmission and plasticity (Sebastião & Ribeiro, 2009). $A_{2A}R$ is highly expressed in neurons in the Str, but less expressed in the Hip, cerebral cortex and glia (Svenningsson *et al*, 1999). Interestingly, the expression of this receptor was increased in the cortex, Hip and Str of aged rats (Cunha *et al*, 1995).

In the context of an injury, the levels of expression and the density of $A_{2A}R$ increase in both neurons (Rebola *et al*, 2005b) and glial cells (Yu *et al*, 2008; Gomes *et al*, 2013). Moreover, the purinergic system is differently modulated according to the injury stimulus, promoting opposing answers by microglia cells (George *et al*, 2015).

1.2.3. Adenosine A_{2A} receptor modulation of microglia

Gyoneva and co-workers showed $A_{2A}R$ to be involved in the control of microglia morphology, since its activation was described to promote an atrophy of these cells (Gyoneva *et al*, 2009).

Studies using LPS to prompt neuroinflammation showed $A_{2A}R$ to be involved in the process and to modulate microglia morphology and the expression of inflammatory mediators. For instance, Orr and colleagues described the exposure to LPS promoting an increase in the levels of adenosine, which binds to $A_{2A}R$ and converts ramified in amoeboid microglia due to the retraction of their processes (Orr *et al*, 2009).

Our group explored the ability of $A_{2A}R$ in modulating the levels of brain-derived neurotrophic factor (BDNF), in an *in vitro* study. We described that LPS-induced activation of $A_{2A}R$, which increases the density of this receptor, promoted a transient decrease in the levels of BDNF. This decrease was prevented by the blockade of $A_{2A}R$ and by adenosine deaminase, an enzyme that removes endogenous adenosine (Gomes *et al*, 2013).

In other study using male Wistar rats, Rebola and colleagues reported the exposure to LPS to increase inflammatory mediators as IL-1β leading to neuronal dysfunction, namely decreased synaptic plasticity, an effect attenuated by the blockade of $A_{2A}R$. Importantly, this study points $A_{2A}R$ activation as essential for LPS-induced neuroinflammation, since its blockade prevented the recruitment of activated microglia and the increase in the levels of IL-1β in the Hip (Rebola *et al*, 2011).

Using the same selective antagonist of $A_{2A}R$, we reported that the chronic blockade of $A_{2A}R$ at adulthood promotes a gender-specific morphological rearrangement of microglia in the PFC of Wistar rats. That blockade of $A_{2A}R$ lead to an atrophy in microglia morphology only in females. The model of chronic anxiety with developmental genesis promoted gender-specific morphological alterations in microglia, more precisely an hypertrophy in males and an atrophy in females. However, the chronic blockade of $A_{2A}R$ was able to revert that effect only in males (Caetano *et al*, 2016).

1.2.4. Adenosine A_{2A} receptor modulation of anxiety

The involvement of $A_{2A}R$ was described in neuropsychiatric disorders (reviewed in (Gomes *et al*, 2011)). The manipulation of this receptor was proposed as a therapeutic target (Cunha *et al*, 2008), since antidepressant effects were described for both the pharmacological blockade and the genetic depletion of $A_{2A}R$ (Yacoubi *et al*, 2001).

Concerning anxiety, the literature is still controversial about the effect of the pharmacological blockade of this receptor. Both pharmacological blockade and genetic

depletion of $A_{2A}R$ were reported to be anxiolytic in animals exposed to an anxiogenic protocol, the chronic unpredictable stress. In that context, $A_{2A}R$ manipulation was proposed not only as therapeutics, but also as preventive (Kaster *et al*, 2015). Under physiological conditions, $A_{2A}R$ knockout (KO) mice developed increased anxious-like behaviour in both the EPM and the Light-dark box tests, (Yacoubi *et al*, 2000; Berrendero *et al*, 2003; Kaster *et al*, 2015).

Yacoubi and colleagues modulated $A_{2A}R$, not only by pharmacology, but also through a genetic approach. The authors showed that both acute and chronic administration of caffeine, a non-selective antagonist of $A_{2A}R$ (Fredholm *et al*, 1999), is anxiogenic, as well as the acute treatment with a selective agonist of $A_{2A}R$, as tested in the EPM test in male mice. Contrastingly, acute and chronic blockade of $A_{2A}R$ through the administration of the selective antagonist did not affect anxious-like behaviour. Concerning the genetic approach, $A_{2A}R$ KO mice display increased anxious-like behaviour compared with wild-type (WT) ones. Moreover, the chronic administration of caffeine promoted lower variation in anxious-like behaviour measures in $A_{2A}R$ KO mice than in the WT ones (Yacoubi *et al*, 2000).

Most of the studies concerning neuropsychiatric disorders and the modulation of $A_{2A}R$ are performed in male rodents. However, mood disorders affect females more than twice, comparing to males (Kessler *et al*, 2005; Bekker & van Mens-Verhulst, 2007). Taking this into account, our group explored the effects of the chronic blockade of $A_{2A}R$ in both genders. We observed that this treatment at adulthood is anxiogenic in female, but not in male Wistar rats. Additionally, in a model of anxiety with developmental genesis (prenatal exposure to DEX) we described the $A_{2A}R$ blockade at adulthood to be anxiolytic in males, but not in females. Moreover, we correlated gender-specific morphological remodelling of microglia in the PFC with the increase in anxious-like behaviour in animals exposed to DEX; however, when treated at adulthood, only in males microglia morphology underwent a recover, recapitulating what happens with anxious-like behaviour (Caetano *et al*, 2016). Importantly, this study highlighted the involvement of $A_{2A}R$ in the pathophysiology of anxiety, not only in a microglial coupled way, but also in a gender-specific manner.

In humans, the consumption of caffeine, a selective antagonist of $A_{2A}R$, has been reported as both beneficial (Smith, 2009; Santos *et al*, 2010; Lieberman *et al*, 1987; Fine *et al*, 1994; Amendola *et al*, 1998) or damaging (Greden *et al*, 1978; Gilliland & Andress, 1981; James & Crosbie, 1987). Panic and phobic disorders, the most frequently diagnosed anxiety disorders, are highly associated with significant psychosocial morbidity (Markowitz, 1989; Schneier *et al*, 1994). In panic disorder, patients possess high

sensitivity to caffeine, whose consumption seems to be more associated to generalized anxiety symptoms than to those related to phobic disorder (Boulenger & Uhde, 1982). In social phobics, caffeine consumption is similar to the general population and this psychostimulant drug did not increase the severity of anxiety symptoms. Conversely, after oral administration of caffeine both individuals with panic disorder and control ones displayed similar electroencephalographic activity (Uhde, 1994). On the other hand, the consumption of caffeine in extremely high doses, or in individuals with anxiety disorders, is prejudicial. Briefly, the consumption of extremely high doses of caffeine was associated with increased anxiety and mood disorders (Cappelletti *et al*, 2015).

Regarding genetic studies exploring $A_{2A}R$ role in anxiety, there are two single nucleotide polymorphisms, rs2236624 and rs5751876, reported as risk factors for increased anxiety (Hohoff *et al*, 2014).

1.3. Testosterone: shaping gender differentiation of brain and behaviour

Neuropsychiatric disorders have gender-specific genesis, clinical presentation and answer to therapeutics. For instance, mood disorders affect females more than twice comparing to males (Kessler *et al*, 2005; Bekker & van Mens-Verhulst, 2007). Moreover, females present an increased prevalence of mood disturbances, anxiety and depression, in periods of hormonal fluctuations, such as puberty, perimenstrual, post-partum and menopause periods (Douma *et al*, 2005; Solomon & Herman, 2009). This suggests the involvement of gonadal hormones in the pathophysiology of these disorders, with male hormones appearing to possess protective effects. In fact, males have about tenfold higher concentrations of testosterone than women, although women are more sensitive to testosterone (reviewed in (Durdiakova *et al*, 2011)).

1.3.1. Gender determination

Gender determination is the processes by which the genes located in sexual chromosomes determine whether an individual will develop testes or ovaries, early in development. Males possess in the Y chromosome a sex-determining region (SRY) gene, which up-regulates the testis determining factor. In the presence of SRY gene, the individuals develop testes and in its absence they develop ovaries (Goodfellow & Lovell-badge 1993). Following the gender determination, it occurs the process of differentiation by which other tissues experience male or female phenotypes (reviewed in (Lenz *et al*, 2012)).

1.3.2. Gender-specific differentiation

Testosterone is primarily synthesized in the gonads and adrenal cortex in both genders. Then, peripheral testosterone transposes the blood brain barrier and directly acts in the brain. In male rodents, an ontogenic peak of testosterone occurs at the day of birth (Konkle & McCarthy 2011). In male primates, that surge of testosterone occurs from the end of the first gestational trimester and during the second gestational trimester, with a novel surge at birth (Forest et al. 1973; Gendrel et al. 1980; Reyes et al. 1973).

The brain is also a source of estrogens since in some regions it possesses the enzymes necessary to synthesize estradiol from cholesterol or steroidal precursors, leading to a process named de novo synthesis (Baulieu *et al*, 2001; Melcangi *et al*, 2008).

Cholesterol, the precursor of all steroid hormones (reviewed in (Ghayee & Auchus, 2007)), is transported from the cytoplasm to the inner mitochondrial membrane, were steroidogenic enzymes are located to synthesize neurosteroids (reviewed in (Sierralta *et al*, 2005; Miller, 2013)).

In male rodents, testosterone is converted into estradiol by p450 enzyme aromatase, an enzyme highly abundant and active in the brain during the gonadal androgen surge (George & Ojeda, 1982; Roselli & Resko, 1993). The action of estrogens on estrogen receptors (ER) triggers two processes in the male developing brain, masculinisation and defeminisation, which promote male-typical behaviours (McCarthy, 2008). These processes occur at slightly different developmental windows (Wallen & Baum, 2002) and are controlled by different mechanisms downstream to estradiol (Todd *et al*, 2005). For instance, masculinisation and defeminisation of brain circuits are driven by estrogens action in two different ER (Kudwa *et al*, 2006). More detailed information regarding the molecular mechanisms of androgens and estrogens at their receptors can be found in (Bennett *et al*, 2010).

In females, the absence of that testosterone surge drives the two opposite processes, feminisation and demasculinisation, promoting the development of female-typical behaviours. However, estradiol is also necessary for the full feminisation of female brain and behaviour, as shown in female mice lacking functional aromatase (Bakker & Baum, 2008).

The long-lasting brain specifications that occur during development due to exposure to sexual hormones are referred as organizational effects of hormones (Phoenix, 2009; Arnold, 2009). In humans and nonhuman primates, the organizational effects of hormones begin prenatally and extend until the early postnatal period (Forest *et al*, 1973; Reyes *et al*, 1973; Gendrel *et al*, 1980). On the other hand, acute and transient effects of gonadal hormones that occur throughout life are called activational effects of hormones (Cooke *et al*, 1998).

The exposure to male-typical hormones during the brain development, a period of extreme plasticity, is responsible for programming some features of the brain as the size of some brain regions, the number of cells, synapses and dendritic spines, the connectivity and signalling among brain regions, as well as gene expression (McCarthy, 2008). These gender differences in brain development are responsible for permanent gender-specific behaviours like copulatory, parental and territorial behaviours, emotionality, cognition and sensory processing (Balthazart *et al*, 1995; Morris *et al*, 2004; McCarthy & Arnold, 2011).

1.3.3. Gender differences in anxiety: the role of testosterone

1.3.3.1. Studies with animal models

Animal studies are imperative to further understand the molecular mechanisms by which testosterone impacts anxiety disorders. It is of interest to discriminate the organizational and the activational effects of this hormone in both males and females, since both anxiolytic and anxiogenic effects have been described according to the context.

I) Organizational effects of testosterone

The perinatal or pubertal exposure to testosterone organizes adult behaviour, producing characteristics that persist throughout life, through its action on androgen receptors (AR) or on ER following aromatization to estrogen. The literature is controversial about the impact of testosterone during organizational periods with reports of both anxiolytic (Zuloaga *et al*, 2008, 2011b; Schulz *et al*, 2009) and anxiogenic (Lucion *et al*, 1996; Zuloaga *et al*, 2011a) effects.

Neonatal gonadectomy (GNX) in male rats is correlated with reduced anxious-like behaviour at adulthood, evaluated in EPM (Lucion *et al*, 1996). The anxiolytic effect of neonatal GNX implies testosterone as being anxiogenic during organizational periods. Zuloaga and colleagues reported that after GNX at PND0 both WT and AR-deficient (Tfm, testicular feminization due to a mutation of the gene for ARs) male rats, present similar decrease in anxious-like behaviour measures comparing with sham-operated controls (Zuloaga *et al*, 2011a). This behavioural similarity between WT and Tfm males suggests that the anxiogenic effect is not mediated by ARs, so it can be due to testosterone aromatization to estrogen and its effect on ER.

In opposition, two other studies from the same lab showed that, in the absence of GNX, Tfm males have higher anxious-like behaviour measures in the dark-light box and in the novel object recognition (NOR) tests, comparing with WT male rats. In the NOR test, both Tfm males and WT females presented an increase in the levels of corticosterone, comparing with WT males (Zuloaga *et al*, 2008, 2011b). Prepubertal GNX was also shown to be anxiogenic in the OF and in male-male social interactions in hamsters (reviewed in (Schulz *et al*, 2009)). In conclusion, these studies show neonatal testosterone to promote an anxiolytic effect at adulthood.

II) Activational effects of testosterone

The activational effects of testosterone is still a controversial issue due to confounding parameters as gender, removal of gonads or the presence of a model of neuropsychiatric disorders.

Bitran and colleagues tested adult male Long-Evans rats (PND60 to PND70) subcutaneously implanted with a capsule filled with testosterone propionate (TP) in the EPM test. They reported an anxiolytic effect of testosterone under physiological conditions, one week, but not two-weeks after TP exposure, comparing to controls (Bitran *et al*, 1993). Moreover, in aged male rodents, which also present lower levels of testosterone, its replacement was also shown to reduce the anxious-like behaviour evaluated in the OF and the light-dark box tests, compared to vehicle-treated (VH) ones (Frye *et al*, 2008). In adult male rodents, GNX has an anxiogenic effect recovered by testosterone replacement therapy, measured in the EPM, OF, and defensive probe-burying (Slob *et al*, 1981; Adler *et al*, 1999; Frye & Seliga, 2001; Fernández-Guasti & Martínez-Mota, 2003; Morsink *et al*, 2007).

After two-weeks of chronic social isolation, a model of anxiety, testosterone replacement restored the physiological levels of testosterone and was anxiolytic in adult GNX male, but not in adult ovariectomized (OVX) female rats (Carrier & Kabbaj, 2012). In the absence of a model of anxiety, adult OVX Long-Evans female rats experienced a transient effect of testosterone, since it is anxiolytic 1 h, but not 24 h after injection (Frye & Lacey, 2001). Furthermore, in two months old female Wistar rats, testosterone enhanced serum testosterone and estradiol levels and reduced defensive burying in a defensive burying task, comparing to controls (Gutiérrez-García *et al*, 2009).

Summarizing the activational effects of testosterone, its administration in adult and aged rodent males under physiological conditions is anxiolytic (Bitran *et al*, 1993). When these males are GNX at adulthood, they experienced increased anxious-like behaviour recovered by testosterone replacement (Slob *et al*, 1981; Adler *et al*, 1999; Frye & Seliga, 2001; Fernández-Guasti & Martínez-Mota, 2003; Morsink *et al*, 2007). In a model of anxiety with genesis at adulthood, testosterone is able to ameliorate the increased anxious-like behaviour only in males (Carrier & Kabbaj, 2012), suggesting that testosterone during organizational periods would be necessary to protect females. Furthermore, in females not exposed to anxiogenic stimuli, OVX at adulthood is anxiogenic and testosterone administration only promoted a transient anxiolytic effect that disappears before 24 h after treatment (Frye & Lacey, 2001).

1.3.3.2. Studies with humans

Hormonal disturbances, as hypogonadism, a condition in which decreased levels of testosterone are produced due to reduced functional activity of the gonads, have been associated with neuropsychiatric disorders. Hypogonadal men present higher prevalence of anxiety disorders and major depressive disorder (Shores *et al*, 2004; Zarrouf *et al*, 2009). In hypogonadal men, testosterone replacement improves mood, ameliorates anxiety and alleviates symptoms of depression (Wang *et al*, 1996; Pope *et al*, 2003; Kanayama *et al*, 2007; Zarrouf *et al*, 2009).

In adolescent males but not females, increased anxiety-like measures were correlated with lower levels of salivary testosterone due to circadian flux (Granger *et al*, 2003). On the other hand, lower salivary levels of testosterone were observed in women with a type of anxiety disorder (Giltay *et al*, 2012).

In women, a single dose of testosterone ameliorates anxiety in the fear-potentiated startle response (Hermans *et al*, 2006). Moreover, transdermal application of testosterone ameliorated mood disturbances following surgical removal of ovaries (Shifren *et al*, 2000) and in age-related declines in androgens (Goldstat *et al*, 2003). However, higher doses of testosterone can contribute to the genesis of major depressive disorder in women (Rohr, 2002).

Transgender individuals present an approximately threefold increased risk for developing anxiety disorders. However, trans women under treatment with male hormones present lower levels of anxiety disorder symptomatology (Cooke *et al*, 1998).

Concluding, the compilation and analysis of these data increase the appeal to further investigate the physiological gender differences in the genesis, clinical presentation and answer to therapeutics of anxiety. The age of surge of this neuropsychiatric disorder was also shown to impact in its recover and the organizational and activational studies led us to inquire about the role of testosterone in that processes. Despite all these knowledge about testosterone and its metabolites, the precise molecular pathways mediating its anxiolytic and anxiogenic effects and the steroid receptors involved in these processes remain to be elucidated. Clinical studies evidenced an anxiolytic and antidepressant role for testosterone in both genders. However, human imaging studies are necessary to clarify the unknown mechanisms behind this protective effect of testosterone.

CHAPTER 2

RATIONALE AND AIMS

Microglia morphology is entirely associated with their function (Hinwood *et al*, 2013; Kreisel *et al*, 2014). Thus, abnormal morphological changes of microglia during neurodevelopment can directly affect the brain sculpture, leading to misconnected neuronal circuits and promoting behavioural disorders (Zhan *et al*, 2014; Sousa, 2016). Morphological alterations of microglia were already described in some neuropsychiatric disorders with developmental genesis in males (Kreisel *et al*, 2014). However, this is known that these developmental disorders possess a gender-specific genesis, clinical presentation and answer to therapeutics, with a gender biased prevalence. Stress stimuli was shown to provoke structural abnormalities in the PFC, a brain region widely associated with anxiety disorders (Shirazi *et al*, 2015). Thus, in a previous study from our group, Caetano and Pinheiro associated gender differences in microglia morphology in the PFC with gender biased anxious-like behaviour in adult rats (Caetano *et al*, 2016).

Briefly, they observed that, under physiological conditions, females possess more complex microglia than males, presenting a higher number of microglial processes, which are longer. When these animals were prenatally exposed to two injections of DEX at E18 and E19, an anxiogenic stimulus, they undergo a gender-specific morphological rearrangement of microglia: atrophy in females and hypertrophy in males, which was paralleled by an increase in anxious-like behaviour in both genders. Moreover, the pharmacological chronic blockade of $A_{2A}R$ at adulthood was able to recover these effects in males but not in females. Interestingly, the chronic blockade of $A_{2A}R$ at adulthood *per se* promoted an atrophy of microglia morphology and an increase in anxious-like behaviour measures only in females (Caetano *et al*, 2016). Of relevance is the fact that this is the first study comprising a morphometric analysis of microglia. Other works explore alterations of microglia morphology only using a dichotomic characterization of these cells, considering the presence or absence of processes.

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

- To analyse the effect of the absence of $A_{2A}R$ from neurodevelopment in microglia morphology and anxious-like behaviour;

- To compare the effects of the pharmacological chronic blockade of $A_{2A}R$ at adulthood and the effects of the genetic depletion of $A_{2A}R$ early in neurodevelopment, regarding microglia morphology and anxious-like behaviour;

- To clarify the time window of surge of the physiological gender differences observed at adulthood in microglia morphology in the PFC and anxious-like behaviour;

- To address whether testosterone is involved in the genesis of gender differences in microglia morphology and anxious-like behaviour observed at adulthood.

CHAPTER 3

EXPERIMENTAL WORK

3.1

Effects of the genetic depletion of $A_{2A}R$ during neurodevelopment on microglia morphology in the PFC: gender specificities

3.1.1. Rationale

Adenosine $A_{2A}R$ were shown to be involved in the control of microglia morphology since their activation promoted an atrophy of these cells (Gyoneva *et al*, 2014). An inflammatory stimulus, such as the exposure to LPS, may trigger that retraction of microglia processes and the treatment with an antagonist of $A_{2A}R$ induces the re-extension of microglia processes (Orr *et al*, 2009).

Despite their capability to modulate microglia morphology, $A_{2A}R$ pharmacological blockade was also shown to possess therapeutic potential in the context of neuropsychiatric disorders (Cunha *et al*, 2008; Gomes *et al*, 2011). Regarding anxiety, the literature is controversial about the benefits of manipulating $A_{2A}R$. The pharmacological blockade and the genetic deletion of this receptor ameliorate the anxious-like behaviour in animals subjected to chronic unpredictable stress (Kaster *et al*, 2015). On the other hand, under physiological conditions, the pharmacological blockade of $A_{2A}R$ at adulthood promoted a gender-biased effect: increased anxious-like behaviour in females, without affecting males (Caetano *et al*, 2016). The fact that $A_{2A}R$ KO mice develop anxious-like behaviour throughout life (Kaster *et al*, 2015) reinforces the role of this receptor during neurodevelopment.

In the present chapter, we characterize microglia morphology of $A_{2A}R$ KO mice, through a morphometric analysis of their processes, and anxious-like behaviour at adulthood, evaluating these animals in the OF and in the EPM, in both genders. Since the analysis was performed in accordance with our aforementioned study (Caetano *et al*, 2016), we might be able to discuss the modulation role of $A_{2A}R$ upon microglia morphology and anxious-like behaviour, considering age and gender differences.

3.1.2. Methods

3.1.2.1. Animals

Mice were housed under standard laboratory conditions (light/dark cycle 12/12 h), at room temperature (RT) and with *ad libitum* food and water. C57BL/6 mice at PND90 were used. All procedures were carried out in accordance to the European Union and National legislation. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.1.2.2. Immunohistochemistry

C57BL/6 mice at PND90 were deeply anesthetized with an intraperitoneal (ip) injection of ketamine (80 mg/kg; Nimatek) and xylazine (5 mg/kg; Ronpum 2%) and transcardially perfused with PBS 1x, followed by 4% paraformaldehyde (PFA). The brains were removed in ice-cold Hank's balanced salt solution (HBSS: 137 nM NaCl, 5.4 nM KCl, 0.45 nM KH_2PO_4, 0.34 nM NaHPO, 4 nM $NaHCO_3$, 5 nM glucose; pH 7.4), and meninges and cerebellum were excluded. Brains were reserved overnight in a 4% PFA solution at 4 ºC and then in a 30% sucrose solution in phosphate buffered saline (PBS: NaCl 137 mM, KCl 2.1 mM, KH_2PO_4 1.8 mM and $Na_2HPO_4.2H_2O$ 10 mM, at pH 7.4) at 4 ºC. After fixation, brains were involved in optimal cutting temperature compound, frozen in dry-ice and stored at - 80 ºC.

Brains were sectioned in a cryostat (Leica CM3050S, Germany) at - 21°C (chamber temperature) and - 24°C (object temperature). Once aligned in the cutting platform, brains were cut in 50 µm slices, stored at 4°C in 24 well plates filled with cryoprotection solution (50 nM $NaH_2PO_4.H_2O$, 50 mM K_2HPO_4, 30% sucrose, 30% ethylene glycol, diluted in MilliQ H_2O, pH 7.2).

A free floating immunohistochemical procedure was performed in brain slices from the PFC located at the stereotaxic coordinates of interaural 6.60 mm and bregma 2.80 mm (Figure 3) (Paxinos & Franklin, 2001). Slices were incubated with blocking solution [5% bovine serum albumin (BSA) and 0.1% Triton X-100] in PBS, for 2 h, at RT under mild agitation. Incubation with the primary antibody Iba-1 from rabbit (1:1000) in blocking solution was performed for 48 h at 4 ºC under mild agitation. Then, slices were washed 3 times with PBS during 10 min and incubated with the secondary antibody anti-rabbit (1:1000) in blocking solution for 2 h, at RT. Once washed 3 times with PBS for 10 min, slices were incubated with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI, 1:5000) in blocking solution for 10 min, at RT, under mild agitation. Slices were washed 3 times with PBS during 10 min and mounted using glycergel (Dako mounting medium).

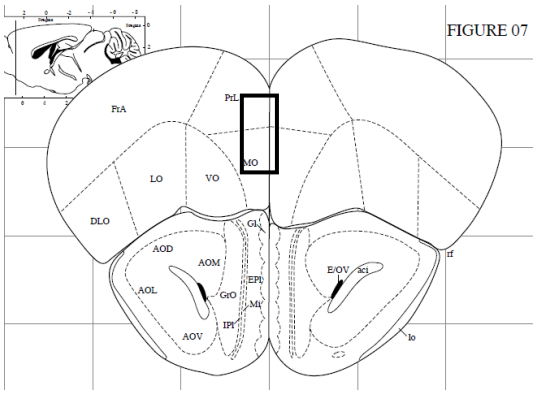


Figure 3 Representative image of the mouse brain in the analysed section. The black rectangle represents the region of acquisition by confocal microscopy after the immunohistochemistry assay. From (Paxinos & Franklin, 2001).

3.1.2.3. Morphometric analysis of microglia

A laser scanning confocal microscope LSM 710 META connected to ZEN Black software (Zeiss Microscopy, Germany) with a 63x objective lens (oil immersed, Plan-Apochromat 63x/1.40 Oil DIC M27) was used to acquire images of 10 random microglial cells from each animal. 4 animals were analysed *per* gender, according to the represented region of the PFC (Figure 3). Settings were chosen to optimize the labelling of microglial processes and maintained in all sections. To perform the tridimensional reconstruction of microglial cells from the PFC, Z-stacks were imported to the Neurolucida software (MBF Biosciences) and 50 microglial cells were manually reconstructed *per* condition. The morphometric analysis was performed using NeuroExplorer software (MBF Biosciences), an extension of Neurolucida software.

Microglial cells were reconstructed by drawing the cell body and the cellular processes. The parameters measured were the number and the length of processes *per* branch order. Processes of order 1 are those who emerge from the cell body, processes of order 2 are those who leave bifurcations of the previous one, and so forth (Figure 4). The mean per condition was considered for further analysis.

Figure 4 Illustrative scheme showing the tridimensional reconstruction of ramified microglia cells. The branch orders emerging from the cell body are represented by numbers (1 to 4).

3.1.2.4. Data analysis

Statistical analysis was performed in GraphPad Prism version 6 (GraphPad Software Inc., USA). All graphic values are expressed as mean ± standard error of the mean (SEM). Shapiro-Wilk normality test was used to assess whether the sample presents a normal distribution. Student's t test was used to compare two independent means when the samples followed a normal distribution and the correspondent non-parametric Mann-Whitney test was used when samples did not follow the normality. Differences were considered significant at $p<0.05$ (*).

3.1.2.5. Team involvement

The morphological reconstruction and analysis of microglia cells in the PFC of males was performed by another member of our group, Helena Pinheiro (MSc). In females, the morphological reconstruction of microglia in the PFC and data analysis were performed by myself. At least two independent researchers reconstructed the same cells, in order to avoid subjective bias inherent to the method used in the manual reconstruction.

3.1.3. Results

3.1.3.1. $A_{2A}R$ KO mice adult females exhibited microglia hypertrophy in the PFC

The absence of $A_{2A}R$ from early neurodevelopment induced a dimorphic effect in microglia morphology in the PFC. In males, the depletion of $A_{2A}R$ did not affect microglia morphology (Figure 5, a) at PND90, considering both the number (Figure 5, b) and the length (Figure 5, c) of their processes. The values referent to the morphometric analysis of microglia processes are detailed in the Supplementary table 1 (number of processes) and 2 (length of processes) in Chapter 6.

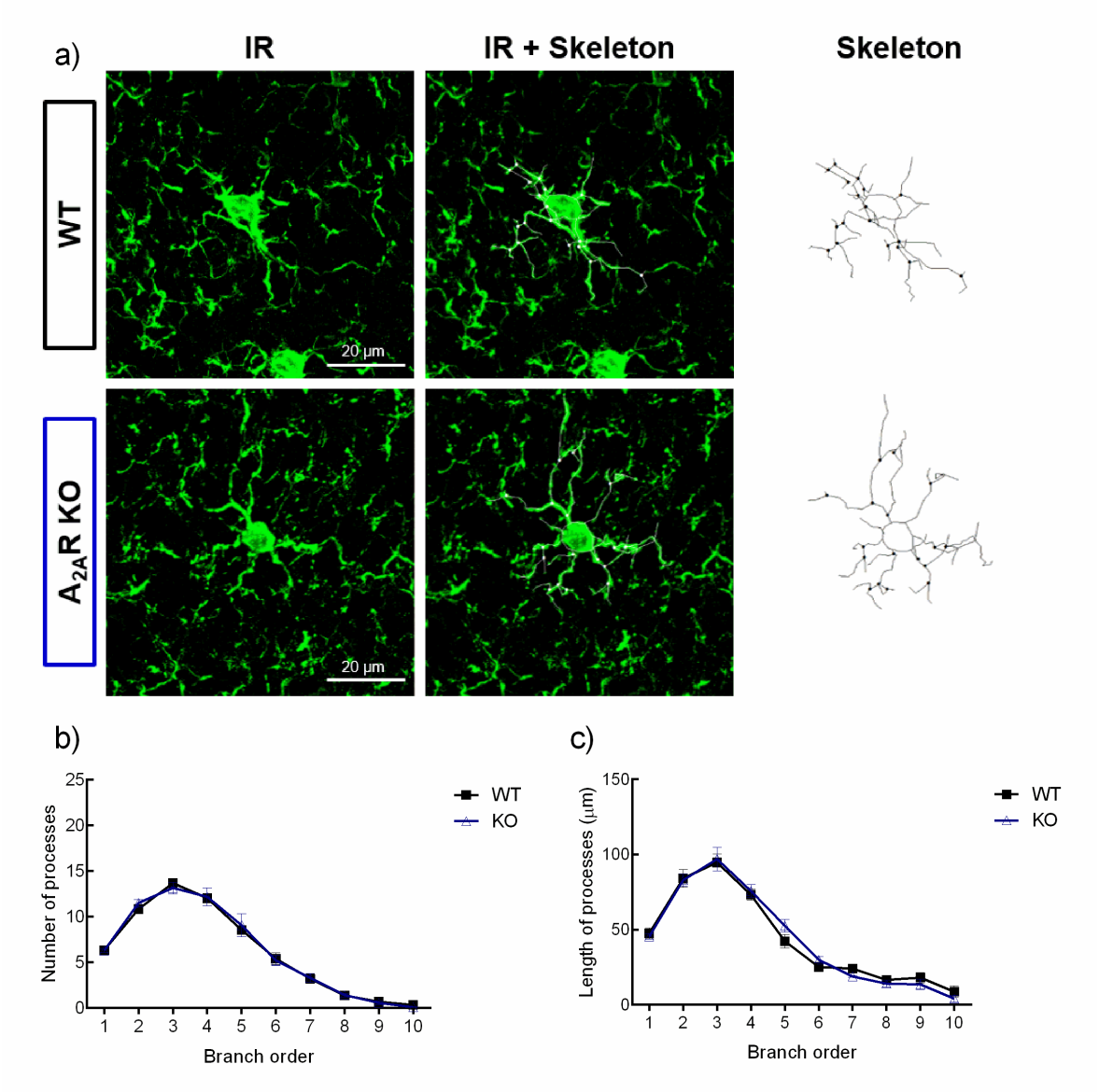


Figure 5 A_{2A}R KO male mice exihibited the number and the lenght of microglia processes similar to thouse in A_{2A}R WT male mice in the PFC at PND90. (a) Microglial morphometric structure was manually reconstructed in the Neurolucida software based on three-dimentional (3D) images of Iba-1 stained microglia (green). Representative images of Iba-1 stained microglia include: Iba-1 immunoreactivity (IR), Iba-1 stained microglia merged with manual reconstruction (IR + skeleton); and isolated manual reconstruction (skeleton) of microglia. (b, c) Number and length of microglial processes resulting from the morphometric analysis of reconstructed cells from the PFC at PND 90 of A_{2A}R KO mice, compared to A_{2A}R WT mice according to branch order. Results are presented as the mean ± SEM of 4 animals; **P*<0.05, comparing with control, calculated using an unpaired Student's *t*-test. KO, knockout; PFC, prefrontal cortex; PND, postnatal day; WT, wild-type.

However, females that not express A_{2A}R experienced a morphological remodelling of microglia (Figure 6, a) visible at PND90, with an increase in the number of processes (Figure 6, b) from the first to the third branch order and an increase in the length of processes (Figure 6, c) in the third branch order, comparing to the WT animals. The values related to the morphometric analysis of microglia processes are detailed in the Supplementary table 1 (number of processes) and 2 (length of processes) in Chapter 6.

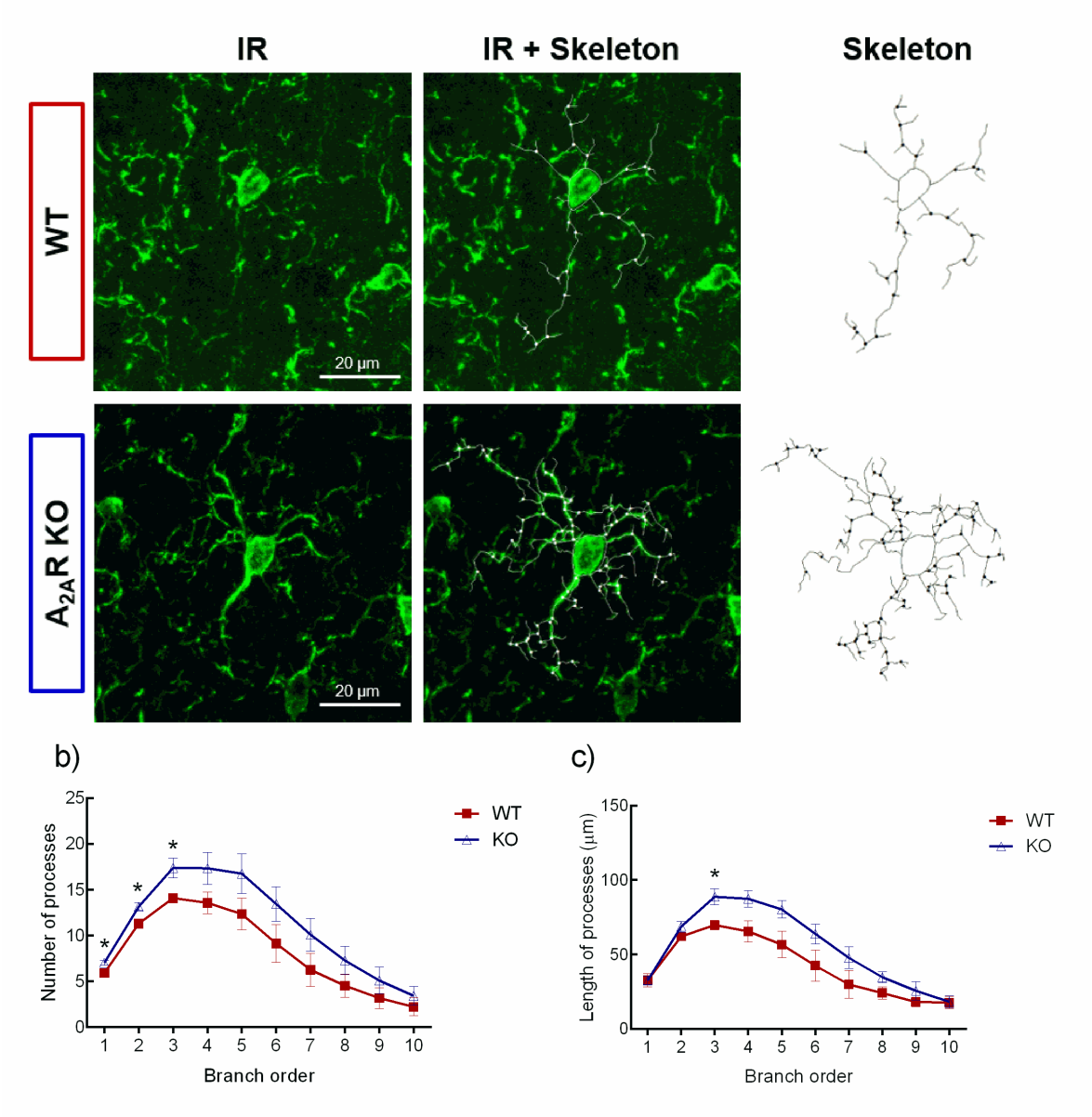


Figure 6 A_{2A}R KO female mice exihibited higher number and length of microglial processes *per order* in the PFC than A_{2A}R WT female mice at PND90. (a) Microglial morphometric structure was manually reconstructed in the Neurolucida software based on 3D images of Iba-1 stained microglia (green). Representative images of Iba-1 stained microglia include: Iba-1 immunoreactivity (IR), Iba-1 stained microglia merged with manual reconstruction (IR + skeleton); and isolated manual reconstruction (skeleton) of microglia. (b, c) Number and length of microglial processes resulting from the morphometric analysis of reconstructed cells from the PFC at PND 90 of A_{2A}R KO mice, compared to A_{2A}R WT mice according to branch order. Results are presented as the mean ± SEM of 4 animals; **P*<0.05, comparing with control, calculated using an unpaired Student's *t*-test. KO, knockout; PFC, prefrontal cortex; PND, postnatal day; WT, wild-type.

3.1.4. Discussion

Microglia cells perform imperative functions in the developing brain, as the pruning of synapses (Paolicelli *et al*, 2011). In these cells, morphology and function are strongly associated (Hinwood *et al*, 2013; Kreisel *et al*, 2014). An aberrant morphological remodelling of microglia may be associated with the impairment of their functions, promoting weak synaptic transmission, decreased functional brain connectivity and behaviour abnormalities (Zhan *et al*, 2014). Ultimately, this can lead to neuropsychiatric disorders (Caetano *et al*, 2016; D'Mello & Stoodley, 2015; Ferrarelli *et al*, 2015; Yirmiya *et al*, 2015; Rial *et al*, 2016).

One of the modulators of microglia morphology and function is the adenosine $A_{2A}R$ (Gyoneva *et al*, 2009; Orr *et al*, 2009). In a previous study, we also reported that the chronic pharmacological blockade of $A_{2A}R$ at adulthood promoted a morphological remodelling of microglia cells in a gender-specific manner: atrophied microglia cells from females, not affecting males. Furthermore, we also observed that the blockade of $A_{2A}R$ was anxiogenic in females, but not in males (Caetano *et al*, 2016). Importantly, we correlated the morphological remodelling of microglia with changes in anxious-like behaviour, although without the inference of a causative effect (Caetano *et al*, 2016).

In the present work, we aimed to characterize the effect of $A_{2A}R$ depletion early in development. In the $A_{2A}R$ KO mice, a model which does not express $A_{2A}R$ from early development, we characterized microglia morphology in the PFC at PND90, the same age in which animals were analysed in the aforecited study (Caetano *et al*, 2016). We observed that the absence of $A_{2A}R$ in these animals affected microglia morphology in a gender-specific manner: hypertrophied microglia cells in females, but had no effect in these cells in males.

Comparing the present work with our previous study (Caetano *et al*, 2016), we used two distinct approaches, a pharmacological and a genetic manipulation of $A_{2A}R$. We observed that microglia morphology was affected only in females, in both cases. Thus, we can report a robust gender effect in the manipulation of this receptor. It can occur due to the organisational effects of hormones, for instance testosterone that is responsible for brain masculinisation (Konkle & McCarthy, 2011), but can also depend on differences in molecules and receptors associated with the purinergic system. For instance, women possess lower levels of adenosine in the total cortex, as shown in a *post mortem* study (Kovács *et al*, 2010). Differences in the availability of adenosine can be responsible for the

gender-specific answer from the adenosinergic system, considering that, despite the blockade or absence of $A_{2A}R$, adenosine may act in other receptors, besides $A_{2A}R$.

The age in which $A_{2A}R$ was manipulated has also an effect, taking into account the present work and our previous study (Caetano *et al*, 2016). Microglia underwent opposite morphological remodelling in females whose $A_{2A}R$ was blocked at adulthood after a normal neurodevelopment (atrophy) and in those whose $A_{2A}R$ was never expressed (hypertrophy). Possibly, it makes sense that females that never expressed $A_{2A}R$ experienced an hypertrophy of these cells, since $A_{2A}R$ activation promotes an atrophy of these cells (Gyoneva *et al*, 2014). Regarding the atrophy of microglia cells reported in our previous study, these females can be under a compensatory mechanism for the absence of $A_{2A}R$ function; however, the molecular machinery and the mechanism involved in this process deserve further attention. Importantly, $A_{2A}R$ blockade has opposite impacts in the brain, considering the age of manipulation. For instance, $A_{2A}R$ blockade is strongly neuroprotector against brain damage at adulthood; however, brain damage is intensified in developing $A_{2A}R$ KO mice (Ådén *et al*, 2003), reinforcing an opposite effect of $A_{2A}R$ during development or at adulthood.

In addition, both the pharmacological blockade and the genetic depletion of $A_{2A}R$ were not confined to microglia cells. Neurons and other cells are also equipped with this receptor (Rebola *et al*, 2005b). Thus, the effects we observed can be mediated by these cells. Moreover, imperative developmental functions of these cells are widely associated with microglia morphology and motility, as the pruning of synapses (Paolicelli *et al*, 2011), reinforcing the possibility of a cumulative effect of $A_{2A}R$ function deprivation in both microglia cells and neurons.

Adenosine $A_{2A}R$ modulation has been proposed as a therapeutic approach in cases of neuropsychiatric disorders (Cunha *et al*, 2008). Regarding microglia morphology in the PFC, females powerfully answer to the modulation of $A_{2A}R$, in opposition to males. Moreover, females possess increased risk to develop some neuropsychiatric disorders, namely anxiety and depression, and microglia have been reported to have an effect in this context in a gender-specific manner (Caetano *et al*, 2016) (reviewed in (Nelson & Lenz, 2017b)). This reinforces the role of microglia in the pathophysiology of neuropsychiatric disorders. We observed different effects of the deprivation of $A_{2A}R$ function, not only between genders (Caetano *et al*, 2016), but also taking in consideration the developmental stage of manipulation. This highlights the importance of a therapeutic

strategy focused on the individual, according with gender and age, considering the whole organism and not only the pathology that requires treatment.

3.2.

Microglia morphology in the PFC during neurodevelopment: screening gender differences

3.2.1. Rationale

A plethora of functions that are essential during neurodevelopment in order to sculpt a healthy brain are attributed to microglia. When exposed to adverse stimuli early in life, irreversible changes may occur in the developing brain and trigger neurodevelopmental disorders. In our previous work, we described physiological gender differences in microglia morphology in the PFC, a brain region widely associated with anxiety, and in the performance in the EPM test, at adulthood (Caetano *et al*, 2016). This physiological dimorphism of microglia can help explaining the gender biased risk for developing this kind of disorders. Thus, it is imperative to further understand whether physiological gender differences in microglia surge in a crucial stage of development, leading to short- and long-term effects in behaviour.

To address this question, we characterized microglia morphology in the PFC of Wistar rats at PND0, PND7 and PND33 under physiological conditions, in both genders, in parallel with behavioural tests for anxious-like behaviour and recognition memory.

3.2.2. Methods

3.2.2.1. Animals

Wistar rats (Charles River, Barcelona, Spain) were housed under standard laboratory conditions (light/dark cycle 12/12 h)), at RT and with *ad libitum* food and water. All procedures were carried out in accordance to the European Union and National legislation. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2.2.2. Behavioural tests

Starting on PND 21, the day of maternal weaning, rats were only handled by the researcher that performed the tests. Animals were submitted to behavioural tests between PND 30 and PND 32, after the pubertal period. Tests were conducted during the light phase of the light/dark cycle, after 1 h of room habituation, always in the same conditions: red light and controlled temperature and ventilation.

The animals were submitted to different tests in the following order: in the first day, the animals were first tested for locomotor activity in the open field (OF), followed by an adaptation to the EPM test; in the second day, the short-term memory was assessed in the NOR1 and NOR2; and in the third day, animals finish with the assessment of anxious-like behaviour in the EPM. A videocamera located overhead was used to record mice behaviour during the test. All apparatus were cleaned with 10% ethanol solution and dried between tests.

I) Locomotor activity: Open field test

The OF apparatus is an empty bright square arena, surrounded by walls to prevent animal from escaping (45 x 45 x 40 cm) (Figure 7). The animal was placed at the center of the arena, facing one of the walls, and the behaviour was recorded for 5 min. The locomotor activity was measured by the distance travelled and the mean speed, as described in (Machado et al. 2016). The test was analysed in the ANY-MAZE software.

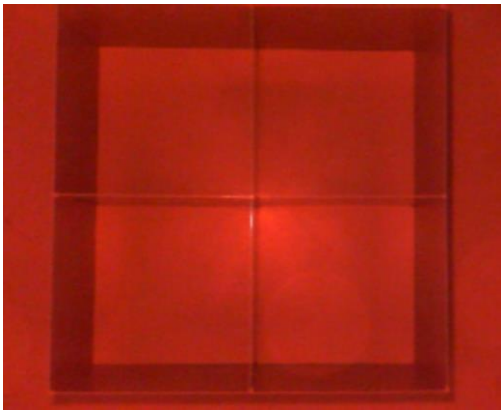


Figure 7 Representation of the open field arena. The apparatus present in the representation possesses four open field arenas.

II) Anxious-like behaviour: Elevated plus maze test

The EPM is an elevated maze (65 cm) with two open (45 x 10 cm) and two closed arms (45 x 10 x 50 cm), that form a plus shape (Figure 8Figure 8). The animal was placed in the intersection of the arms, facing a corner between open and closed arms, and the behaviour was recorded for 5 min. The assessment of anxious-like behaviour was performed based on the number of entries in open arms (OA) and the time spent in OA in function of total time, as described in (Machado et al. 2016). The test was manually analysed with Observador software.

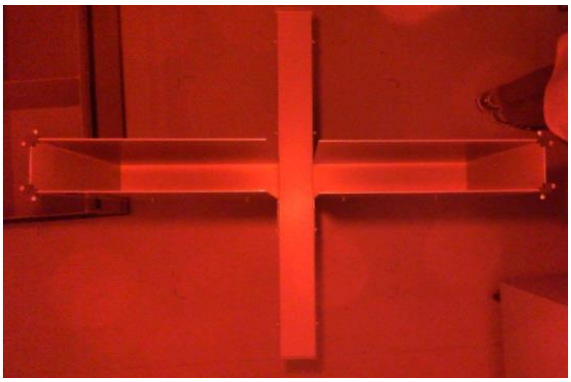


Figure 8 Representation of the elevated plus maze.

III) Short-term recognition memory: Novel object recognition test

The NOR test was performed to evaluate short-term recognition memory, by assessing the capability of the animal to distinguish between a familiar and a novel object. In the NOR1, the animals were placed in the OF apparatus in the presence of two equal objects in colour, shape and size (Figure 9, a). The test begins with the rat positioned back to objects and facing the wall, being recorded for 5 min. After 2 h, in the NOR2, one of the previous objects persists in the apparatus (familiar object) and the other is replaced by a different one (novel object 1) in colour and shape, but not in size (Figure 9Figure 9, b). The recognition index was measured by the time spent exploring the new object in function of the time spent exploring both objects, as described in (Machado et al. 2016). The test was manually analysed with the software Observador.

a) b)

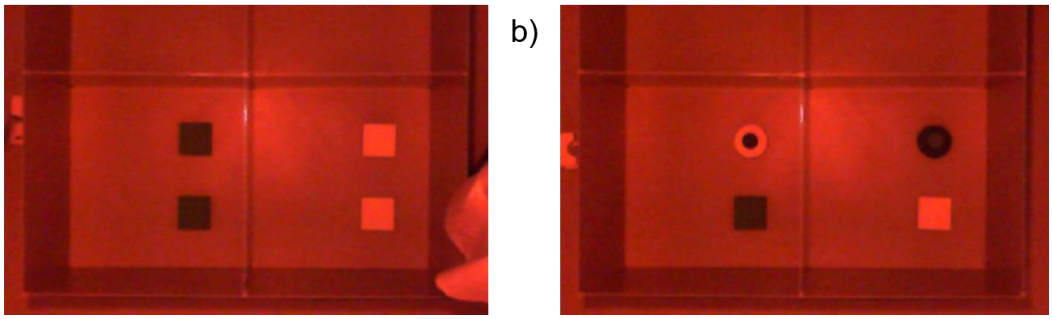


Figure 9 Representation of the arenas from the novel object recognition test. Short-term recognition memory was evaluated using the NOR test. In the training phase of the test (NOR1), mice were exposed to two similar objects during 5 min (a). The test session was performed two hours latter (NOR2), with a familiar object and a novel object (b).

The NOR1 was performed with a total of 43 animals, 20 males and 23 females. From these animals, 3 males were excluded because they did not explore one of the familiar objects and 6 males and 14 females were excluded because they show preference for one of the objects. From the 11 males and 9 females whose behaviour in NOR2 was analysed, 2 males and 1 female were excluded because they did not explore the novel object. Finally, 9 males and 8 females were used for statistical analysis of this test.

3.2.2.3. Immunohistochemistry

Wistar rats were sacrificed by decapitation at PND0 and PND7 or deeply anesthetized with an ip injection of ketamine (90 mg/kg; Nimatek) and xylazine (10 mg/kg; Ronpum 2%) and transcardially perfused with PBS 1x, followed by 4% PFA at PND 33. The brains were removed, reserved and sectioned according to the protocol described in Section 3.1.2.2 from Chapter 3, page 32.

A free floating immunohistochemical procedure was performed in brain slices from the PFC located at the stereotaxic coordinates of interaural 5.00 mm and bregma 1.60 mm in PND 0 rats (Khazipov *et al*, 2015), interaural 8.00 mm and bregma 2.60 mm in PND 7 rats (Khazipov *et al*, 2015) and interaural 12.72 mm and bregma 3.72 mm in PND 30 rats (Paxinos & Watson, 1998), in accordance with the protocol described in Section 3.1.2.2 from Chapter 3, page 32.

3.2.2.4. Morphometric analysis of microglia

Morphometric analysis was conducted in accordance with the method described in Section 3.1.2.3 from Chapter 3, page 33. 5 animals were analysed *per* age and gender, according to the represented region of the PFC (Figure 10). Settings were chosen to optimize the labelling of microglial processes and kept throughout all sets of acquisitions for animals with the same age.

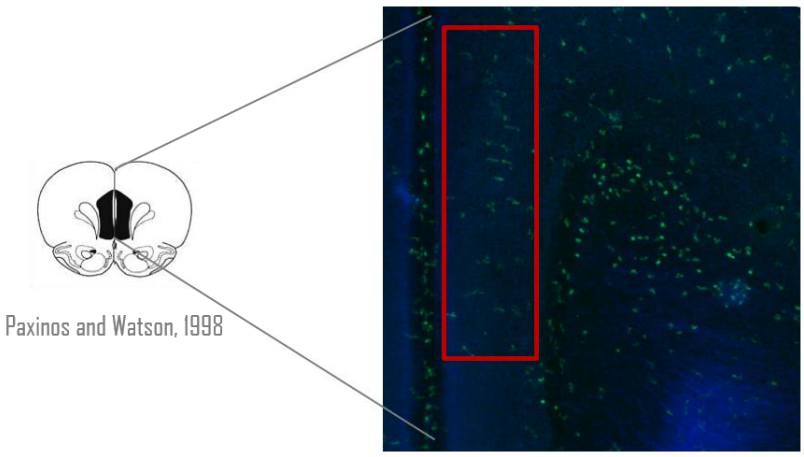


Figure 10 Schematic representation of the region from the PFC where microglia cells were acquired for 3D reconstruction. a) Illustration of a slice from the rat brain, with the PFC in black, adapted from (Paxinos & Watson, 1998). b) Representative image of the region where the images for tridimensional reconstruction of microglia were acquired (red), obtained by the author in fluorescence microscope with 20x magnification. Nuclei were stained with DAPI (blue), and microglia with Iba-1 (green).

To perform the tridimensional reconstruction of microglial cells from the PFC, Z-stacks were imported to the Neurolucida software (MBF Biosciences) and 50 microglial cells were manually reconstructed *per* condition. The morphometric analysis was performed using NeuroExplorer software (MBF Biosciences), an extension of Neurolucida software. At PND 0 microglial cells present an amoeboid morphology, but at PND7 and PND33 differentiated processes can be observed. Taking this into account, we evaluated different morphological parameters in these ages: at PND0, microglial cells were reconstructed by contouring the entire cell in each Z-stack (Figure 11) and the parameters analysed were the cell volume and the surface area. From PND 7 onwards, microglial cells were reconstructed according to the described in Section 3.1.2.3 from Chapter 3, page 33. The mean *per* condition was considered for further analysis.

Figure 11 Illustrative scheme showing the tridimensional reconstruction of microglia at PND0. Microglia cells were reconstructed by contouring the entire cell in each Z-stack from the laser scanning

confocal microscope LSM 710 META connected to ZEN Black software (Zeiss Microscopy, Germany) with a 63x objective lens (oil immersed, Plan-Apochromat 63x/1.40 Oil DIC M27).

3.2.2.5. Data analysis

Statistical analysis was performed in GraphPad Prism version 6 (GraphPad Software Inc., USA). All graphic values are expressed as mean ± SEM. Shapiro-Wilk normality test was used to assess whether the sample presents a normal distribution. Student's t test was used to compare two independent means when the samples followed a normal distribution, and the non-parametric Mann-Whitney test was used when samples do not follow the normality. Differences were considered significant at $p<0.05$ (*) and $p<0.01$ (**).

Figure 12 Schematic overview of the analysis of behaviour and microglia morphology of Wistar rats under physiological conditions. EPM, elevated plus maze; Mic. Morph., microglia morphology; NOR, novel object recognition; OF, open field; PND, postnatal day.

3.2.3. Results

3.2.3.1. Microglia morphology in the PFC is similar between genders in neonates

At PND0, we did not observe any gender effect in microglia morphology in the PFC (Figure 13, a), regarding cell volume (NT males: 2749 ± 391.2 µm^3; NT females: 2376 ± 198.9 µm^3, n=3, p>0.05) (Figure 13, b) or surface area (NT males: 1654 ± 158.3 µm^3; NT females: 1543 ± 146.4 µm^3, n=3, p>0.05) (Figure 13, c).

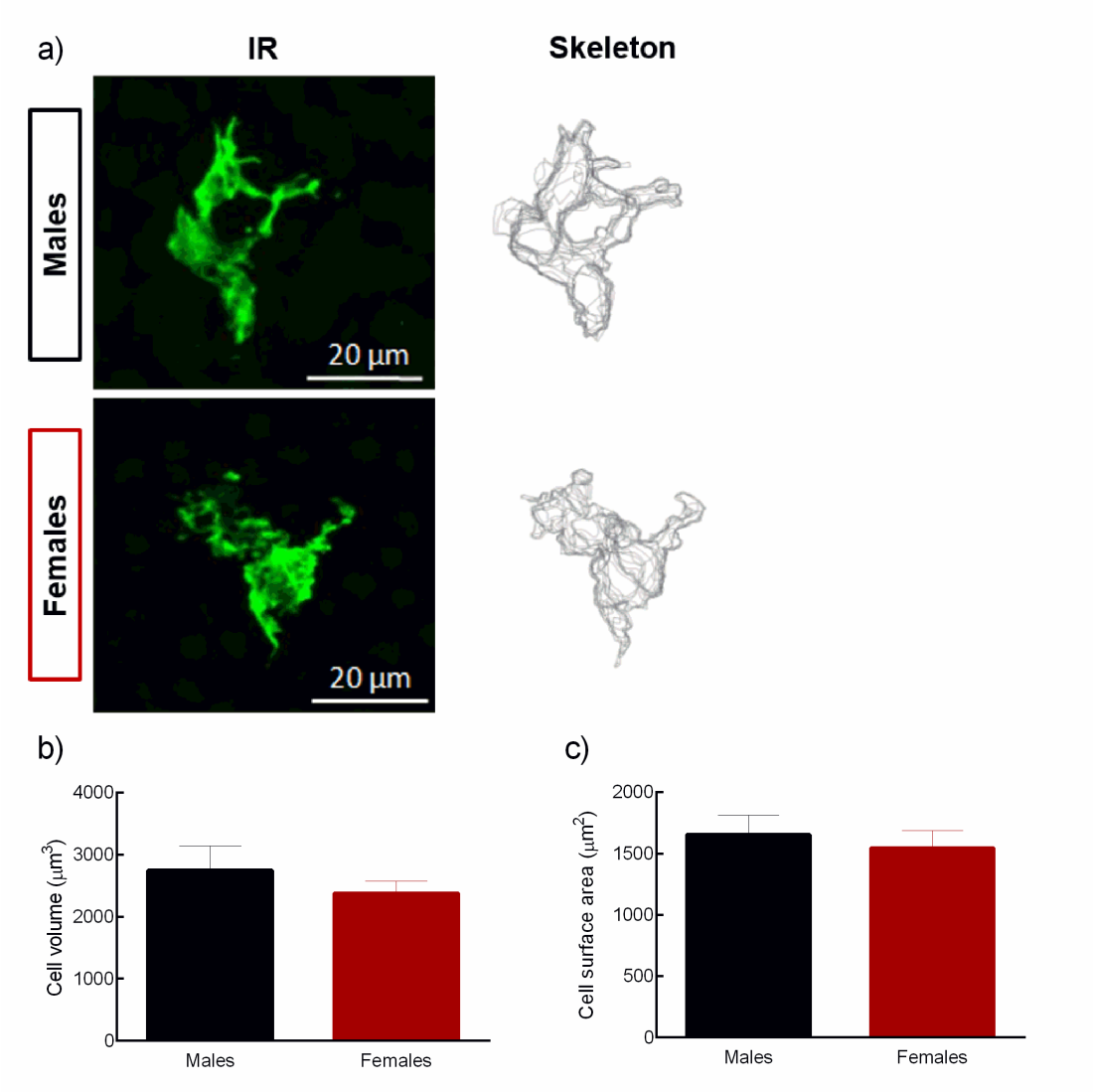


Figure 13 Morphometric analysis and statistics of microglia in the PFC at PND0. Male and female Wistar rats were sacrificed at PND0. Male and female Wistar rats were sacrificed at PND0. Brain slices were stained with Iba-1 and microglia were manually reconstructed in 3D using Neurolucida Software. Representative

images were obtained from Iba-1 stained microglia from males (top) and females (bottom): Iba-1 immunoreactivity (IR, green); isolated manual reconstruction (skeleton) (a). Morphometric analysis of microglia was aquired in Neurolucida Explorer, considering the volume (b) and the surface area (c). Results are presented as mean ± SEM of 3 animals. Student's t test was used to compare two independent means: * $p<0.05$, comparing males with females.

3.2.3.2. Microglia morphology in the PFC is similar between genders at PND7

Childhood is a stage of neurodevelopment in which the sculpture of the brain is a very plastic process, well-regulated to originate a functional healthy brain. At PND7, it occurs a peak of expression of $A_{2A}R$ (Weaver, 1993), a receptor well described as modulating microglia morphology (Gyoneva *et al*, 2009; Orr *et al*, 2009). Considering the essential roles of microglia throughout the developmental process, as well as the narrow relation between their morphology and function, we evaluated microglia morphology in the PFC at PND7 to understand whether gender differences in these cells surge prior or after the peak of expression of $A_{2A}R$.

At PND7, we did not observe gender differences in microglia morphology in the PFC (Figure 14, a), regarding the number (Figure 14, b) and the length (Figure 14, c) of their processes in the first ten branch orders. The values related to the morphometric analysis of microglia processes are detailed in the Supplementary table 3, in Chapter 6.

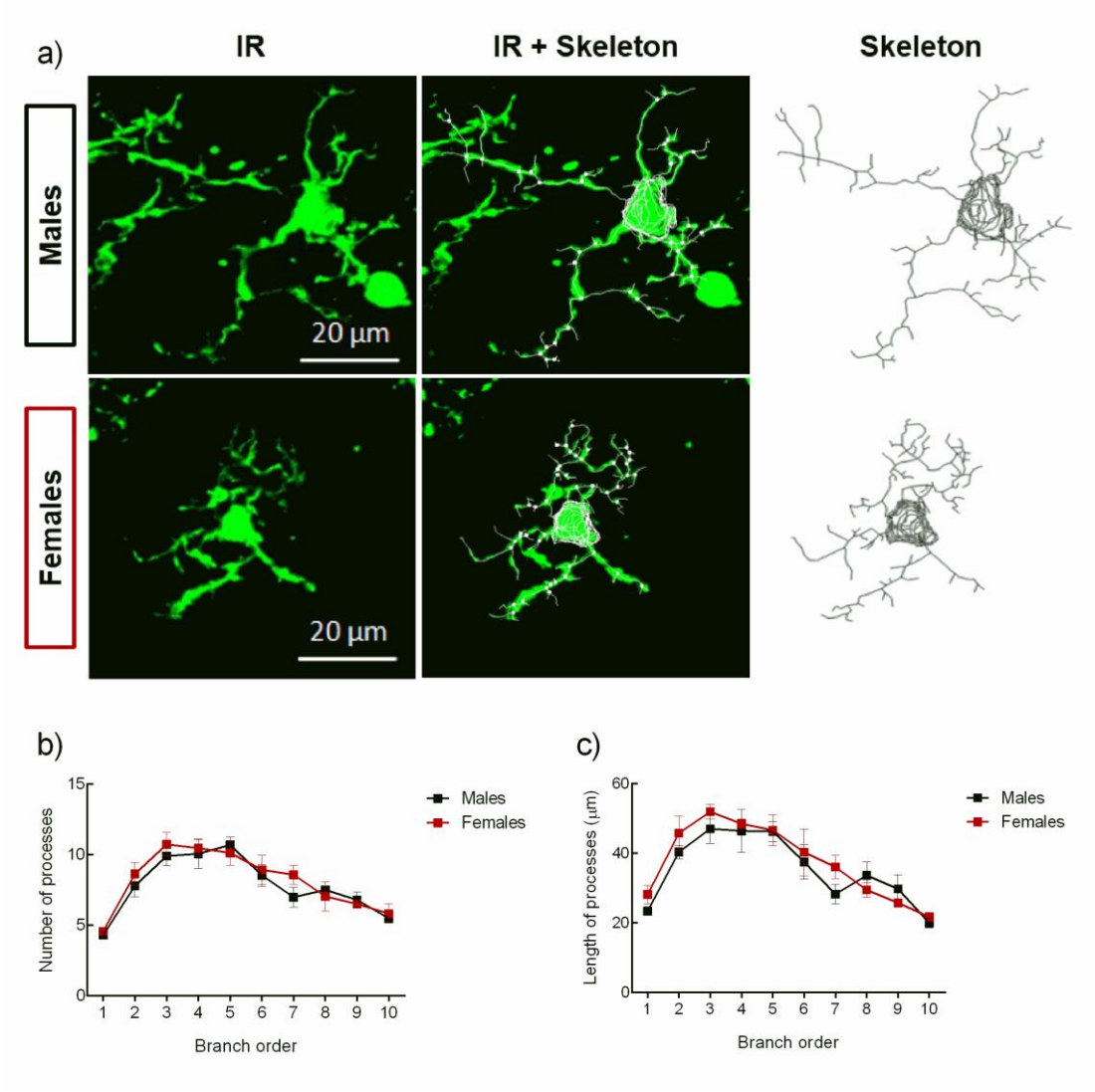


Figure 14 Morphometric analysis and statistics of microglia in the PFC at PND7. Male and female Wistar rats were sacrificed at PND7. Brain slices were stained with Iba-1 and microglia were manually reconstructed in 3D using Neurolucida Software. Representative images were obtained from Iba-1 stained microglia from males (top) and females (buttom): Iba-1 immunoreactivity (IR, green); IR merged with isolated manual reconstruction (skeleton) (white); skeleton (black) (a). Morphometric analysis of microglia was aquired in Neurolucida Explorer, considering the number of processes (b) and the length of processes (c) *per* branch order. Results are presented as mean ± SEM of 3 animals. Student's t test was used to compare two independent means: * $p<0.05$, comparing males with females.

3.2.3.3. Microglia morphology in the PFC is similar between genders at PND33

During juvenile, after the early neurodevelopmental process, we hypothesized that the gender differences observed in microglia morphology in the PFC at adulthood may be already present.

At PND33, we did not observe gender differences in microglia morphology in the PFC (Figure 15, a), regarding the number (Figure 15, b) and the length (Figure 15, c) of their processes in the first ten branch orders. The values related to morphometric analysis of microglia processes are detailed in the Supplementary table 4, in Chapter 6.

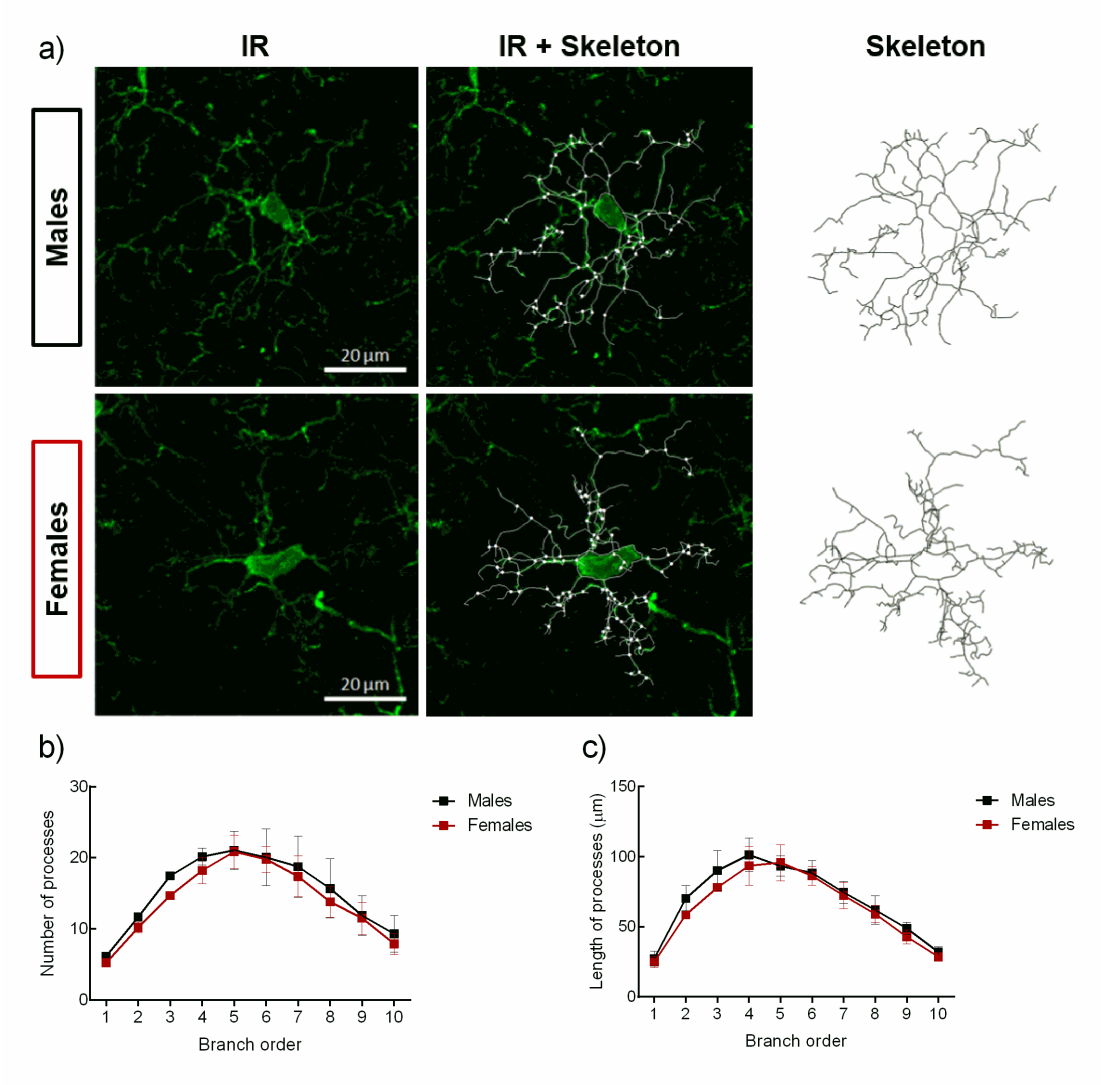


Figure 15 Morphometric analysis and statistics of microglia in the PFC at PND33. Male and female Wistar rats were sacrificed at PND33. Brain slices were stained with Iba-1 and microglia were manually reconstructed in 3D using Neurolucida Software. Representative images were obtained from Iba-1 stained microglia from males (top) and females (buttom): Iba-1 immunoreactivity (IR, green); IR merged with isolated manual reconstruction (skeleton) (white); skeleton (black) (a). Morphometric analysis of microglia was aquired in Neurolucida Explorer, considering the number of processes (b) and the length of processes (c) *per* branch order. Results are presented as mean ± SEM of 3 animals. Student's t test was used to compare two independent means: * $p<0.05$, comparing males with females.

3.2.3.4. Females are more disinhibited than males in the EPM at PND32

We evaluated locomotor activity in both genders by performing the OF test at PND30. We observed that males and females displayed similar locomotor activity, concerning the distance travelled in the maze (Males: 10.02 ± 1.16, N=20; Females: 11.80 ± 0.81, N=17; m) (Figure 16, a) and the mean speed (Males: 0.03 ± 0.004, N=20; Females: 0.04 ± 0.003, N=17; m/s) (Figure 16, b).

Data from the performance of these animals in the EPM showed females to be more disinhibited than males at PND32, under physiological conditions, concerning the time spent in OA in function of total time of the test (Males: 0.05 ± 0.01, N=20; Females: 0.10 ± 0.02, N=24; $p<0.01$; Figure 16, c) and the number of entries in OA (Males: 2.00 ± 0.33, N=21; Females: 3.30 ± 0.46, N=23; $p<0.05$; Figure 16, d).

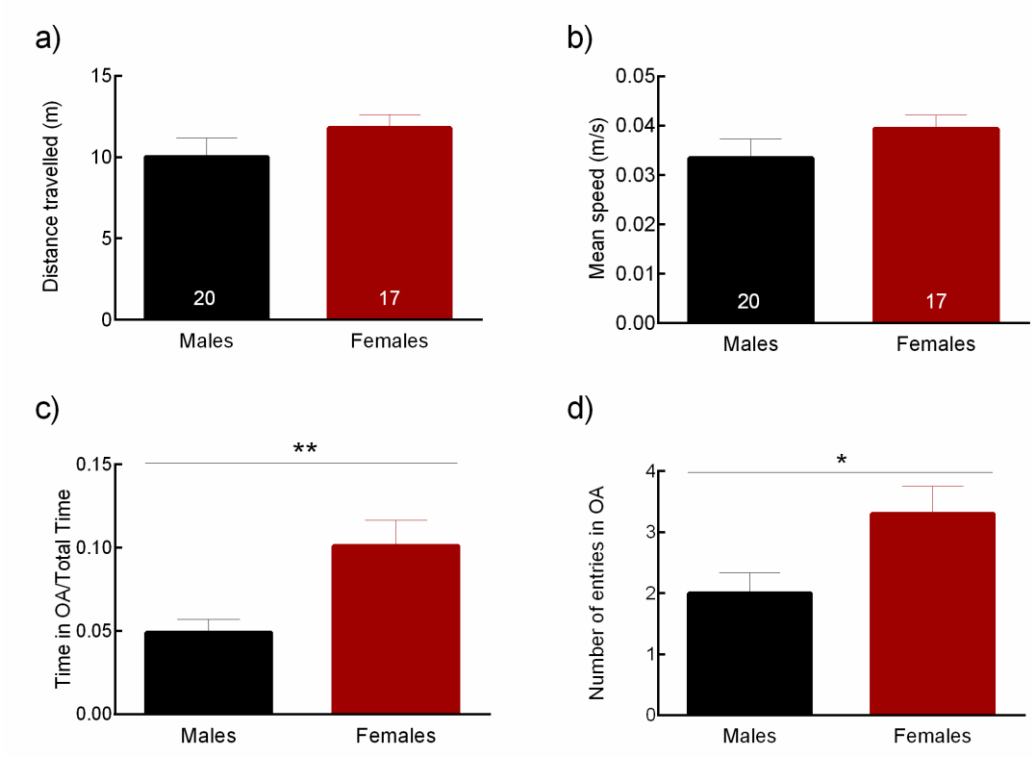


Figure 16 Analysis of locomotor activity and anxious-like behaviour in open field (OF) and elevated plus maze (EPM) tests. Male and female Wistar rats were tested in the OF at PND30 and in the EPM at PND32, under physiological conditions. The tests were recorded with a videocamera. Recordings from OF tests were analysed with ANY-MAZE software and recordings from EPM were manually analysed with the software Observador. The distance travelled (a) and the mean speed (b) in the OF were used as a measure of locomotor activity. The quocient between the time spent in open arms (OA) of the EPM and the total time (c), and the number of entries in OA (d) were used as a measure of anxious-like behaviour. Results are presented as mean ± SEM, for the indicated number of animals. Student's t test was used to compare two independent means when the samples followed a normal distribution and the correspondent non-parametric Mann-Whitney

test was used when samples do not follow the normality: * $p<0.05$ and ** $p<0.01$, comparing males with females.

3.2.3.5. Short-term recognition memory is not dependent on the gender at PND31

Considering the gender differences observed in the behaviour, as analysed in the EPM, we also evaluated the short-term recognition memory at PND31 by performing the NOR test, under physiological conditions. We observed that there are no gender differences in short-term recognition memory at this age (Males: 0.57 ± 0.12, N=9; Females: 0.48 ± 0.09, N=8; $p>0.05$; Figure 17), as analysed in the NOR2 test.

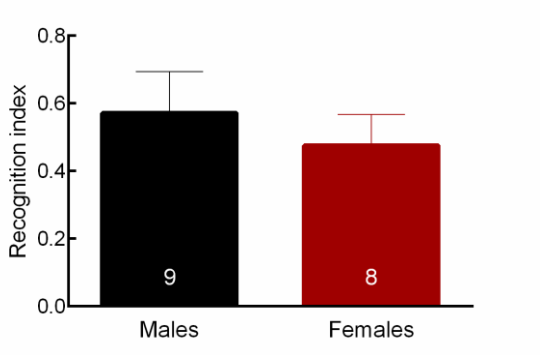


Figure 17 Analysis of the short-term recognition memory in males and females. The NOR test was performed to evaluate gender differences in cognition at PND31, under physiological conditions.
The tests were recorded with a videocamera and the recordings analysed manually with Observador software. The graphis represent the time spent exploring the novel object *per* total time exploring both objects (recognition index). Results are presented as mean ± SEM, for the indicated number of animals. Student's t test was used to compare two independent means.

3.2.1. Discussion

Microglia cells have been described as actively participating in the process of brain sculpture (Cunningham *et al*, 2013; Wake *et al*, 2009; Paolicelli *et al*, 2011; Peri & Nüsslein-Volhard, 2008). Importantly, microglia morphology is widely associated with their function (Hinwood *et al*, 2013; Kreisel *et al*, 2014), due to the ability for constantly retract and extend their processes, that allow these cells to survey the surrounding environment (Nimmerjahn *et al*, 2005; Davalos *et al*, 2005) and actively contact with synaptic structures (Tremblay *et al*, 2010). The impairment of these cells can lead to neuropsychiatric disorders, namely mood disorders, as anxiety (Caetano *et al*, 2016) or depression (Yirmiya *et al*, 2015; Rial *et al*, 2016). Moreover, these pathologies are known to possess gender-specific genesis, clinical presentation and answer to therapeutics. For instance, mood disorders affect females more than twice comparing to males (Kessler *et al*, 2005; Bekker & van Mens-Verhulst, 2007). Thus, it is imperative to explore the pathophysiology of anxiety in a gender-integrated manner.

In our previous study, microglia morphology in the PFC and anxious-like behaviour were analysed at PND90, in Wistar rats under physiological conditions. We reported microglia cells from females to possess a higher number of processes and longer processes *per* branch order, comparing to males. Moreover, we observed that females displayed a more disinhibited performance in the EPM test than males, staying a longer time in the OA, not due to differences in locomotor activity. Thus, these gender differences in microglia morphology were correlated with gender-specific performance in behavioural tests for anxiety at adulthood (Caetano *et al*, 2016).

In the present work, we aimed to clarify the developmental stage of surge of these morphological differences in the PFC, a brain region widely associated with anxiety, considering the imperative functions of these cells during neurodevelopment, as well as in the context of an injury. We evaluated 3 timepoints associated with events that contribute for the normal brain development, as the surge of testosterone in males at PND0 (Konkle & McCarthy, 2011), the peak of expression of $A_{2A}R$, a well characterized modulator of microglia morphology and function, at PND7 (Weaver, 1993), or the post pubertal period, following imperative hormonal fluctuations, at PND33. In parallel, we analysed behavioural performance in these animals between PND30 and PND32, under physiological conditions.

Unexpectedly, we did not observe gender differences in microglia morphology in the PFC in any of the evaluated timepoints, leading us to conclude that the gender differences in microglia morphology observed at adulthood surge after PND33. Other groups reported gender differences in the preoptic area, paraventricular nucleus, Hip, Amy and cortex throughout development in microglia morphology and number. However, these studies were based in a dichotomic morphological evaluation of these cells, only considering the presence or absence of processes (Schwarz *et al*, 2012; Lenz *et al*, 2013). Briefly, Schwarz and colleagues described variations in microglia number and morphology along life in the PFC. These authors reported a higher number of amoeboid microglia in the PFC of males at PND4; a higher number of microglia cells in females at PND30; and a higher number of microglia with thick and thin, long processes in females at PND60, comparing with males (Schwarz *et al*, 2012). Considering the gender differences we described in our previous work at PND90 (Caetano *et al*, 2016) and the aforementioned report from Schwarz and co-workers, we can anticipate an earlier time window for the surge of those gender differences in microglia morphology in the PFC, between PND30 and PND60. However, we should consider that two distinct methodologies of evaluation were used. Considering that, despite females possessing a higher number of ramified microglia than males at PND60, these processes can be similar to those in males regarding morphometric parameters.

Importantly, not only the PFC is associated with anxious-like behaviour, but also the Amy (highly involved in fear-related anxiety) (Rauch *et al*, 2003) and the bed nucleus of stria terminalis (involved in the control of anxiety-related emotionality) (Walker *et al*, 2003), among other brain regions. Thus, morphometric differences in microglia cells may possibly exist in other brain regions related with anxious-like behaviour prior to their appearance in the PFC. The colonization of the brain by microglia and the maturation of these cells are processes that depend not only on the gender, but also on the brain region (Schwarz *et al*, 2012).

In the behavioural analysis, we observed gender differences in the performance in the EPM test at PND32, with females exploring more the OA than males. Despite the EPM test is a well described test to measure anxious-like behaviour, we are not in the presence of a model of disease. Thus, we can not report our observations as males being more anxious than females; in this case, we should consider that females are more “disinhibited”, not due to differences in locomotor activity. Regarding the short-term recognition memory, analysed by the NOR test, the recognition index was similar between genders, leading us to conclude that males and females developed similarly their ability to

recognize a familiar object and to explore the novel one, as assessed by this particular test.

In our previous study (Caetano *et al*, 2016), we correlated gender differences in microglia morphology with anxious-like behaviour at adulthood, under physiological conditions. However, we did not show a causative relation between anxious-like behaviour and morphometric characteristics of microglia in the PFC. At youth, we observed that the gender-specific performance in the EPM test was not paralleled by differences in microglia morphology in the PFC. Thus, we are not able to perform the same assumption. There is the possibility that changes in microglia morphology do not directly affect or are affected by behavioural alterations, for instance after a stressful stimulus. Furthermore, other cell types can be involved in this process, namely neurons. For instance, the interaction between microglia and neurons in both healthy neurodevelopment (Tremblay *et al*, 2010; Paolicelli *et al*, 2011) and in the injured brain (Cristovao *et al*, 2014) lead us to hypothesize that the effects observed in behaviour, can be directly driven by neurons, or mediated by neuronal action in microglia cells, or even the opposite. The molecular mechanisms underlying these gender-specific effects of brain modulation on behaviour is still unknown.

In a different context, Nelson and Lenz showed that the depletion of microglia during the first two weeks of life decreased “anxious-like behaviour” in both genders at youth. However, only females are still affected at adulthood (Nelson & Lenz, 2017a). This study irrefutably showed the importance of microglia in neonatal period in the construction of behavioural characteristics in a gender-specific manner. Altogether, these data reinforce the capability of microglia cells in sculpting both brain and behaviour during development. The molecular mechanisms underlying these gender-specific effects of brain modulation on behaviour are still unknown.

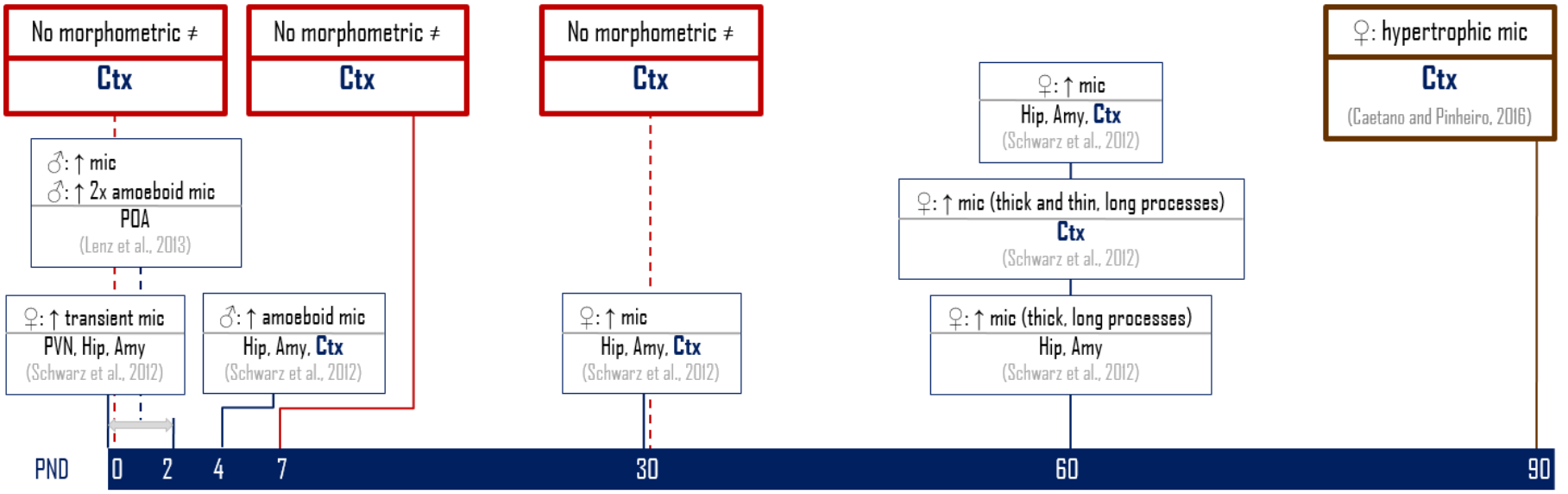


Figure 18 Physiological gender differences in microglia morphology between brain regions throughout neurodevelopment: a timeline. The abbreviations on scheme mean: amygdala (Amy), cortex (Ctx), hippocampus (Hip), microglia (mic), paraventricular nucleus (PVN) of the hypothalamus, preoptic area (POA), similar to the opposite gender (=), different from the opposite gender (≠). The arrows represent higher (↑) number than in the opposite gender, males (♂) and females (♀). The colours mean: studies from other groups (blue), published studies from our group (brown), and data from the present thesis (red). Based on (Schwarz *et al*, 2012; Lenz *et al*, 2013; Caetano *et al*, 2016).

3.3

Impact of neonatal injection of testosterone in female microglia morphology in the PFC

3.3.1. Rationale

The masculinisation of brain and behaviour is triggered by the ontogenic peak of testosterone that occurs in males during the first day of life (Konkle & McCarthy, 2011). Interestingly, this gender specific aspect of neurodevelopment could be reflected at adulthood in microglia morphology and anxious-like behaviour. Testosterone seems to produce a protective effect against neuropsychiatric disorders as anxiety, what is reinforced by the fact that females possess a higher risk for developing these pathologies. Taking this into account, we hypothesized that the protective role of testosterone against anxiety could be reflected in microglia morphology in the PFC, a brain region tightly involved in anxiety.

3.3.2. Methods

3.3.2.1. Animals and pharmacological treatment

Wistar rats (Charles River, Barcelona, Spain) were handled according to the European Union and National legislation. Wistar females received an ip injection with Testosterone Propionate (TP) compound (100 µg) (Sigma-Aldrich, Portugal - 86541-5G) in peanut oil (25 µl) (Sigma-Aldrich, Portugal - P2144-250ML) at PND0 (Hisasue *et al*, 2010). Other cohort of male rats was injected with vehicle (VH) (25 µl). Non-treated (NT) animals were also analysed. Animals were placed under standard laboratory conditions (RT; food and water *ad libitum*; light/dark cycle 12/12 h). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Figure 19 Schematic overview of the pharmacological treatment of female Wistar rats and microglia morphology and behaviour analysis. NT male and NT female Wistar rats were used as a control. EPM, elevated plus maze; Mic. Morph., microglia morphology; NOR, novel object recognition; OF, open field; PND, postnatal day; sc, subcutaneous; TP, testosterone propionate.

3.3.2.2. Behavioural tests

Behavioural tests were conducted as described in Section 3.2.2.2 from Chapter 3, page 43.

Regarding the NOR test, a total of 50 animals performed the NOR1: 20 males, 23 females and 7 TP females. From these animals, 3 males were excluded because they did not explore one of the familiar objects and 6 males, 14 females and 2 TP females were excluded because they show preference for one of the objects. From the 11 males, 9 females and 5 TP females whose behaviour was analysed in NOR2, 2 males and 1 female were excluded because they did not explore the novel object and 1 TP female was excluded because it is a statistical outlier. Finally, 9 males, 8 females and 4 TP females were evaluated considering the time spent exploring novel object *per* total time exploring both objects (recognition index).

3.3.2.3. Immunohistochemistry

Wistar rats deeply were anesthetized with of ketamine (90 mg/kg; Nimatek) and xylazine (10 mg/kg; Ronpum 2%) by ip injection and transcardially perfused with PBS 1x, followed by 4% PFA at PND 33. The immunohistochemistry procedure was performed as described in Section 3.2.2.3 from Chapter 3, page 46.

3.3.2.4. Morphometric analysis of microglia

Morphometric analysis of microglia was conducted as described in Section 3.2.2.4 from Chapter 3, page 46. According to the represented region of the PFC (Figure 10), 3 animals were analysed per condition and gender, and microglia cells were reconstructed according to (Figure 4). The number and length of processes per branch order were analysed. The mean per condition was considered for further analysis.

3.3.2.5. Data analysis

Statistical analysis was performed in GraphPad Prism version 6 (GraphPad Software Inc., USA). All graphic values are expressed as mean ± SEM. Shapiro-Wilk normality test was used to assess the normality of the sample. One-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test were used to compare

all groups when the samples followed a normal distribution. The correspondent non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test were used since samples do not follow the normality: * $p<0.05$, comparing males and females under physiological conditions; # $p<0.05$, ## $p<0.01$, comparing TP females with NT males and females.

3.3.3. Results

3.3.3.1. Neonatal female androgenisation did not alter microglia morphology in the PFC at PND33

In the previous chapter, we observed that microglia morphology in the PFC is similar between genders at youth. Now, we report that neonatal exposure to TP did not promote morphological changes in microglia morphology in the PFC of females at PND33 (Figure 20, a), neither in the number (Figure 20, b) nor in the length (Figure 20, c) of their processes in the first ten branch orders. The values from the morphometric analysis of microglia processes are detailed in in Supplementary table 4, in Chapter 6.

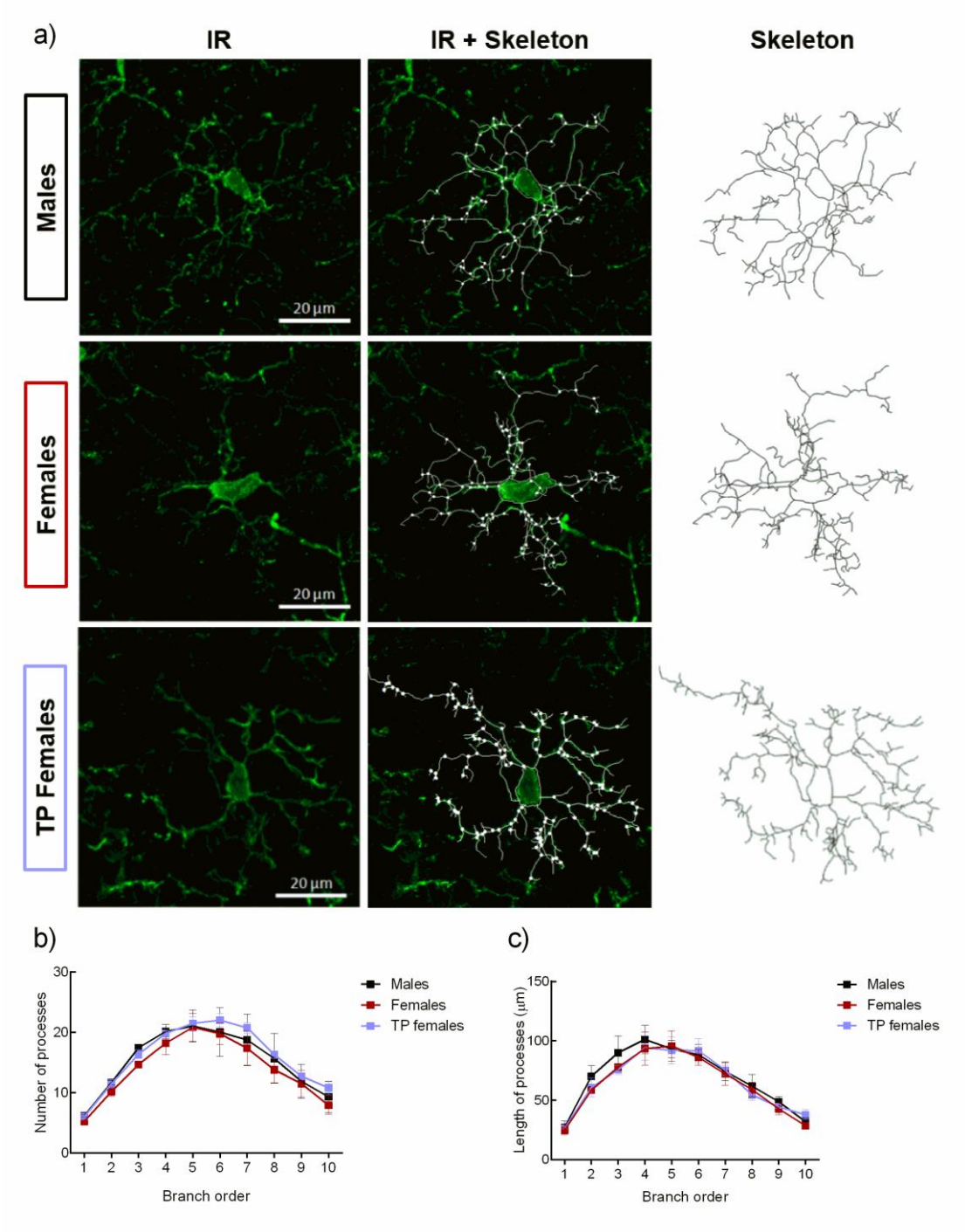


Figure 20 Effect of neonatal androgenisation of female Wistar rats in microglia morphology in the PFC at youth. NT male, NT female and TP female Wistar rats were sacrificed at PND33. Brain slices were stained with Iba-1 and microglia were manually reconstructed in 3D using Neurolucida Software. Representative images were obtained from Iba-1 stained microglia from males (top), females (center) and masculinised females (buttom): Iba-1 immunoreactivity (IR, green); IR merged with isolated manual reconstruction (skeleton) (white); skeleton (black) (a). Morphometric analysis of microglia was aquired in Neurolucida Explorer, concerning the number of processes (b) and the length of processes (c) per branch order. Results are presented as mean ± SEM of 3 animals. Kruskal-Wallis test was used to perform multiple comparitions.

3.3.3.2. Neonatal female androgenisation did not alter locomotor activity, but revealed a trend to an anxiolytic effect in the PFC at PND32

We evaluated locomotor activity in both genders under physiological conditions, as well as in females treated neonatally with TP (TP females), by performing the OF test at PND30. We did not observe a gender effect nor an effect of androgenisation in females, concerning the distance travelled in the field (TP females: 13.73 ± 1.24 m, N=7, n.s. as compared with controls [Males: 10.02 ± 1.16, N=20; Females: 11.80 ± 0.81, N=17; m]; Figure 21, a) and the mean speed (TP females: 0.05 ± 0.004 m/s, N=7; n.s. as compared with controls [Males: 0.03 ± 0.004, N=20; Females: 0.04 ± 0.003, N=17; m/s]; Figure 21, b).

Anxious-like behaviour was evaluated in the same animals by performing the EPM test at PND32. In this test, statistical analysis using non-parametric tests did not show an effect of the treatment in EPM test performance, regarding the time spent in OA *per* total time of the test (TP females: 0.06 ± 0.004, N=6; n.s. as compared with controls [Males: 0.05 ± 0.008, N=20; Females: 0.10 ± 0.02, N=24]; Figure 21, c). However, an effect of gender was observed (Figure 21, c). Concerning the number of entries in OA, neither treatment nor gender effects were observed (TP females: 2.00 ± 0.37, N=6, n.s. as compared with controls [Males: 2.00 ± 0.33, N=21; Females: 3.30 ± 0.46, N=23]; Figure 21, d).

Although a significant effect of TP was not observed as compared with NT females, a clear-cut trend anticipates an anxiolytic effect of neonatal androgenisation, as previously described by others.

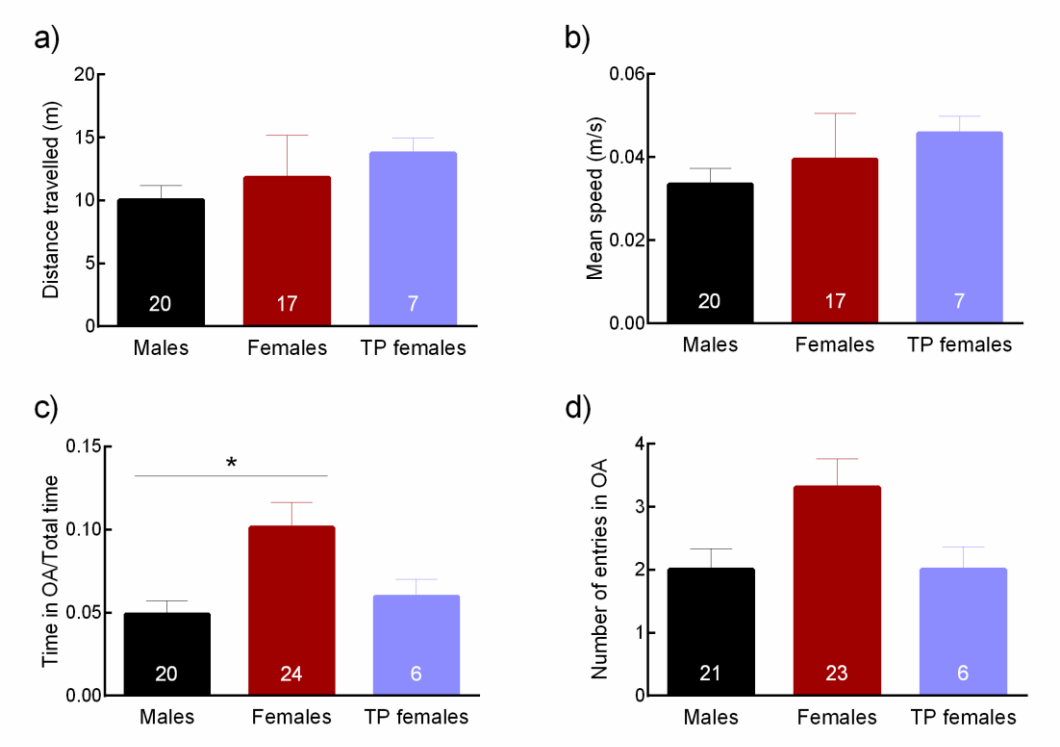


Figure 21 Effect of neonatal androgenisation in the locomotor activity and the anxious-like behaviour. NT Male, NT female and neonatally androgenised female Wistar rats (TP) were tested in the OF at PND30 and in the EPM at PND32. The tests were recorded with a videocamera. Recordings from OF tests were analysed with ANY-MAZE software and recordings from EPM were manually analysed with Observador software. The distance travelled (a) and the mean speed (b) in the OF were used as a measure of locomotor activity. The quocient between the time spent in open arms (OA) of the EPM and the total time (c), and the number of entries in OA (d) were used as a measure of anxious-like behaviour. Results are presented as mean ± SEM, for the indicated number of animals. Non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test were used since samples do not follow the normality: * $p<0.05$, comparing NT males with NT females.

3.3.3.3. Neonatal female androgenisation did not alter short-term recognition memory at PND31

Considering the similarities in short-term recognition memory between genders observed in the last chapter of results, we aimed to evaluate whether this behavioural measurement is influenced by neonatal androgenisation in females. We observed that there was no treatment effect in short-term recognition memory at this age (TP females: 0.39 ± 0.02, N=4, n.s. as compared with controls [Males: 0.57 ± 0.12, N=9; Females: 0.48 ± 0.09, N=8]; Figure 22).

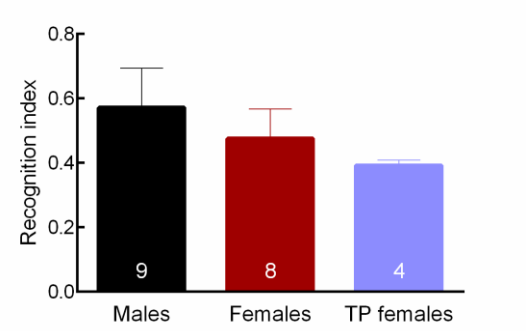


Figure 22 Effect of neonatal female androgenisation in short-term recognition memory. The NOR test was performed to evaluate the impact of neonatal androgenisation in female cognition at PND31. The tests were recorded with a videocamera and the recordings analysed manually with Observador software. The graphis represents the time spent exploring the novel object *per* total time exploring both objects. Results are presented as mean ± SEM, for the indicated number of animals. Kruskal-Wallis test followed by Dunn's multiple comparisons test were used since samples do not follow the normality.

3.3.4. Discussion

Nowadays, gender differences in anxiety disorders (Kessler *et al*, 2005; Bekker & van Mens-Verhulst, 2007) and microglia are one of the most discussed issues with both original studies (Caetano *et al*, 2016; Nelson & Lenz, 2017a) and reviews (Nelson & Lenz, 2017b; Gobinath *et al*, 2017) being published in the field. An hypothesis to explain gender differences in brain and behaviour is the involvement of testosterone (McHenry *et al*, 2014).

In the present study, we aimed to clarify the involvement of testosterone in the masculinization of brain, more precisely in microglia morphology and behaviour, namely anxious-like behaviour and recognition memory. For that, female Wistar rats were subcutaneously injected with testosterone at the day of birth to mimic the testosterone surge that occurs in males at PND0 (Konkle & McCarthy, 2011). Mid-term effects of neonatal exposure to testosterone were analysed at youth (PND30 - PND33).

We observed that neonatal androgenisation did not alter microglia morphology in the PFC at PND33, comparing with males and females whose microglia morphology was previously shown to be similar at that age, in Section 3.2.3.3, page 51. These observations suggest that neonatal testosterone surge that occurs in males (Konkle & McCarthy, 2011) is not sufficient to promote morphological differences in microglia at that age, reinforcing the absence of gender differences reported in Section 3.2.3.3, page 51. Moreover, as discussed in the mentioned section, gender differences described in the PFC until youth (Schwarz *et al*, 2012) are not supported by a morphometric analysis as in the case of the present study. Possibly, gross morphological characteristics of microglia that we did not evaluate or even their number can be different at PND33. Neonatal androgenisation may trigger other effects in the developing brain, namely changes in the size of some brain regions, as reported in the BNST from mice at 6 months old (Seney *et al*, 2012), alterations in signalling and connectivity between brain regions, number of cells, synapses and dendritic spines, and also gene expression (McCarthy, 2008). Since we did not find gender differences in microglia morphology in the PFC at youth, we consider evaluating the long- and not the mid-term effects of testosterone in brain and behaviour, for instance at PND90, an age in which we reported the existence of these gender differences (Caetano *et al*, 2016).

Regarding the behavioural analysis of these animals, in the present section of results, we reported that neonatal androgenisation did not promote significant differences

in anxious-like behaviour, comparing with both NT males and females. We could report gender differences in the performance in the EPM test, regarding the time spend in OA *per* total time of the test ($p<0.05$), but not the number of entries in OA (n.s.). Locomotor activity and short-term recognition memory were not affected by neonatal androgenisation.

In Section 3.2.3.4, page 53, we observed significant differences in the performance in the EPM test, regarding both the time spend in OA *per* total time of the test ($p<0.01$) and the number of entries in OA ($p<0.05$): females presented a higher disinhibition than males. Comparing the results from these two sections, we detected a reduction in statistical significance when comparing control males and females under physiological conditions, and the absence of statistical significance comparing androgenised females with control females. That reduction in statistical significance when comparing both genders are probably due to the alteration of the chosen statistical test: in the present section, we performed the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test due to the dimension of neonatal androgenised females sample (N=7), while in the Section 3.2.3.4 we applied the parametric Student's *t* test. Considering a visible effect of neonatal androgenisation mirrored in the graphics, which show that TP females underwent an intermediate phenotype comparing with control animals, we also performed the statistical analysis according to the methodology used in the Section 3.2.3.4, in order to understand whether the statistical results would be similar between statistical tests. As expected, the use of Student's *t* test to compare TP females with both NT males and females led us to report differences in anxious-like behaviour between TP females and control females, but not between TP females and control males. Importantly, an increase in the sample of TP females in order to confirm these results is needed.

Other study explored alterations in anxious-like behaviour, using the same model of neonatal androgenisation (Seney *et al*, 2012). Seney and colleagues evaluated 6 months old female mice under physiological conditions and at 8 months old after a protocol of 8 weeks of unpredictable chronic mild stress. Under physiological conditions, neonatal androgenisation did not alter anxious-like behaviour; however, females displayed higher locomotor activity than males, as observed by the distance travelled in the OF. When exposed to a stressful stimulus at adulthood, females displayed higher anxious-like behaviour than males, with neonatal androgenised females displaying an intermediate phenotype, not due to differences in locomotor activity. Thus, the anxious-like behaviour was partially masculinised in neonatal androgenised females (Seney *et al*, 2012). In this case, the report of an effect of neonatal androgenisation in female anxious-like behaviour

was restricted to those exposed to a stressful stimulus at adulthood, reinforcing the protective role of testosterone in a stressful context. Considering that, since we previously described gender differences in both anxious-like behaviour and microglia morphology at PND90 (Caetano *et al*, 2016), it would be interesting to test the effect of neonatal androgenisation at that age. Moreover, regarding the morphological rearrangement microglia cells underwent in a model of anxiety with developmental genesis (Caetano *et al*, 2016), it would be of interest to study the ability of testosterone to protect females from both the morphological rearrangement of microglia cells and the increased anxious-like behaviour.

Importantly, not only neonatal, but also pubertal testosterone promotes organisational effects in the neural circuits and behaviour (Primus & Kellogg, 1990). Sisk and colleagues proposed the two-stage model of social behaviour development in rats: a perinatal period of gender differentiation of neural circuits (Konkle & McCarthy, 2011), followed by pubertal surge of testosterone, between PND28 and PND49, which also promotes organisational effects to finish the process that began neonatally (Sisk *et al*, 2003; Schulz *et al*, 2004). The same group showed that pubertal hormones organise the adolescent brain specifically during puberty, leading to long-lasting behavioural phenotypes observable at adulthood (Schulz & Sisk, 2006). Taking this into account, we can speculate that the partial masculinisation of behaviour that we observed can be due to the lack of pubertal testosterone in TP females. Possibly, pubertal testosterone may be the missing trigger to completely masculinise anxious-like behaviour.

Altogether, these studies provide evidence for an organisational anxiolytic role of testosterone. Regarding females, neonatal testosterone is necessary, but not sufficient to trigger gender differences in anxious-like behaviour at youth, possibly due to the missing of pubertal testosterone. Thus, to a complete masculinisation of behaviour, maybe we need to use a model in which both testosterone surges are present. Importantly, under stressful stimulus, neonatal testosterone appears to masculinise anxious-like behaviour (Seney *et al*, 2012), emphasizing the protective role of this hormone. Focusing on our evaluation of behaviour under physiological conditions, not only pubertal testosterone could be a missing piece of the puzzle, but also the “genetic gender”, the presence of an Y chromosome, that is responsible for gender determination. To further study these questions, an imperative tool is the four core genotype mice, that are mice in which genetic and gonadal gender can be differentially modulated to unravel the precise contribution of each parameter.

CHAPTER 4

GENERAL CONCLUSIONS

4.1. General conclusions

The main objectives of the present study were to evaluate the developmental role of $A_{2A}R$ in remodelling microglia morphology in the PFC; to disclose the moment of surge of gender differences in microglia morphology in the PFC; and to correlate morphological remodelling of microglia cells in the PFC with alterations in anxious-like behaviour and short-term recognition memory, under physiological conditions.

Adenosine $A_{2A}R$ are well described modulators of microglia morphology and function (Orr *et al*, 2009; Gyoneva *et al*, 2009, 2014; George *et al*, 2015; Gomes *et al*, 2013; Caetano *et al*, 2016). Regarding the genetic depletion of adenosine $A_{2A}R$ (Section 3.1.) we reported that there was a gender effect in our observations of microglia morphology in the PFC: microglia cells underwent an hypertrophy in females, but were not changed in males. In our previous study (Caetano *et al*, 2016), we observed the same gender effect by chronically blocking $A_{2A}R$ at adulthood. However, in that case, microglia morphology in females became atrophied (Caetano *et al*, 2016). The precise correlation between these morphological refashioning and anxious-like behaviour still elusive, since we did not evaluate the performance in the EPM test in both genders in the present work. In Section 3.1., we reinforced the role of $A_{2A}R$ in remodelling microglia morphology in the PFC in a gender-specific manner. We can also conclude that, under physiological conditions, $A_{2A}R$ have an effect in microglia morphology in the PFC that is dependent of the developmental stage of modulation, considering the different nature of changes observed by pharmacologic and genetic modulation of this receptor. However, the molecular mechanisms behind these “contradictory” effects of $A_{2A}R$ modulation in microglia morphology remain unknown.

In Section 3.2., we attempted to screen the moment of surge of gender differences in microglia morphology in the PFC. We analysed these cells in 3 timepoints of development, from the day of birth until PND33. We did not observe differences in microglia morphology in the PFC until PND33. Thus, considering our previous study, in which we reported these differences in microglia morphology in the PFC at PND90 (Caetano *et al*, 2016), we concluded that the differences likely appear between PND33 and PND90. Moreover, we analysed anxious-like behaviour and short-term recognition memory in these animals. We reported gender differences in the performance in the EPM test, with females being more “disinhibited” than males in the exploration of the whole maze, not due to differences in locomotor activity. Concerning the performance in the NOR test, we did not observe differences in short-term recognition memory between

genders. Concluding, we reported that gender differences in anxious-like behaviour at PND32 are not paralleled by differences in microglia morphology in the PFC. This led us to conclude that microglia morphology in the PFC does not directly affect anxious-like behaviour, but it could be a causative effect involving an effect in other cell types, namely neurons, that display an active interaction with microglia cells throughout life.

In Section 3.3., we used an hormonal approach to study the effect of the neonatal testosterone surge, which occurs in males at the day of birth (Konkle & McCarthy, 2011), in masculinising brain and behaviour. We analysed microglia morphology in the PFC, anxious-like behaviour and short-term recognition memory in neonatal androgenised females. We observed that neonatal testosterone partially masculinised anxious-like behaviour, but did not affect microglia morphology in the PFC, locomotor activity, or short-term recognition memory. We concluded that, at PND32, neonatal androgenisation is necessary, but nor sufficient to fully masculinise female behaviour, probably due to the missing of the pubertal surge of testosterone and the genetic gender.

Concluding, the present work provides insights regarding gender differences in neurodevelopment, considering the relation between the resident brain immune cells, microglia, and behavioural phenotypes. Our main conclusions were:

- The genetic depletion of the adenosine $A_{2A}R$ in the early development promote gender-specific morphological remodelling of microglia cells in the PFC;

- When blocking the adenosine $A_{2A}R$ at adulthood, the gender-biased remodelling of microglia morphology in the PFC is maintained, however this morphological rearrangement occurs in an opposite fashion in females;

- Physiological gender differences in microglia morphology in the PFC surge between PND33 and PND90, in Wistar rats;

- Physiological gender differences in anxious-like behaviour are observable at PND32, but gender differences in locomotor activity and short-term recognition memory are not observable;

- Physiological gender differences in anxious-like behaviour are observable before the surge of gender differences in microglia morphology in the PFC;

- Neonatal female androgenisation did not change microglia morphology in the PFC;

- Neonatal female androgenisation promotes an intermediate phenotype of anxious-like behaviour between males and females, not affecting locomotor activity and short-term recognition memory.

4.2. Future perspectives

Regarding the results we reported in the present study, those from our previous work (Caetano *et al*, 2016), and other from studies that we discussed, several experiments need to be performed:

- Evaluate anxious-like behaviour in $A_{2A}R$ KO mice in both genders, under physiological conditions;

- Induce the developmental model of anxiety (prenatal exposure to DEX) in $A_{2A}R$ KO mice to evaluate the effect of developmental blockade of $A_{2A}R$, in the context of neuropsychiatric disorder, between genders;

- Explore the impact of $A_{2A}R$ blockade, specifically in microglia cells from the PFC, in anxious-like behaviour: using $A_{2A}R$ KO mice that do not express $A_{2A}R$ only in microglia cells, not affecting other cell types as neurons.

- Improve our hormonal approach, by administering testosterone in females not only neonatally, but also during the pubertal period;

- Explore the effect of female androgenisation in the model of chronic anxiety with developmental genesis.

CHAPTER 5

REFERENCES

Ådén U, Halldner L, Lagercrantz H, Dalmau I, Ledent C & Fredholm BB (2003) Aggravated brain damage after hypoxic ischemia in immature adenosine A2A knockout mice. *Stroke* **34:** 739–744

Ådén U, Herlenius E, Tang L-Q & Fredholm BB (2000) Maternal Caffeine Intake Has Minor Effects on Adenosine Receptor Ontogeny in the Rat Brain. *Pediatr. Res.* **48:** 177–183

Adler A, Vescovo P, Robinson JK & Kritzer MF (1999) Gonadectomy in adult life increases tyrosine hydroxylase immunoreactivity in the prefrontal cortex and decreases open field activity in male rats. *Neuroscience* **89:** 939–54

Amendola CA, Gabrieli JDE & Lieberman HR (1998) Caffeine's Effects on Performance and Mood are Independent of Age and Gender. *Nutr. Neurosci.* **1:** 269–280

Andjelkovic AV V., Nikolic B, Pachter JSS & Zecevic N (1998) Macrophages/microglial cells in human central nervous system during development: An immunohistochemical study. *Brain Res.* **814:** 13–25

Arnold AP (2009) The organizational–activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. *Horm. Behav.* **55:** 570–578

Arnoux I, Hoshiko M, Mandavy L, Avignone E, Yamamoto N & Audinat E (2013) Adaptive phenotype of microglial cells during the normal postnatal development of the somatosensory 'Barrel' cortex. *Glia* **61:** 1582–1594

Askew K, Li K, Olmos-Alonso A, Garcia-Moreno F, Liang Y, Richardson P, Tipton T, Chapman MA, Riecken K, Beccari S, Sierra A, Molnár Z, Cragg MS, Garaschuk O, Perry VH & Gomez-Nicola D (2017) Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain. *Cell Rep.* **18:** 391–405

Avitsur R, Stark JL & Sheridan JF (2001) Social Stress Induces Glucocorticoid Resistance in Subordinate Animals. *Horm. Behav.* **39:** 247–257

Bakker J & Baum MJ (2008) Role for estradiol in female-typical brain and behavioral sexual differentiation. *Front. Neuroendocrinol.* **29:** 1–16

Balthazart J, Arnold AP & Adkins-Regan E (1995) Sexual differentiation of brain and behavior in birds. *Trends Endocrinol. Metab.* **6:** 21–29

Baulieu EE, Robel P & Schumacher M (2001) Neurosteroids: beginning of the story. *Int. Rev. Neurobiol.* **46:** 1–32

Bekker MHJ & van Mens-Verhulst J (2007) Anxiety Disorders: Sex Differences in Prevalence, Degree, and Background, But Gender-Neutral Treatment. *Gend. Med.* **4:** Suppl B:S178-93

Bennett NC, Gardiner RA, Hooper JD, Johnson DW & Gobe GC (2010) Molecular cell biology of androgen receptor signalling. *Int. J. Biochem. Cell Biol.* **42:** 813–827

Berrendero F, Castañé A, Ledent C, Parmentier M, Maldonado R & Valverde O (2003) Increase of morphine withdrawal in mice lacking A2a receptors and no changes in CB1/A2a double knockout mice. *Eur. J. Neurosci.* **17:** 315–324

Bertrand JY, Jalil A, Klaine M, Jung S, Cumano A & Godin I (2005) Three pathways to mature macrophages in the early mouse yolk sac. *Blood* **106:** 3004–3011

Bitran D, Kellogg CK & Hilvers RJ (1993) Treatment with an anabolic-androgenic steroid affects anxiety-related behavior and alters the sensitivity of cortical GABAA receptors in the rat. *Horm. Behav.* **27:** 568–583

Boulenger JP & Uhde TW (1982) Caffeine consumption and anxiety: preliminary results of a survey comparing patients with anxiety disorders and normal controls. *Psychopharmacol. Bull.* **18:** 53–7

Caetano L, Pinheiro H, Patrício P, Mateus-Pinheiro a, Alves ND, Coimbra B, Baptista FI, Henriques SN, Cunha C, Santos a R, Ferreira SG, Sardinha VM, Oliveira JF, Ambrósio a F, Sousa N, Cunha R a, Rodrigues a J, Pinto L & Gomes C a (2016) Adenosine A2A receptor regulation of microglia morphological remodeling-gender bias in physiology and in a model of chronic anxiety. *Mol. Psychiatry***:** 1–9

Cappelletti S, Daria P, Sani G & Aromatario M (2015) Caffeine: Cognitive and Physical Performance Enhancer or Psychoactive Drug? *Curr. Neuropharmacol.* **13:** 71–88

Carrier N & Kabbaj M (2012) Testosterone and imipramine have antidepressant effects in socially isolated male but not female rats. *Horm. Behav.* **61:** 678–685

Casano AM, Albert M & Peri F (2016) Developmental Apoptosis Mediates Entry and Positioning of Microglia in the Zebrafish Brain. *Cell Rep.* **16:** 897–906

Chen Z, Jalabi W, Hu W, Park H-J, Gale JT, Kidd GJ, Bernatowicz R, Gossman ZC, Chen JT, Dutta R & Trapp BD (2014) Microglial displacement of inhibitory synapses provides neuroprotection in the adult brain. *Nat. Commun.* **5:** 1–12

Chen Z & Trapp BD (2016) Microglia and neuroprotection. *J. Neurochem.* **136:** 10–17

Chung S, Son GH & Kim K (2011) Circadian rhythm of adrenal glucocorticoid: Its

regulation and clinical implications. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1812:** 581–591

Claypoole LD, Zimmerberg B & Williamson LL (2017) Neonatal lipopolysaccharide treatment alters hippocampal neuroinflammation, microglia morphology and anxiety-like behavior in rats selectively bred for an infantile trait. *Brain. Behav. Immun.* **59:** 135–146

Cooke B, Hegstrom CD, Villeneuve LS & Breedlove SM (1998) Sexual Differentiation of the Vertebrate Brain: Principles and Mechanisms. *Front. Neuroendocrinol.* **19:** 323–362

Crain JM, Nikodemova M & Watters JJ (2009) Expression of P2 nucleotide receptors varies with age and sex in murine brain microglia. *J. Neuroinflammation* **6:** 24

Crain JM & Watters JJ (2015) Microglial P2 Purinergic Receptor and Immunomodulatory Gene Transcripts Vary By Region, Sex, and Age in the Healthy Mouse CNS. *Transcr. Open Access* **3 (2):** 124

Cristovao G, Pinto MJ, Cunha RA, Almeida RD & Gomes CA (2014) Activation of microglia bolsters synapse formation. *Front Cell Neurosci* **8:** 153

Cunha RA, Constantino MD, Sebastião AM & Ribeiro JA (1995) Modification of Ai and A2a adenosine receptor binding in aged striatum, hippocampus and cortex of the rat. *Neuro Rep.* **6:** 1583–1588

Cunha RA, Ferré S, Vaugeois J-M & Chen J-F (2008) Potential therapeutic interest of adenosine A2A receptors in psychiatric disorders. *Curr. Pharm. Des.* **14:** 1512–1524

Cunningham CL, Martinez-Cerdeno V & Noctor SC (2013) Microglia Regulate the Number of Neural Precursor Cells in the Developing Cerebral Cortex. *J. Neurosci.* **33:** 4216–4233

D'Mello AM & Stoodley CJ (2015) Cerebro-cerebellar circuits in autism spectrum disorder. *Front. Neurosci.* **9:** 408

Dalmau I, Finsen B, Tønder N, Zimmer J, González B & Castellano B (1997) Development of microglia in the prenatal rat hippocampus. *J. Comp. Neurol.* **377:** 70–84

Dalmau I, Finsen B, Zimmer J, González B & Castellano B (1998) Development of microglia in the postnatal rat hippocampus. *Hippocampus* **8:** 458–474

Daré E, Schulte G, Karovic O, Hammarberg C & Fredholm BB (2007) Modulation of glial

cell functions by adenosine receptors. *Physiol. Behav.* **92:** 15–20

Davalos D, Grutzendler J, Yang G, Kim J V, Zuo Y, Jung S, Littman DR, Dustin ML & Gan W-B (2005) ATP mediates rapid microglial response to local brain injury in vivo. *Nat. Neurosci.* **8:** 752–8

Doorn KJ, BrevÃ© JJP, Drukarch B, Boddeke HW, Huitinga I, Lucassen PJ & van Dam A-M (2015) Brain region-specific gene expression profiles in freshly isolated rat microglia. *Front. Cell. Neurosci.* **9:** 1–11

Douma SL, Husband C, O'Donnell ME, Barwin BN & Woodend AK (2005) Estrogen-related mood disorders - Reproductive life cycle factors. *Adv. Nurs. Sci.* **28:** 364–375

Durdiakova J, Ostatnikova D & Celec P (2011) Testosterone and its metabolites - modulators of brain functions. *Acta Neurobiol. Exp. (Wars).* **71:** 434–454

Fernández-Guasti A & Martínez-Mota L (2003) Orchidectomy sensitizes male rats to the action of diazepam on burying behavior latency: Role of testosterone. *Pharmacol. Biochem. Behav.* **75:** 473–479

Ferrarelli F, Riedner BA, Peterson MJ & Tononi G (2015) Altered prefrontal activity and connectivity predict different cognitive deficits in schizophrenia. *Hum. Brain Mapp.* **36:** 4539–4552

Fine BJ, Kobrick JL, Lieberman HR, Marlowe B, Riley RH & Tharion WJ (1994) Effects of caffeine or diphenhydramine on visual vigilance. *Psychopharmacology (Berl).* **114:** 233–238

Forest MG, Cathiard AM & Bertrand JA (1973) EVIDENCE OF TESTICULAR ACTIVITY IN EARLY INFANCY. *J. Clin. Endocrinol. Metab.* **37:** 148–151

Fowden AL, Li J & Forhead AJ (1998) Glucocorticoids and the preparation for life after birth: Are there long-term consequences of the life insurance? *Proc. Nutr. Soc.* **57:** 113–122

Fredholm BB, Bättig K, Holmén J, Nehlig a & Zvartau EE (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* **51:** 83–133

Fredholm BB, Chen J-F, Cunha RA, Svenningsson P & Vaugeois J-M (2005) Adenosine and Brain Function. *Int. Rev. Neurobiol.* **63:** 191–270

Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN & Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors.

Pharmacol.Rev. **53:** 527–552

Frye CA, Edinger K & Sumida K (2008) Androgen Administration to Aged Male Mice Increases Anti-Anxiety Behavior and Enhances Cognitive Performance. *Neuropsychopharmacology* **33:** 1049–1061

Frye CA & Lacey EH (2001) Posttraining androgens' enhancement of cognitive performance is temporally distinct from androgens' increases in affective behavior. *Cogn Affect Behav Neurosci* **1:** 172–182

Frye CA & Seliga AM (2001) Testosterone increases analgesia, anxiolysis, and cognitive performance of male rats. *Cogn. Affect. Behav. Neurosci.* **1:** 371–381

Gendrel D, Chaussain JL, Roger M & Job JC (1980) Simultaneous postnatal rise of plasma LH and testosterone in male infants. *J. Pediatr.* **97:** 600–602

George FW & Ojeda SR (1982) Changes in aromatase activity in the rat brain during embryonic, neonatal, and infantile development. *Endocrinology* **111:** 522–529

George J, Gonçalves F, Cristóvão G, Rodrigues L, Meyer Fernandes J, Gonçalves T, Cunha R & Gomes C (2015) Different danger signals differently impact on microglial proliferation through alterations of ATP release and extracellular metabolism. *Glia* **63:** 1636–1645

Ghayee HK & Auchus RJ (2007) Basic concepts and recent developments in human steroid hormone biosynthesis. *Rev. Endocr. Metab. Disord.* **8:** 289–300

Gilliland K & Andress D (1981) Ad lib caffeine consumption, symptoms of caffeinism, and academic performance. *Am. J. Psychiatry* **138:** 512–514

Giltay EJ, Enter D, Zitman FG, Penninx BWJH, van Pelt J, Spinhoven P & Roelofs K (2012) Salivary testosterone: Associations with depression, anxiety disorders, and antidepressant use in a large cohort study. *J. Psychosom. Res.* **72:** 205–213

Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM & Merad M (2010) Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science (80-.).* **330:** 841–845

Ginhoux F, Lim S, Hoeffel G, Low D & Huber T (2013) Origin and differentiation of microglia. *Front. Cell. Neurosci.* **7:** 1–14

Gobinath AR, Choleris E & Galea LAM (2017) Sex, hormones, and genotype interact to influence psychiatric disease, treatment, and behavioral research. *J. Neurosci. Res.*

95: 50–64

Goldstat R, Briganti E, Tran J, Wolfe R & Davis SR (2003) Transdermal testosterone therapy improves well-being, mood, and sexual function in premenopausal women. *Menopause* **10:** 390–398

Gomes C, Ferreira R, George J, Sanches R, Rodrigues DI, Gonçalves N & Cunha RA (2013) Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: A2A receptor blockade prevents BDNF release and proliferation of microglia. *J. Neuroinflammation* **10:** 16

Gomes CAR V., Simões PF, Canas PM, Quiroz C, Sebastião AM, Ferré S, Cunha RA & Ribeiro JA (2009) GDNF control of the glutamatergic cortico-striatal pathway requires tonic activation of adenosine A 2A receptors. *J. Neurochem.* **108:** 1208–1219

Gomes C V, Kaster MP, Tomé AR, Agostinho PM & Cunha RA (2011) Adenosine receptors and brain diseases : Neuroprotection and neurodegeneration. *Biochim. Biophys. Acta* **1808:** 1380–1399

Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, Garner H, Trouillet C, de Bruijn MF, Geissmann F & Rodewald H-R (2014) Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* **518:** 547–551

Granger DA, SHIRTCLIFF EA, ZAHN–WAXLER C, USHER B, KLIMES–DOUGAN B & HASTINGS P (2003) Salivary testosterone diurnal variation and psychopathology in adolescent males and females: Individual differences and developmental effects. *Dev. Psychopathol.* **15 (2):** 431–49

Greden JF, Fontaine P, Lubetsky M & Chamberlin K (1978) Anxiety and depression associated with caffeinism among psychiatric inpatients. *Am. J. Psychiatry* **135:** 963–966

Gutiérrez-García AG, Contreras CM, Vásquez-Hernández DI, Molina-Jiménez T & Jacome-Jacome E (2009) Testosterone reduces cumulative burying in female Wistar rats with minimal participation of estradiol. *Pharmacol. Biochem. Behav.* **93:** 406–412

Gyoneva S, Orr AG & Traynelis SF (2009) Differential regulation of microglial motility by ATP/ADP and adenosine. *Parkinsonism Relat. Disord.* **15:** S195–S199

Gyoneva S, Shapiro L, Lazo C, Garnier-Amblard E, Smith Y, Miller GW & Traynelis SF (2014) Adenosine A2A receptor antagonism reverses inflammation-induced

impairment of microglial process extension in a model of Parkinson's disease. *Neurobiol. Dis.* **67:** 191–202

Harry GJ (2013) Microglia during development and aging. *Pharmacol. Ther.* **139:** 313–326

Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, Becker CD, See P, Price J, Lucas D, Greter M, Mortha A, Boyer SW, Forsberg EC, Tanaka M, van Rooijen N, García-Sastre A, Stanley ER, Ginhoux F, Frenette PS, et al (2013) Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* **38:** 792–804

Hermans EJ, Putman P, Baas JM, Koppeschaar HP & van Honk J (2006) A Single Administration of Testosterone Reduces Fear-Potentiated Startle in Humans. *Biol. Psychiatry* **59:** 872–874

Hinwood M, Tynan RJ, Charnley JL, Beynon SB, Day TA & Walker FR (2013) Chronic stress induced remodeling of the prefrontal cortex: Structural re-organization of microglia and the inhibitory effect of minocycline. *Cereb. Cortex* **23:** 1784–1797

Hisasue S, Seney ML, Immerman E & Forger NG (2010) Control of cell number in the bed nucleus of the stria terminalis of mice: role of testosterone metabolites and estrogen receptor subtypes. *J. Sex. Med.* **7:** 1401–9

Hoeffel G, Chen J, Lavin Y, Low D, Almeida FF, See P, Beaudin AE, Lum J, Low I, Forsberg EC, Poidinger M, Zolezzi F, Larbi A, Ng LG, Chan JKY, Greter M, Becher B, Samokhvalov IM, Merad M & Ginhoux F (2015) C-Myb^{+} Erythro-Myeloid Progenitor-Derived Fetal Monocytes Give Rise to Adult Tissue-Resident Macrophages. *Immunity* **42:** 665–678

Hoeffel G & Ginhoux F (2015) Ontogeny of tissue-resident macrophages. *Front. Immunol.* **6:** 486

Hohoff C, Garibotto V, Elmenhorst D, Baffa A, Kroll T, Hoffmann A, Schwarte K, Zhang W, Arolt V, Deckert J & Bauer A (2014) Association of Adenosine Receptor Gene Polymorphisms and In Vivo Adenosine A1 Receptor Binding in The Human Brain. *Neuropsychopharmacology* **39:** 2989–2999

James JE & Crosbie J (1987) Somatic and Psychological Health Implications of Heavy Caffeine Use. *Br. J. Addict.* **82:** 503–509

Johansson B, Georgiev V & Fredholm BB (1997) Distribution and postnatal ontogeny of adenosine A2A receptors in rat brain: comparison with dopamine receptors. **80:** 1187–1207

Jones EA V (2011) The initiation of blood flow and flow induced events in early vascular development. *Semin. Cell Dev. Biol.* **22:** 1028–1035

Kanayama G, Amiaz R, Seidman S & Pope HG (2007) Testosterone supplementation for depressed men: current research and suggested treatment guidelines. *Exp. Clin. Psychopharmacol.* **15:** 529–38

Kaster MP, Machado NJ, Silva HB, Nunes A, Ardais AP, Santana M, Baqi Y, Müller CE, Rodrigues ALS, Porciúncula LO, Chen JF, Tomé ÂR, Agostinho P, Canas PM & Cunha R a. (2015) Caffeine acts through neuronal adenosine A 2A receptors to prevent mood and memory dysfunction triggered by chronic stress. *Proc. Natl. Acad. Sci.* **112:** 201423088

Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR & Walters EE (2005) Lifetime Prevalence and Age-of-Onset Distributions of. *Arch Gen Psychiatry* **62:** 593–602

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

Khazipov R, Zaynutdinova D, Ogievetsky E, Valeeva G, Mitrukhina O, Manent J-B & Represa A (2015) Atlas of the Postnatal Rat Brain in Stereotaxic Coordinates. *Front. Neuroanat.* **9:** 1–5

Kierdorf K, Erny D, Goldmann T, Sander V, Schulz C, Perdiguero EG, Wieghofer P, Heinrich A, Riemke P, Hölscher C, Müller DN, Luckow B, Brocker T, Debowski K, Fritz G, Opdenakker G, Diefenbach A, Biber K, Heikenwalder M, Geissmann F, et al (2013) Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nat. Neurosci.* **16:** 273–280

Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ & Popovich PG (2009) Identification of Two Distinct Macrophage Subsets with Divergent Effects Causing either Neurotoxicity or Regeneration in the Injured Mouse Spinal Cord. *J. Neurosci.* **29:** 13435–13444

Kinsey SG, Bailey MT, Sheridan JF, Padgett DA & Avitsur R (2007) Repeated social defeat causes increased anxiety-like behavior and alters splenocyte function in C57BL/6 and CD-1 mice. *Brain. Behav. Immun.* **21:** 458–466

Konkle ATM & McCarthy MM (2011) Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain. *Endocrinology* **152:** 223–235

Kovács Z, Juhász G, Dobolyi Á, Bobest M, Papp V, Takáts L & Kékesi KA (2010) Gender-

and age-dependent changes in nucleoside levels in the cerebral cortex and white matter of the human brain. *Brain Res. Bull.* **81:** 579–584

Kreisel T, Frank MG, Licht T, Reshef R, Ben-Menachem-Zidon O, Baratta M V, Maier SF & Yirmiya R (2014) Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis. *Mol. Psychiatry* **19:** 699–709

Kudwa AE, Michopoulos V, Gatewood JD & Rissman EF (2006) Roles of estrogen receptors α and β in differentiation of mouse sexual behavior. *Neuroscience* **138:** 921–928

Lawson LJ, Perry VH, Dri P & Gordon S (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience* **39:** 151–170

Lenz KM, Nugent BM, Haliyur R & McCarthy MM (2013) Microglia Are Essential to Masculinization of Brain and Behavior. *J. Neurosci.* **33:** 2761–2772

Lenz KM, Nugent BM & McCarthy MM (2012) Sexual differentiation of the rodent brain: Dogma and beyond. *Front. Neurosci.* **6:** 1–13

Li Y, Du XF, Liu CS, Wen ZL & Du JL (2012) Reciprocal Regulation between Resting Microglial Dynamics and Neuronal Activity In Vivo. *Dev. Cell* **23:** 1189–1202

Lieberman HR, Wurtman RJ, Emde GG & Coviella IL (1987) The effects of caffeine and aspirin on mood and performance. *J. Clin. Psychopharmacol.* **7:** 315–320

Lucion AB, Charchat H, Pereira GAM & Rasia-Filho AA (1996) Influence of Early Postnatal Gonadal Hormones on Anxiety in Adult Male Rats. *Physiol. Behav.* **60:** 1419–1423

Markowitz JS (1989) Quality of Life in Panic Disorder. *Arch. Gen. Psychiatry* **46:** 984

McCarthy MM (2008) Estradiol and the Developing Brain. *Physiol. Rev.* **88:** 91–134

McCarthy MM & Arnold AP (2011) Reframing sexual differentiation of the brain. *Nat. Neurosci.* **14:** 677–683

McHenry J, Carrier N, Hull E & Kabbaj M (2014) Sex differences in anxiety and depression: Role of testosterone. *Front. Neuroendocrinol.* **35:** 42–57

Melcangi RC, Garcia-Segura LM & Mensah-Nyagan AG (2008) Neuroactive steroids: State of the art and new perspectives. *Cell. Mol. Life Sci.* **65:** 777–797

Mesquita AR, Wegerich Y, Patchev A V., Oliveira M, Le?o P, Sousa N & Almeida OFX (2009) Glucocorticoids and neuro- and behavioural development. *Semin. Fetal Neonatal Med.* **14:** 130–135

Miller WL (2013) Steroid hormone synthesis in mitochondria. *Mol. Cell. Endocrinol.* **379:** 62–73

Mittelbronn M, Dietz K, Schluesener HJ & Meyermann R (2001) Local distribution of microglia in the normal adult human central nervous system differs by up to one order of magnitude. *Acta Neuropathol.* **101:** 249–255

Mizutani M, Pino PA, Saederup N, Charo IF, Ransohoff RM & Cardona AE (2012) The Fractalkine Receptor but Not CCR2 Is Present on Microglia from Embryonic Development throughout Adulthood. *J. Immunol.* **188:** 29–36

Monier A, Adle-Biassette H, Delezoide A-L, Evrard P, Gressens P & Verney C (2007) Entry and Distribution of Microglial Cells in Human Embryonic and Fetal Cerebral Cortex. *J. Neuropathol. Exp. Neurol.* **66:** 372–382

Monier A, Evrard P, Gressens P & Verney C (2006) Distribution and differentiation of microglia in the human encephalon during the first two trimesters of gestation. *J. Comp. Neurol.* **499:** 565–582

Moore MA & Metcalf D (1970) Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Br. J. Haematol.* **18:** 279–296

Morris JA, Jordan CL & Breedlove SM (2004) Sexual differentiation of the vertebrate nervous system. *Nat. Neurosci.* **7:** 1034–1039

Morsink LFJ, Vogelzangs N, Nicklas BJ, Beekman ATF, Satterfield S, Rubin SM, Yaffe K, Simonsick E, Newman AB, Kritchevsky SB & Penninx BWJH (2007) Associations between sex steroid hormone levels and depressive symptoms in elderly men and women: Results from the Health ABC study. *Psychoneuroendocrinology* **32:** 874–883

Mosser CA, Baptista S, Arnoux I & Audinat E (2017) Microglia in CNS development: Shaping the brain for the future. *Prog. Neurobiol.* **149**–**150:** 1–20

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

Nelson LH & Lenz KM (2017a) Microglia depletion in early life programs persistent changes in social, mood-related, and locomotor behavior in male and female rats. *Behav. Brain Res.* **316:** 279–293

Nelson LH & Lenz KM (2017b) The immune system as a novel regulator of sex differences in brain and behavioral development. *J. Neurosci. Res.* **95:** 447–461

Nikodemova M, Kimyon RS, De I, Small AL, Collier LS & Watters JJ (2015) Microglial numbers attain adult levels after undergoing a rapid decrease in cell number in the third postnatal week. *J. Neuroimmunol.* **278:** 280–288

Nimmerjahn A, Kirchhoff F & Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Neuroforum* **11:** 95–96

Nissl F (1899) Über einige Beziehungen zwischen Nervenzellerkrankungen und gliösen Erscheinungen bei verschiedenen Psychosen. *Arch f Psychiatr* **32:** 656–676

Orr AG, Orr AL, Li X, Gross RE & Traynelis SF (2009) Adenosine A2A receptor mediates microglial process retraction. *Nat. Neurosci.* **12:** 872–878

Pagani F, Paolicelli RC, Murana E, Cortese B, Angelantonio S Di, Zurolo E, Guiducci E, Ferreira TA, Garofalo S, Catalano M, Dâ€™Alessandra G, Porzia A, Peruzzi G, Mainiero F, Limatola C, Gross CT & Ragozzino D (2015) Defective microglial development in the hippocampus of Cx3cr1 deficient mice. *Front. Cell. Neurosci.* **9:** 1–14

Palis J, Robertson S, Kennedy M, Wall C & Keller G (1999) Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126:** 5073–5084

Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, Ragozzino D & Gross CT (2011) Synaptic Pruning by Microglia Is Necessary for Normal Brain Development. *Science (80-.).* **333:** 1456–1458

Paxinos G & Franklin KBJ (2001) The Mouse Brain in Stereotaxic Coordinates Second. Academic Press

Paxinos G & Watson C (1998) The Rat Brain in Stereotaxic Coordinates San Diego: Academic Press

Peri F & Nüsslein-Volhard C (2008) Live Imaging of Neuronal Degradation by Microglia Reveals a Role for v0-ATPase a1 in Phagosomal Fusion In Vivo. *Cell* **133:** 916–927

Phoenix CH (2009) Organizing action of prenatally administered testosterone propionate on the Tissues mediating mating behavior in the female guinea pig. *Horm. Behav.* **55:** 566

Pogledic I, Kostovic I, Fallet-bianco C, Adle-biassette H & Gressens P (2014) Involvement of the Subplate Zone in Preterm Infants with. *Brain Pathol.* **24:** 128–141

Pope HG, Cohane GH, Kanayama G, Ph D, Siegel AJ, Hudson JI & Sc D (2003) Testosterone Gel Supplementation for Men With Refractory Depression: A Randomized, Placebo-Controlled Trial. *Am. J. Psychiatry* **160:** 105–111

Primus RJ & Kellogg CK (1990) Gonadal hormones during puberty organize environment-related social interaction in the male rat. *Horm. Behav.* **24:** 311–323

Ramón y Cajal S (1897) Algo sobre la significacióin fisiológica de la neuroglía. *Rev.Trimes.Microgr.* **2:** 33–47

Ramón y Cajal S (1913) Contribución al conocimiento de la neuroglia del cerebro humano. *Trab. Lab. Invest. Biol.* **XI:** 225–315

Rauch SL, Shin LM & Wright CI (2003) Neuroimaging Studies of Amygdala Function in Anxiety Disorders. *Ann. N. Y. Acad. Sci.* **985:** 389–410

Rebola N, Canas PM, Oliveira CR & Cunha RA (2005a) Different synaptic and subsynaptic localization of adenosine A2A receptors in the hippocampus and striatum of the rat. *Neuroscience* **132:** 893–903

Rebola N, Porciuncula LO, Lopes L V., Oliveira CR, Soares-da-Silva P & Cunha RA (2005b) Long-term Effect of Convulsive Behavior on the Density of Adenosine A1 and A2A Receptors in the Rat Cerebral Cortex. *Epilepsia* **46:** 159–165

Rebola N, Simões AP, Canas PM, Tomé AR, Andrade GM, Barry CE, Agostinho PM, Lynch MA & Cunha RA (2011) Adenosine A2A receptors control neuroinflammation and consequent hippocampal neuronal dysfunction. *J. Neurochem.* **117:** 100–111

Reyes FI, Winter JSD & Faiman C (1973) Studies on Human Sexual Development. I. Fetal Gonadal and Adrenal Sex Steroids. *J. Clin. Endocrinol. Metab.* **37:** 74–78

Rezaie P, Dean A, Male D & Ulfig N (2005) Microglia in the cerebral wall of the human telencephalon at second trimester. *Cereb. Cortex* **15:** 938–949

Rial D, Lemos C, Pinheiro H, Duarte JM, Gonçalves FQ, Real JI, Prediger RD, Gonçalves N, Gomes CA, Canas PM, Agostinho P & Cunha RA (2016) Depression as a Glial-Based Synaptic Dysfunction. *Front. Cell. Neurosci.* **9:** 1–11

Ribeiro JA, Sebastião AM & de Mendonça A (2002) Adenosine receptors in the nervous system: Pathophysiological implications. *Prog. Neurobiol.* **68:** 377–392

Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, Phillip K & Peterson PK (2004) Role of Microglia in Central Nervous System Infections Role. *Clin. Microbiol. Rev.* **17:** 942–64

Rohr UD (2002) The impact of testosterone imbalance on depression and women's health. *Maturitas* **41**, **Supple:** 25–46

Roselli CE & Resko J a (1993) Aromatase activity in the rat brain: hormonal regulation and sex differences. *J. Steroid Biochem. Mol. Biol.* **44:** 499–508

Roumier A, Pascual O, Béchade C, Wakselman S, Poncer J-C, Réal E, Triller A & Bessis A (2008) Prenatal Activation of Microglia Induces Delayed Impairment of Glutamatergic Synaptic Function. *PLoS One* **3:** e2595

Santos C, Lunet N, Azevedo A, De Mendonça A, Ritchie K & Barros H (2010) Caffeine intake is associated with a lower risk of cognitive decline: A cohort study from Portugal. *J. Alzheimer's Dis.* **20:** 175–185

Schafer D, Lehrman E, Kautzman A, Koyama R, Mardinly A, Yamasaki R, Ransohoff R, Greenberg M, Barres B & Stevens B (2012a) Microglia sculpt postnatal neuronal circuits in an activivty and complement-dependent manner. *Neuron* **74:** 691–705

Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, Ransohoff RM, Greenberg ME, Barres BA & Stevens B (2012b) Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron* **74:** 691–705

Schneier FR, Heckelman LR, Garfinkel R, Campeas R, Fallon BA, Gitow A, Street L, Del Bene D & Liebowitz MR (1994) Functional impairment in social phobia. *J. Clin. Psychiatry* **55:** 322–331

Schulz C, Perdiguero EG, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, Prinz M, Wu B, Jacobsen SEW, Pollard JW, Frampton J, Liu KJ & Geissmann F (2012) A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells. *Science (80-.).* **336:** 86–90

Schulz KM, Molenda-Figueira HA & Sisk CL (2009) Back to the future: The organizational–activational hypothesis adapted to puberty and adolescence. *Horm. Behav.* **55:** 597–604

Schulz KM, Richardson HN, Zehr JL, Osetek AJ, Menard TA & Sisk CL (2004) Gonadal hormones masculinize and defeminize reproductive behaviors during puberty in the male Syrian hamster. *Horm. Behav.* **45:** 242–249

Schulz KM & Sisk CL (2006) Pubertal hormones, the adolescent brain, and the maturation of social behaviors: Lessons from the Syrian hamster. *Mol. Cell. Endocrinol.* **254–255:** 120–126

Schwarz JM, Sholar PW & Bilbo SD (2012) Sex differences in microglial colonization of the developing rat brain. *J. Neurochem.* **120:** 948–963

Sebastião AM & Ribeiro JA (2009) Tuning and Fine-Tuning of Synapses with Adenosine. *Curr. Neuropharmacol.* **7:** 180–194

Seney ML, Walsh C, Stolakis R & Sibille E (2012) Neonatal testosterone partially organizes sex differences in stress-induced emotionality in mice. *Neurobiol. Dis.* **46:** 486–496

Shaw C, Hall SE & Cynader M (1986) Characterization, distribution, and ontogenesis of adenosine binding sites in cat visual cortex. *J Neurosci* **6:** 3218–3228

Sheng J, Ruedl C & Karjalainen K (2015) Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells. *Immunity* **43:** 382–393

Sheridan GK & Murphy KJ (2013) Neuron-glia crosstalk in health and disease: fractalkine and CX3CR1 take centre stage. *Open Biol.* **3:** 130181–130181

Shifren JL, Braunstein GD, Simon JA, Casson PR, Buster JE, Redmond GP, Burki RE, Ginsburg ES, Rosen RC, Leiblum SR, Caramelli KE, Jones KP, Daugherty CA & Mazer NA (2000) Transdermal Testosterone Treatment in Women with Impaired Sexual Function after Oophorectomy. *N. Engl. J. Med.* **343:** 682–688

Shirazi SN, Friedman AR, Kaufer D & Sakhai SA (2015) Glucocorticoids and the Brain: Neural Mechanisms Regulating the Stress Response. In pp 235–252.

Shores MM, Sloan KL, Matsumoto AM, Moceri VM, Felker B & Kivlahan DR (2004) Increased Incidence of Diagnosed Depressive Illness in HypogonadalOlder Men. *Arch. Gen. Psychiatry* **61:** 162

Sierralta WD, Kohen P, Castro O, Muñoz A, Strauss JF & Devoto L (2005) Ultrastructural and biochemical evidence for the presence of mature steroidogenic acute regulatory protein (StAR) in the cytoplasm of human luteal cells. *Mol. Cell. Endocrinol.* **242:** 103–110

Silva CG, Metin C, Fazeli W, Machado NJ, Darmopil S, Launay P-S, Ghestem A, Nesa M-P, Bassot E, Szabo E, Baqi Y, Muller CE, Tome AR, Ivanov A, Isbrandt D, Zilberter Y, Cunha RA, Esclapez M & Bernard C (2013) Adenosine Receptor Antagonists Including Caffeine Alter Fetal Brain Development in Mice. *Sci. Transl. Med.* **5:** 197ra104-197ra104

Sisk CL, Schulz KM & Zehr JL (2003) Puberty: A Finishing School for Male Social Behavior. *Ann. N. Y. Acad. Sci.* **1007:** 189–198

Slob AK, Bogers H & Van Stolk MA (1981) Effects of gonadectomy and exogenous gonadal steroids on sex differences in open field behaviour of adult rats. *Behav. Brain Res.* **2:** 347–362

Smith AP (2009) Caffeine, cognitive failures and health in a non-working community sample. *Hum. Psychopharmacol.* **24:** 29–34

Smith SM & Vale WW (2006) The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin. Neurosci.* **8:** 383–395

Solomon MB & Herman JP (2009) Sex differences in psychopathology: Of gonads, adrenals and mental illness. *Physiol. Behav.* **97:** 250–258

Sousa N (2016) The dynamics of the stress neuromatrix. *Mol. Psychiatry* **21:** 302–312

Stephan AH, Barres BA & Stevens B (2012) The Complement System: An Unexpected Role in Synaptic Pruning During Development and Disease. *Annu. Rev. Neurosci.* **35:** 369–389

Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SWM & Barres BA (2007) The Classical Complement Cascade Mediates CNS Synapse Elimination. *Cell* **131:** 1164–1178

Svenningsson P, Le Moine C, Fisone G & Fredholm BB (1999) Distribution, biochemistry and function of striatal adenosine A(2A) receptors. *Prog. Neurobiol.* **59:** 355–396

Swinnen N, Smolders S, Avila A, Notelaers K, Paesen R, Ameloot M, Brône B, Legendre P & Rigo JM (2013) Complex invasion pattern of the cerebral cortex bymicroglial cells during development of the mouse embryo. *Glia* **61:** 150–163

Thorburn GD, Challis RC & Currie WB (1977) Control of Parturition in Domestic Animals. *Biol. Reprod.***:** 18–27

Todd BJ, Schwarz JM & McCarthy MM (2005) Prostaglandin-E2: A point of divergence in estradiol-mediated sexual differentiation. *Horm. Behav.* **48:** 512–521

Tremblay M-ève, Lecours C, Samson L & Sánchez-zafra V (2015) From the Cajal alumni Achúcarro and Río-Hortega to the rediscovery of never-resting microglia. *Front. Neuroanat.* **9:** 1–10

Tremblay MĚ, Lowery RL & Majewska AK (2010) Microglial interactions with synapses are modulated by visual experience. *PLoS Biol.* **8:** 11

Uhde TW (1994) Anxiety and growth disturbance: is there a connection? A review of

biological studies in social phobia. *J. Clin. Psychiatry* **55 Suppl:** 17–27

Verney C, Monier A, Fallet-Bianco C & Gressens P (2010) Early microglial colonization of the human forebrain and possible involvement in periventricular white-matter injury of preterm infants. *J. Anat.* **217:** 436–448

Verney C, Pogledic I, Adle-biassette H, Fallet-bianco C & Gressens P (2012) Microglial Reaction in Axonal Crossroads Is a Hallmark of Noncystic Periventricular White Matter Injury in Very Preterm Infants. *J Neuropathol Exp Neurol* **71:** 251–264

Waffarn F & Davis EP (2012) Effects of antenatal corticosteroids on the hypothalamic-pituitary-adrenocortical axis of the fetus and newborn: experimental findings and clinical considerations. *Am. J. Obstet. Gynecol.* **207:** 446–454

Wake H, Moorhouse AJ, Jinno S, Kohsaka S & Nabekura J (2009) Resting Microglia Directly Monitor the Functional State of Synapses In Vivo and Determine the Fate of Ischemic Terminals. *J. Neurosci.* **29:** 3974–3980

Wakselman S, Bechade C, Roumier A, Bernard D, Triller A & Bessis A (2008) Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAP12 immunoreceptor. *J Neurosci* **28:** 8138–8143

Walker DL, Toufexis DJ & Davis M (2003) Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *Eur. J. Pharmacol.* **463:** 199–216

Wallen K & Baum MJ (2002) Masculinization and Defeminization in Altricial and Precocial Mammals: Comparative Aspects of Steroid Hormone Action. *Horm. Brain Behav.***:** 385–423

Wang C, Berman N, Davidson T, Steiner B, Hull L, Callegari C & Swerdloff S (1996) Testosterone Hypogonadal Replacement Therapy Improves Mood in Clinical Research Center Study *. *J. Clin. Endocrinol. Metab.* **31:** 3578–3583

Weaver DR (1993) A2a adenosine receptor gene expression in developing rat brain. *Brain Res Mol Brain Res* **20:** 313–327

Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, Nelson RJ, Godbout JP & Sheridan JF (2011) -Adrenergic Receptor Antagonism Prevents Anxiety-Like Behavior and Microglial Reactivity Induced by Repeated Social Defeat. *J. Neurosci.* **31:** 6277–6288

Wolf SA, Boddeke HWGM & Kettenmann H (2017) Microglia in Physiology and Disease. *Annu. Rev. Physiol.* **79:** 619–643

Yacoubi M El, Ledent C, Parmentier M, Bertorelli R, Ongini E, Costentin J & Vaugeois J (2001) Adenosine A2A receptor antagonists are potential antidepressants: evidence based on pharmacology and A2A receptor knockout mice. *Br. J. Pharmacol.* **134:** 68–77

Yacoubi M, Ledent C, Parmentier M, Costentin J & Vaugeois JM (2000) The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective A(2A) adenosine receptor antagonists. *Psychopharmacology (Berl).* **148:** 153–63

Yirmiya R, Rimmerman N & Reshef R (2015) Depression as a Microglial Disease. *Trends Neurosci.* **38:** 637–658

Yu L, Shen H, Coelho JE, Rebola N, Canas PM, Rapp EK, Ferrara J, Taylor D, Mu CE, Linden J, Cunha RA & Chen J (2008) Adenosine A2A Receptor Antagonists Exert Motor and Neuroprotective Effects by Distinct Cellular Mechanisms. *Ann. Neurol.* **63:** 338 –346

Zarrouf FA, Artz S, Griffith J, Sirbu C & Kommor M (2009) Testosterone and depression: systematic review and meta-analysis. *J. Psychiatr. Pract.* **15:** 289–305

Zhan Y, Paolicelli RC, Sforazzini F, Weinhard L, Bolasco G, Pagani F, Vyssotski AL, Bifone A, Gozzi A, Ragozzino D & Gross CT (2014) Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. *Nat. Neurosci.* **17:** 400–406

Zuloaga DG, Jordan CL & Breedlove SM (2011a) The organizational role of testicular hormones and the androgen receptor in anxiety-related behaviors and sensorimotor gating in rats. *Endocrinology* **152:** 1572–1581

Zuloaga DG, Morris JA, Jordan CL & Breedlove SM (2008) Mice with the testicular feminization mutation demonstrate a role for androgen receptors in the regulation of anxiety-related behaviors and the hypothalamic-pituitary-adrenal axis. *Horm. Behav.* **54:** 758–766

Zuloaga DG, Poort JE, Jordan CL & Breedlove SM (2011b) Male rats with the testicular feminization mutation of the androgen receptor display elevated anxiety-related behavior and corticosterone response to mild stress. *Horm. Behav.* **60:** 380–388

CHAPTER 6

SUPPLEMENTARY DATA

Supplementary table 1 Summary of the morphometric analysis of the number of microglia processes in the PFC from male and female A_{2A}R WT/KO mice at PND90. Descriptive data from Section 3.1.3.1, page 35. Values are presented as mean ± SEM from 4 animals.

Branch order	WT males	A_{2A}R KO males	WT females	A_{2A}R KO females
1	6.4 ± 0.2	6.3 ± 0.2	5.9 ± 0.3	7.0 ± 0.3
2	10.9 ± 0.4	11.5 ± 0.4	11.3 ± 0.3	13.2 ± 0.4
3	13.7 ± 0.2	13.2 ± 0.6	14.1 ± 0.5	17.4 ± 1.1
4	12.0 ± 04	12.2 ± 1.0	13.6 ± 1.2	17.4 ± 1.8
5	8.6 ± 0.3	9.1 ± 1.3	12.4 ± 1.8	16.8 ± 2.2
6	5.4 ± 0.6	5.2 ± 0.6	9.1 ± 2.1	13.5 ± 1.9
7	3.2 ± 0.2	3.3 ± 0.5	6.3 ± 1.8	10.1 ± 1.8
8	1.4 ± 0.3	1.4 ± 0.2	4.5 ± 1.3	7.3 ± 1.5
9	0.7 ± 0.2	0.6 ± 0.2	3.2 ± 1.1	5.1 ± 1.5
10	0.4 ± 0.2	0.1 ± 0.1	2.2 ± 1.0	3.4 ± 1.0

Supplementary table 2 Summary of the morphometric analysis of the length (µm) of microglia processes in the PFC from male and female A_{2A}R WT/KO mice at PND90. Descriptive data from from Section 3.1.3.1, page 35. Values are presented as mean ± SEM from 4 animals.

Branch order	WT males	A_{2A}R KO males	WT females	A_{2A}R KO females
1	47.5 ± 3.6	45.4 ± 1.7	32.8 ± 4.5	32.7 ± 2.8
2	84.1 ± 5.9	83.0 ± 3.8	62.1 ± 2.9	68.8 ± 3.7
3	94.7 ± 5.6	96.9 ± 8.0	69.8 ± 3.3	88.8 ± 5.3
4	73.1 ± 3.6	75.6 ± 4.2	65.6 ± 7.2	87.4 ± 5.6
5	42.3 ± 4.4	52.6 ± 4.1	56.7 ± 8.8	80.3 ± 5.9
6	25.2 ± 1.5	29.9 ± 2.1	42.5 ± 10.6	63.9 ± 6.7
7	24.0 ± 1.6	18.8 ± 0.6	29.9 ± 93	47.8 ± 7.3
8	16.6 ± 2.0	14.1 ± 2.4	24.2 ± 4.1	34.8 ± 3.7
9	18.0 ± 3.5	13.6 ± 3.4	18.2 ± 3.2	25.7 ± 6.2
10	8.7 ± 3.8	4.1 ± 2.4	17.8 ± 4.0	18.3 ± 3.9

Supplementary table 3 Summary of the morphometric analysis of the number and length (µm) of microglia processes in the PFC from male and female Wistar rats at PND7. Descriptive data from Section 3.2.3.2, page 50. Values are presented as mean ± SEM from 3 animals.

	Number of processes		Length of processes	
Branch order	**Males**	**Females**	**Males**	**Females**
1	4.3 ± 0.21	4.6 ± 0.27	23.4 ± 0.89	28.2 ± 2.69
2	7.8 ± 0.78	8.6 ± 0.81	40.4 ± 1.94	45.8 ± 4.90
3	9.9 ± 0.70	10.7 ± 0.86	47.1 ± 4.20	52.0 ± 2.06
4	10.1 ± 1.05	10.5 ± 0.58	46.5 ± 6.24	48.5 ± 1.35
5	10.7 ± 0.56	10.1 ± 0.88	46.3 ± 2.82	46.7 ± 4.47
6	8.5 ± 0.77	8.9 ± 1.04	37.5 ± 4.96	40.3 ± 6.73
7	7.0 ± 0.72	8.6 ± 0.68	28.3 ± 2.83	36.1 ± 3.38
8	7.5 ± 0.22	7.0 ± 1.02	33.7 ± 3.86	29.5 ± 2.06
9	6.8 ± 0.54	6.5 ± 0.21	29.8 ± 4.09	25.8 ± 125
10	5.5 ± 0.33	5.8 ± 0.67	20.0 ± 1.36	21.7 ± 1.24

Supplementary table 4 Effect of neonatal androgenisation of female Wistar rats in microglia morphology in the PFC at PND33. Descriptive data from Section 3.2.3.3, page 51, and Section 3.3.3.1, page 64. Values are presented as mean ± SEM from 3 animals.

	Number of processes			Length of processes (µm)		
Branch order	**Males**	**Females**	**TP females**	**Males**	**Females**	**TP females**
1	4.3 ± 0.21	4.6 ± 0.27	6.0 ± 0.20	23.4 ± 0.89	28.2 ± 2.69	26,5 ± 0,73
2	7.8 ± 0.78	8.6 ± 0.81	11,5 ± 0,67	40.4 ± 1.94	45.8 ± 4.90	60,8 ± 7,98
3	9.9 ± 0.70	10.7 ± 0.86	16,4 ± 0,72	47.1 ± 4.20	52.0 ± 2.06	75,7 ± 3,73
4	10.1 ± 1.05	10.5 ± 0.58	19,8 ± 0,45	46.5 ± 6.24	48.5 ± 1.35	94,2 ± 10,20
5	10.7 ± 0.56	10.1 ± 0.88	21,5 ± 1,15	46.3 ± 2.82	46.7 ± 4.47	92,0 ± 11,36
6	8.5 ± 0.77	8.9 ± 1.04	22,0 ± 1,15	37.5 ± 4.96	40.3 ± 6.73	91,8 ± 10,16
7	7.0 ± 0.72	8.6 ± 0.68	20,8 ± 0,93	28.3 ± 2.83	36.1 ± 3.38	75,3 ± 4,305
8	7.5 ± 0.22	7.0 ± 1.02	16,3 ± 0,62	33.7 ± 3.86	29.5 ± 2.06	54,6 ± 4,56
9	6.8 ± 0.54	6.5 ± 0.21	12,7 ± 0,55	29.8 ± 4.09	25.8 ± 125	44,4 ± 1,34
10	5.5 ± 0.33	5.8 ± 0.67	10,8 ± 1,14	20.0 ± 1.36	21.7 ± 1.24	37,8 ± 4,29

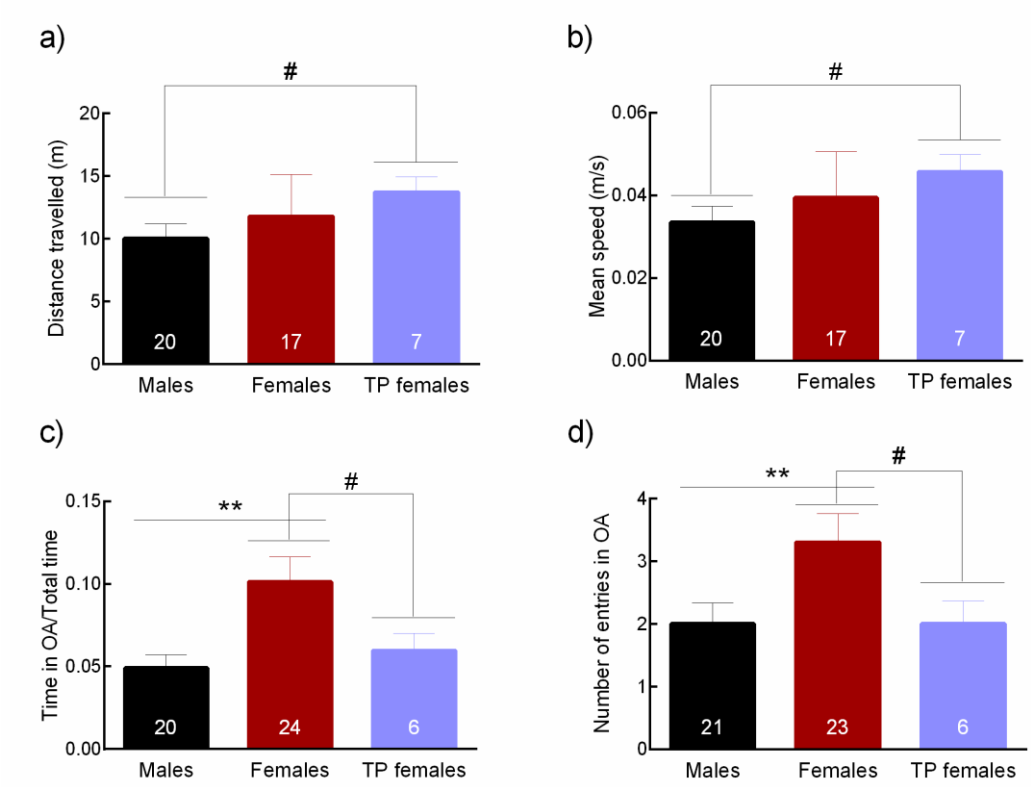


Figure 23 Analysis of locomotor activity in open field (OF) test and anxious-like behaviour in the elevated plus maze (EPM) test. NT male, NT female and female Wistar rats androgenised (TP) at PND0 were tested in the OF at PND30 and in the EPM at PND32. The tests were recorded with a videocamera. Recordings from OF tests were analysed with ANY-MAZE software and recordings from EPM were manually analysed with Observador software. The distance travelled (a) and the mean speed (b) in the OF were used as a measure of locomotor activity. The quocient between the time spent in open arms (OA) of the EPM and the total time (c), and the number of entries in OA (d) were used as a measure of anxious-like behaviour. Results are presented as mean ± SEM, for the indicated number of animals. Student's t test was used to compare two independent means: * $p<0.05$, ** $p<0.01$ comparing NT males with NT females; # $p<0.05$, ## $p<0.01$, comparing TP females with NT males and females.