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José Manuel Nunes Carreira

IMPACT OF CHRONIC STRESS IN BLOOD-BRAIN BARRIER:

FOCUS ON C3 COMPLEMENT PATHWAY AND SEX DIFFERENCES

Dissertação no âmbito do Mestrado em Neurociências Molecular e de Translação, orientada pela Doutora Ana Paula Pereira da Silva Martins e pela Doutora Filipa Isabel Cabaço Baptista, e apresentada à Faculdade de Medicina da Universidade de Coimbra

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Support

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"*There must be a beginning of any great matter, but the continuing unto the end until it be thoroughly finished yields the true glory.*"

Francis Drake

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Abbreviations list

ACTH - Adrenocorticotropic hormone

AMPA - α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

AMY - Amygdala

ANOVA - Analysis of Variance

BBB - Blood-brain barrier

BCA - Bicinchoninic acid

BSA - Bovine serum albumin

CA - Cornu ammonis

CAPS - 3-(cyclohexylamino)-1-propanesulfonic acid

CNS - Central Nervous System

CRH - Corticotropin-releasing hormone

DAPI - 4′,6-diamidino-2-phenylindole

DG - Dentate gyrus

dHIP - Dorsal hippocampus

DOC - Deoxycholate

DTT - Dithiothreitol

ECF - Enhanced chemofluorescent

ECL - Enhanced chemiluminescence

ECs - Endothelial cells

EDTA - Ethylene diamine tetraacetic acid

EGTA - Ethylene glycol tetraacetic acid

ELISA - Enzyme-linked immunosorbent assay

EPM - Elevated plus maze

fB - Factor B

fD - Factor D

GABA - γ-aminobutyric acid

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GCs - Glucocorticoids

GFAP - Glial fibrillary acidic protein

GLUT-1- Glucose transporter-1

GR - Glucocorticoid receptor

HIP - Hippocampus

HPA - Hypothalamic-pituitary-adrenal

HRP - Horseradish peroxidase

ICAM-1 -Intercellular adhesion molecule-1

MAC - Membrane attack complex

MASPs - MBL-associated serine proteases

MBL - Mannose-binding lectin

MMPs - Matrix metalloproteinases

mPFC - Medial prefrontal cortex

MR - Mineralocorticoid receptor

NMDA - N-methyl-D-aspartate

NOR - Novel object recognition

NVU - Neurovascular unit

OFC - Orbitofrontal cortex

PBS - Phosphate buffered saline

PFA - Paraformaldehyde

PFC - Prefrontal cortex

PVDF - Polyvinylidene fluoride

PVN - Paraventricular nucleus

RIPA - Radio-immunoprecipitation assay

RT - Room temperature

SPT - Sucrose preference test

TBS - Tris-buffered saline

uCMS - Unpredictable chronic mild stress

VCAM-1 - Vascular cell adhesion molecule-1

vHIP - Ventral hippocampus

Abstract

Chronic stress is a significant burden in our society, increasing the risk for the development of neuropsychiatric disorders. In fact, prolonged exposure to stress can contribute to the damage of multiple brain regions associated with emotional-cognitive functions, such as the prefrontal cortex (PFC) and the hippocampus (HIP). Recent evidence has been shown that stress is linked with cerebrovascular diseases, particularly with blood-brain barrier (BBB) dysfunction. The BBB is a selective and dynamic barrier that regulates the bidirectional transport in/out the brain, including the trafficking of peripheral immune cells, microorganisms, and several compounds into the brain parenchyma. However, little is known about the mechanisms underlying stress-induced BBB disruption. Moreover, the complement pathway plays an important role in innate immunity, but its abnormal activation has been also associated with several Central Nervous System (CNS) pathologies. Taking into consideration the crucial role of the BBB on brain protection and homeostasis, the present study aimed to assess the possible involvement of BBB dysfunction in stress-induced behavioral alterations by investigating the neurovascular alterations induced by unpredictable chronic mild stress (uCMS) exposure for 6 weeks, with focus on the C3 complement pathway. Additionally, sex-dependent alterations were also explored.

The present results demonstrated that uCMS impaired body weight gain in males, but not in females. However, the results suggest that uCMS did not induce an imbalance in the hypothalamic-pituitary-adrenal (HPA) axis activity in both sexes. In terms of behavior, uCMS induced depressive-like behavior in males, while females did not display any behavioral alterations. Following uCMS, C3 protein levels in the PFC, HIP, and serum were not altered in both sexes. Similarly, no significant changes in the C3aR protein levels in the PFC and HIP were found, although a tendency to decrease was detected in the PFC of males exposed to uCMS. Regarding BBB properties, no significant alterations were observed in the PFC of males after uCMS. Curiously, exposure to uCMS was associated with an increase in claudin-5 protein levels in the PFC of females. Furthermore, astrocytic reactivity was not altered in the PFC of both sexes a result of uCMS exposure. In sum, the results obtained in this work revealed that males and females are differently affected by chronic mild stress: males are more affected at the behavior level, while females cope better and eventually develop a stress-resilient

phenotype. Furthermore, the results suggest that uCMS has no major effects on BBB properties, but future studies are needed to clarify other possible brain vascular alterations induced by uCMS.

Keywords: chronic stress; blood-brain barrier; C3 complement pathway; sex differences

Resumo

O stress crónico tem um impacto significativo na nossa sociedade, aumentando o risco para o desenvolvimento de distúrbios neuropsiquiátricos. A exposição prolongada a stress pode contribuir para danos em múltiplas regiões cerebrais associadas a funções emocionais e cognitivas, como o córtex pré-frontal (CPF) e o hipocampo (HIP). Evidências recentes têm demostrado que o stress está associado a doenças cerebrovasculares, podendo ocorrer a disfunção da barreira hematoencefálica (BHE). A BHE é uma barreira seletiva e dinâmica que regula o transporte bidirecional para dentro/fora do cérebro, incluindo o tráfico de células imunitárias periféricas, microrganismos e vários compostos no parênquima cerebral. No entanto, pouco se sabe sobre os mecanismos subjacentes à perturbação da BHE induzida pelo stress. Além disso, a via do complemento desempenha um papel importante na imunidade inata, tendo a sua ativação anormal sido associada a várias patologias do Sistema Nervoso Central (SNS). Considerando o papel crucial da BHE na proteção do cérebro e na sua homeostase, o presente estudo teve como objetivo avaliar o possível envolvimento da disfunção da BHE em alterações comportamentais induzidas pelo stress, investigando possíveis alterações neurovasculares induzidas pela exposição a stress crónico moderado e imprevisível (uCMS) durante 6 semanas, com foco na via do complemento C3. Adicionalmente, foram também exploradas alterações dependentes do sexo.

Os resultados atuais demonstraram que o uCMS afetou o aumento do peso corporal nos machos, mas não nas fêmeas. No entanto, os resultados sugerem que o uCMS não induziu um desequilíbrio na atividade do eixo hipotálamo-pituitária-adrenal (HPA) em ambos os sexos. Em termos de comportamento, o uCMS induziu um comportamento do tipo depressivo em machos, enquanto as fêmeas não apresentaram alterações comportamentais. Após o uCMS, os níveis da proteína C3 no CPF, HIP e soro não se encontravam alterados em ambos os sexos. Da mesma forma, não foram detetadas alterações significativas nos níveis da proteína C3aR no CPF e HIP, embora tenha sido detetada uma tendência para a sua diminuição no CPF dos machos expostos a uCMS. No que diz respeito às propriedades da BHE, não foram observadas alterações significativas no CPF dos machos após o uCMS. Curiosamente, a exposição ao uCMS foi associada a um aumento dos níveis da proteína claudin-5 no CPF das fêmeas. Além disso, a exposição a uCMS não induziu reatividade astrocítica no CPF de ambos os sexos. Em

suma, os resultados obtidos neste trabalho revelaram que os machos e as fêmeas são diferentemente afetados pelo stress crónico moderado e imprevisível: os machos são mais afetados a nível comportamental, enquanto as fêmeas lidam melhor com o stress e eventualmente desenvolvem um fenótipo resiliente. Além disso, os resultados sugerem que o uCMS não tem efeitos significativos nas propriedades da BHE. No seguimento deste trabalho, estudos futuros são necessários para esclarecer outras possíveis alterações vasculares cerebrais induzidas pelo uCMS.

Palavras-chave: stress crónico; barreira hematoencefálica; via do complemento C3; diferenças de sexo

Chapter 1

Introduction

1. Introduction

1.1 Stress

1.1.1 Overview

The origin of stress research dated of 1936 when the endocrinologist Hans Selye in a short letter to *Nature* described for the first time, "A syndrome produced by diverse nocuous agents", based on his early experiments with rats. The author observed that after injecting rats with sublethal doses of distinct drugs (adrenaline, atropine, morphine, formaldehyde, among others), or exposing them to cold, surgical injury, excessive muscular exercise or production of spinal shock, animals presented a typical syndrome that included gastric ulcerations, enlargement of the adrenal gland, and atrophy of the thymus, spleen and other lymphoid tissue (Selye, 1936). Moreover, this syndrome was divided into three stages: a first one that consisted in an alarm reaction, followed by a period of resistance and a final stage of exhaustion (Perdrizet, 1997; Selye, 1936). Considering his findings, Selye developed the concept of "general adaptation syndrome", which is known today as stress response (Szabo et al., 2012, 2017).

Stress is presently defined as a state of any real or perceived threat to brain homeostasis or well-being (Chrousos, 2009; Herman, 2013; Smith & Vale, 2006). In addition, numerous systems, including the endocrine, autonomic, behavioral and immune system, are activated to promote a coordinated stress response and to reestablish brain homeostasis (Herman, 2013; Lucassen et al., 2014). A stress response can be triggered by exposure to distinct stressors (e.g., making an oral presentation, living in poverty or in a broken family, the loss of a loved one, bereavement), which can be distinguished by its duration, intensity, predictability, controllability, and individual susceptibility (Lucassen et al., 2014; Sousa, 2016). Therefore, stress is not a single entity and multiple types of stress can be differentiated: stress can be acute or chronic, anticipated, unpredictable or uncontrollable, mild or severe, and occur in or out of a specific context (Lucassen et al., 2014). Moreover, the perception of an event as stressful is variable between individuals and can have important outcomes depending on their level of stress, resilience or vulnerability (Franklin et al., 2012).

1.1.2 The role of the hypothalamic-pituitary-adrenal (HPA) axis in stress response

The stress response is coordinated by different systems, with the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.1) being the primary hormonal response to a homeostatic challenge (Myers et al., 2012). The activated HPA axis regulates peripheral functions, including metabolism and immunity, but also impacts on the brain (Pariante & Lightman, 2008). Under stress conditions, neurons in the medial parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing hormone (CRH) and arginine vasopressin (Vyas et al., 2016). In turn, CRH elicits anterior pituitary gland secretion of adrenocorticotropic hormone (ACTH), which leads to the release of ACTH into the bloodstream (Hill & Tasker, 2012). Then, ACTH stimulates the synthesis and release of glucocorticoids (GCs) (cortisol in humans and corticosterone in rodents) by the cortex of the adrenal glands (de Kloet et al., 2005; Kudielka & Kirschbaum, 2005). GCs are the major stress hormones and regulate metabolic, immune, cardiovascular, and nervous systems (de Kloet et al., 1998; Sapolsky et al., 2000). Additionally, GCs can cross the blood-brain barrier (BBB) due to their liposoluble properties and bind to two types of GC receptors: the mineralocorticoid receptor (MR or Type I) and the glucocorticoid receptor (GR or Type II) (Marin et al., 2011). MRs are mainly found in the hippocampus and septal neurons, but also in cortical neurons (Sousa et al., 2008), while GRs are ubiquitously expressed throughout the brain (Reul & de Kloet, 1985; Weinstock, 2008). MRs have a 10-fold higher affinity to endogenous GCs, which results in a basal activation of these receptors, whereas GRs are only activated when GCs levels increase either by stress or during the circadian peak of GC secretion (de Kloet et al., 1998; Reul & de Kloet, 1985). Moreover, both MRs and GRs act on the brain as transcriptional factors, regulating the expression of distinct genes (Myers et al., 2012).

The activity of the HPA axis is controlled by negative feedback mechanism, whereby GCs act in the pituitary and in the hypothalamus to inhibit their own secretion and prevent harmful effects to the brain resulting from chronic exposure to high levels of GCs (Herman et al., 2016; Marin et al., 2011). The HPA axis follows a circadian rhythm, characterized by secretion peak levels of GCs during the active phase (daytime in humans, nighttime in rodents), returning then rapidly to their basal levels (Spiga et al.,

2014). Importantly, disruption of HPA axis circadian rhythmicity is often associated with changes in stress resilience, which can increase the susceptibility to neuropsychiatric disorders (Rao & Androulakis, 2019; Walker et al., 2020).

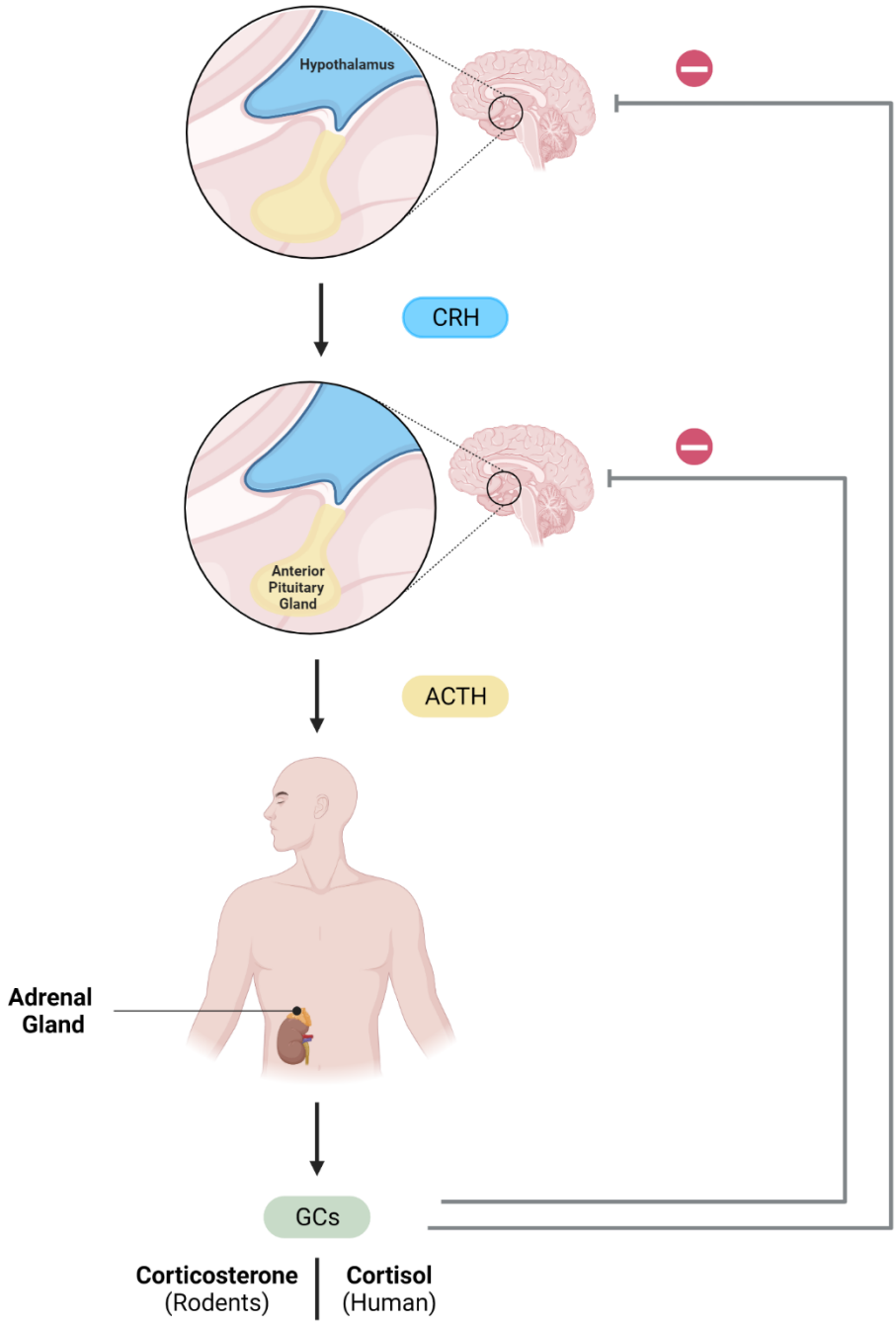

Figure 1.1. Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis. Stress promotes the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN). Then, CRH induces the secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland, followed by its release into the blood circulation and stimulation of glucocorticoids (GCs) (cortisol in humans and corticosterone in rodents) from the adrenal gland. In turn, GCs will stimulate brain glucocorticoid (GR) and mineralocorticoid (MR) receptors. After, GCs act through a negative feedback mechanism to prevent the continuous secretion of GCs and to regulate HPA axis activity. Image created with BioRender.com

1.1.3 Chronic stress and neuropsychiatric disorders

Although stress triggers adjustments of multiple biological systems and promotes adaptation of the organism to different challenges (allostasis), overactivation of the allostatic systems (allostatic load) can lead to maladaptive responses and impact both physical and mental health (McEwen, 2017; McEwen & Gianaros, 2011). Additionally, chronic stress can cause an imbalance of the neural circuits involved in decision making, cognition, anxiety and mood that can lead to behavioral repercussions (McEwen, 2017). Therefore, stress is able to increase susceptibility to the development of various neuropsychiatric disorders (Hall et al., 2015; Sanacora et al., 2022), that have become prevalent worldwide (Bale, 2005) and represent a significant economical and health issue in our society (Lucassen et al., 2014; Pêgo et al., 2010). According to the World Health Organization (2019), 1 in every 8 people, or 970 million people globally are estimated to suffer from a mental disorder, with depressive and anxiety disorders as the most common. Additionally, in the last 2 years due to COVID-19 pandemic these numbers highly increased among all population with a particular incidence in children and adolescents. Interestingly, there are also sex differences in stress prevalence and response in stress-related disorders (Bangasser & Valentino, 2014; Bekhbat & Neigh, 2018). For instance, two-thirds of patients with stress-related disorders are women, but over two-thirds of suicide completers are men (Brivio et al., 2020).

1.1.4 The effect of stress in the brain structure and function

The brain is a key organ involved in stress response and determines the physiological and behavioral response to a stressor. Brain cortico-limbic and hypothalamic structures including prefrontal cortex (PFC), anterior cingulate, amygdala (AMY), insula, hippocampus (HIP), and striatum are highly interconnected and sensitive to stress, making them the main targets of stress hormones and mediators (Bottaccioli et al., 2019; McLaughlin et al., 2009). Therefore, chronic exposure to stress has a significant impact on cellular integrity and function of these brain areas, particularly in dendritic and synaptic reorganization, and glial remodeling (Sanacora et al., 2022; Sousa & Almeida, 2012). In the next subsections, the impact of chronic stress in the PFC and HIP will be detailed.

1.1.4.1 The prefrontal cortex and stress

The PFC is anatomically and functionally divided into two main subregions: the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC) (Wellman et al., 2020). The mPFC includes the AC, prelimbic and infralimbic cortex, that corresponds to the human dorsolateral and medial PFC (Cerqueira et al., 2008; Jacobs & Moghaddam, 2021), whereas the OFC comprises the ventral and lateral regions that are homologous to primate orbitofrontal cortex (Dalley et al., 2004; Wellman et al., 2020). The PFC has an important role in cognitive control, working memory, executive function and self-regulatory behaviors (McEwen & Morrison, 2013; Radley et al., 2015). Additionally, the PFC plays a crucial role in modulating endocrine and autonomic functions during the stress response (Cerqueira et al., 2008; Czéh et al., 2008). Considering that PFC regulates a variety of cognitive and homeostatic functions, the impact of chronic stress on PFC structure and function, and its possible influence in stress-related disorders has received increasing attention from the neuroscience community in recent years (Radley et al., 2015). Moreover, it is important to note that most of the studies focus on the mPFC (McEwen & Morrison, 2013).

Chronic stress induces neuroplastic changes in the mPFC including dendritic retraction (Cook & Wellman, 2004; Liston et al., 2006; Radley et al., 2004), spine loss and altered synaptic transmission (Goldwater et al., 2009; Woo et al., 2021) in the pyramidal neurons (layer II/III and layer V). These alterations are associated with impairments in working memory and behavioral flexibility (Cerqueira et al., 2007) and in the recall of fear conditioning and extinction (Miracle et al., 2006). Furthermore, other studies also reported dendritic alterations in the mPFC due to acute stress exposure (Brown et al., 2005; Izquierdo et al., 2006). Curiously, these effects are described to be reversible when stress exposure is terminated (Radley & Morrison, 2005). Changes in neuronal morphology and function as a result of chronic stress are also accompanied by significant alterations in the PFC glutamatergic system (Wellman et al., 2020). For instance, chronic stress impairs α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)- and N-methyl-D-aspartate (NMDA) receptor-mediated synaptic transmission and cell surface expression in mPFC (Yuen et al., 2012), reduces NMDA and AMPA currents and alters the activity of γ-aminobutyric acid (GABA) interneurons, which inhibit pyramidal neurons (Jacobs & Moghaddam, 2021). Notably, these stress-induced

effects were only described in males, since females were not evaluated in these studies. In fact, chronic stress impacts mPFC in a sex-dependent manner, differentially remodeling neuronal morphology and influencing behavior (Wellman et al., 2018). Accordingly, male rats present a decrease in dendritic arbors following chronic stress exposure, whereas female rats either show no dendritic changes (Moench & Wellman, 2017) or dendritic hypertrophy (Garrett & Wellman, 2009). Moreover, chronic stress leads to deficits in behaviors that involve activation of PFC, including working memory, attentional set-shifting and cognitive flexibility in male rats (Holmes & Wellman, 2009), while female rats do not exhibit these stress-induced alterations (Wei et al., 2014). Glial morphology is also affected by chronic stress in a sex-specific manner. Since astrocytes are essential for the correct functioning of synaptic connectivity, synaptogenesis, and maintenance of synaptic function (Codeluppi et al., 2021), alterations in the morphology of astrocytes can impair their activity. Chronic stress causes PFC astrocytic atrophy in male rats (Codeluppi et al., 2021; Tynan et al., 2013) and hypertrophy in female rats (Bollinger et al., 2019). Moreover, in male rats, chronic stress increases microglial density and activation, which is demonstrated in the morphology and expression of immune molecules in PFC (Walker et al., 2013). Specifically, chronic stress results in an increase of Iba-1 immunoreactivity (Tynan et al., 2010) and microglial branching, process number and length in this brain region (Hinwood et al., 2013) In addition, these alterations in microglial activation were associated with deficits in spatial working memory (Hinwood et al., 2012; Hinwood et al., 2013). However, these stress-induced changes in male microglia reactivity were not reproduced in a more recent study (Bollinger et al., 2016). On the other hand, stress exposure decreased female microglia processes ramification, suggesting a reduced activated state (Bollinger et al., 2016).

1.1.4.2 The hippocampus and stress

The HIP is a brain region located at the medial temporal lobe and responsible for cognitive function and emotional processing (Fanselow & Dong, 2010; Strange et al., 2014). It includes the cornu ammonis (CA) fields (CA1, CA2 and CA3), the dentate gyrus (DG), the subiculum, presubiculum and parasubiculum fields, and the entorhinal cortex (Hartley et al., 2014). The HIP extends along a dorsal (septal) to ventral (temporal) axis in rodents, which corresponds to a posterior-anterior axis in humans

(Strange et al., 2014). Moreover, the dorsal and ventral subregions are associated with distinct behaviors. The dorsal hippocampus (dHIP) is involved with cognitive functions, whereas the ventral hippocampus (vHIP) is related to stress, emotion, and affection (Fanselow & Dong, 2010). The HIP is involved in the regulation of stress response, since it encodes environmental contextual information related to the perceived threat (Herman et al., 2005), and is involved in the inhibition of the HPA axis (Herman et al., 1995; Jacobson & Sapolsky, 1991). Lesions experiments in the HIP produce elevated levels of GCs in basal conditions, further suggesting that the HIP is a key brain region in the regulation of the HPA axis (Herman et al., 2016).

The HIP is a particularly sensitive and vulnerable brain structure to damage by repeated stress (Sapolsky, 1992). Accordingly, chronic stress causes alterations in neuronal morphology, including atrophy of dendrites on CA3 (Bessa et al., 2009; Magariños et al., 1997; Stewart et al., 2005; Watanabe et al., 1992) and CA1 pyramidal neurons (Sousa et al., 2000). These stress-induced changes are usually associated with deficits in hippocampal-dependent learning tasks (Conrad, 2010). Curiously, more recent data showed that chronic stress affects differently the morphology of hippocampal neurons. Accordingly, chronic stress induces a dendritic retraction of CA3 and CA1 pyramidal neurons in dHIP, while in the vHIP, pyramidal neurons exhibited a neuronal hypertrophy in CA3 region (Pinto et al., 2015). Moreover, chronic stress exposure produces impairments in long-term potentiation in DG (Shors & Dryver, 1994) and CA3 region of the HIP (Pavlides et al., 2002). Importantly, these alterations in neuronal morphology are correlated with the decreased hippocampal volume observed in depressed patients (McKinnon et al., 2009; Videbech & Ravnkilde, 2004) and in rats exposed to chronic stress (Schoenfeld et al., 2017). Similar to the PFC, stress also impacts hippocampal neurons morphology in a sex-specific manner. Exposure to chronic stress fails to cause CA3 dendritic retraction in females (Galea et al., 1997; Liu et al., 2006), as well as to impair hippocampal dependent memory (Luine et al., 2007). At glial level and specifically astrocytes, chronic stress reduced glial fibrillary acidic protein (GFAP) mRNA expression and protein levels in male rats (Araya-Callís et al., 2012). Moreover, in females, early life stress decreased the astrocytic population in the CA3 (Saavedra et al., 2021). Regarding microglial cells, no differences were observed in males following chronic stress exposure, while females presented an increase of the total number and length of microglia processes (Gaspar et al., 2021).

1.2. Blood-brain barrier

The blood-brain barrier (BBB) is a selective and dynamic structure that acts as a interface between the Central Nervous System (CNS) and the periphery (Campos-Bedolla et al., 2014; Cardoso et al., 2010). The BBB plays an essential role in the maintenance of the homeostasis of the CNS through the regulation of ion balance and brain nutrition, as well as in the protection of the neural tissue from toxins, pathogens, and immune cells (Abbott et al., 2010; Keaney & Campbell, 2015). In opposition, the specific properties of the BBB are a major obstacle for drug delivery to the brain, since almost 98% of small molecules and 100% of large molecules are not able to cross the BBB (Pardridge, 2003). The BBB is composed by endothelial cells (ECs) that are surrounded by basement membrane, pericytes, neurons, astrocytes, and microglia, which altogether compose the neurovascular unit (NVU) (Figure 1.2) (Daneman & Prat, 2015; Serlin et al., 2015). As a result, any impairment of BBB components may result in the loss of barrier integrity and function, which could promote the entry of pathogens, toxins, or/and autoantibodies into the brain significantly affecting its normal function and originating several diseases (Obermeier et al., 2016).

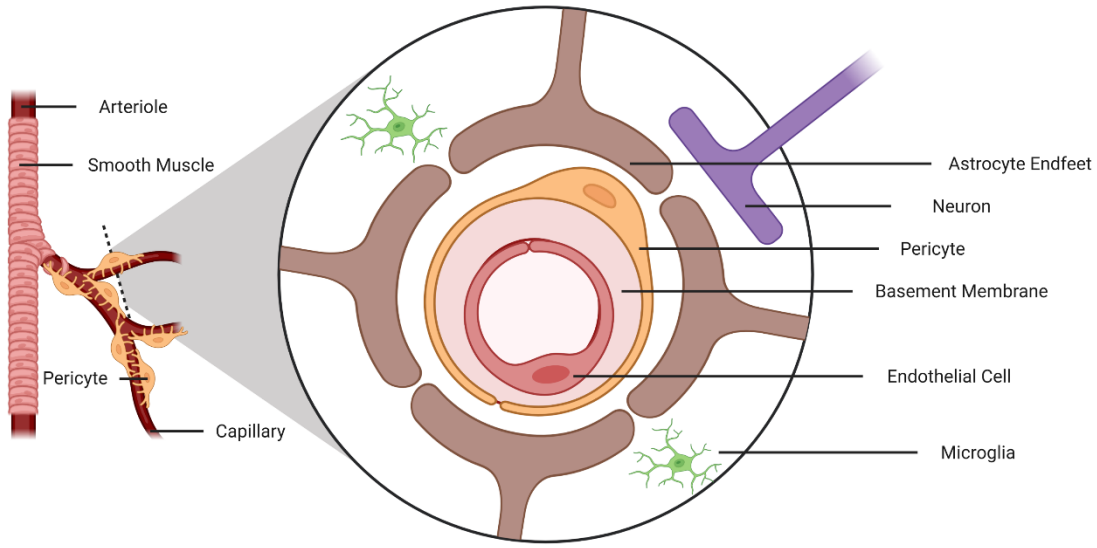

Figure 1.2. Schematic representation of the Neurovascular Unit (NVU). The NVU is composed by endothelial cells, basement membrane, pericytes, astrocytes, microglia, and neurons, that together provide unique features to the blood-brain barrier (BBB). Pericytes are distributed along the microvessel endothelium. Both brain endothelial cells and pericytes are enclosed by basement membrane, and astrocyte endfeet surround the capillaries. Neurons innervate brain vasculature and can induce vessels to adjust their tone based on incoming neuronal afferent signals. Microglial cells are the immunosurveillance cells of the brain that also contribute to barrier properties.

1.2.1 Cellular components of the neurovascular unit

1.2.1.1 Endothelial cells

The brain ECs are considered the core component of the BBB and form the inner layer of brain capillaries (Wilhelm et al., 2011). Additionally, these cells have unique characteristics that differentiate them from other ECs, including a higher number and volume of mitochondria (required for energy demands and transport of nutrients), presence of multiple and selective transporters (allowing the exchange of molecules between the brain and the periphery), and absence of fenestrations (limiting the transendothelial transit of fluid and small solutes) and low pinocytosis/transcytosis activity (preventing movement of molecules through cells- transcellular flux) (Cardoso et al., 2010; Iadecola, 2004; Obermeier et al., 2016; Persidsky et al., 2006). Furthermore, ECs are also characterized by a high transendothelial electrical resistance (TEER), which reflects BBB highly restricted permeability to ions and other molecules (Cardoso et al., 2010). ECs are held together by intercellular junctions, namely tight (TJs) and adherens junctions (AJs) (Figure 1.3), that are responsible to maintain barrier structure and properties (McConnell et al., 2017; Tietz & Engelhardt, 2015). Curiously, gap junctions have also been found at the BBB (Nagasawa et al., 2006), however their role in the barrier is not fully understood (Zlokovic, 2008).

1.2.1.1.1 Tight junctions

Tight junctions (TJs) are composed by transmembrane proteins, including claudins, occludin and junctional adhesion molecules (JAMs), and also by cytoplasmic accessory proteins, such as zonula occludens (ZO), cingulin, AF-6 and 7H6 (Benarroch, 2012; Persidsky et al., 2006; Serlin et al., 2015). The cytoplasmic accessory proteins are essential to link the transmembrane proteins to the actin cytoskeleton (Ballabh et al., 2004; Zlokovic, 2008). Moreover, TJs are responsible for the separation of the luminal and abluminal sides of the plasma membrane (Abbott et al., 2010; Cardoso et al., 2010), which contribute to the polarization of the cell (Wilhelm et al., 2011), and to limit the paracellular permeability between the ECs to ions and other polar solutes, leading to a high TEER of the BBB (Bazzoni & Dejana, 2004; Butt et al., 1990).

a. Claudins

Claudins are a family of four-transmembrane domain proteins that comprises 27 members identified so far, and with a molecular weight around 20-34 kDa (Gonçalves et al., 2013; Tsukita et al., 2019). These proteins are the main components of TJs (Kadry et al., 2020) and are indispensable both for the formation of TJs and to regulate the paracellular permeability (Gonçalves et al., 2013; Tsukita et al., 2019). Moreover, claudin-1, -3, and -5 are the most abundant at brain endothelium (Gonçalves et al., 2013). Curiously, claudin-5 seems to have a crucial role in the maintenance of a normal BBB function, since knockout animals exhibited a compromised BBB and die shortly after birth (Nitta et al., 2003). Additionally, BBB dysfunction due to the loss of claudin-3 has also been noticed in experimental autoimmune encephalomyelitis and human glioblastoma (Wolburg et al., 2003).

b. Occludin

Occludin is a 65 kDa phosphoprotein, with four transmembrane domains and two extracellular loops (Ballabh et al., 2004; Kadry et al., 2020). Occludin was the first transmembrane TJ protein identified (Furuse et al., 1993) and it is highly expressed in brain ECs compared to nonneural tissues, (Hirase et al., 1997; Vorbrodt & Dobrogowska, 2003). Moreover, high levels of occludin contribute to a decrease in paracellular permeability (Huber et al., 2001) and high electrical resistance of ECs monolayers (McCarthy et al., 1996). Although occludin plays an important role in barrier structure and function (Brown & Davis, 2005; Huber et al., 2002), it is not vital for TJs formation (Hawkins & Davis, 2005). In fact, occludin-deficient animals did not present morphological alterations in TJs, but only impairments in development and breeding (Saitou et al., 2000). Interestingly, the absence of occludin can be compensated by other junctional proteins (Zlokovic, 2008). Therefore, occludin acts more in a regulatory context than as a key protein to maintain BBB properties, since it is responsible for the connection to the actin cytoskeleton through ZO proteins (Wolburg & Lippoldt, 2002).

1.2.1.1.2 Adherens junctions

The adherens junctions (AJs) are localized in the basal region of lateral plasma membrane, more close to the abluminal side of ECs (Petty & Lo, 2002) and mediate cell-

cell adhesion in a Ca^{2+}-dependent manner, contact inhibition, paracellular permeability and cell polarity (Hawkins & Davis, 2005; Kadry et al., 2020). These junctions are formed by the membrane protein cadherin, particularly vascular endothelial (VE-) cadherin and neural (N-) cadherin, which are the most prevalent cadherins in brain microvessels and in the brain tissue, respectively (Vorbrodt & Dobrogowska, 2003). Nevertheless, N-cadherin is also present on other cell types, including vascular smooth muscle cells, and myocytes (Navarro et al., 1998). Furthermore, cadherins are connected to the actin cytoskeleton through a group of proteins named catenins (Cardoso et al., 2010). Catenins can be subdivided in four different types (α, β, δ and γ), with β-catenin being crucial for vascular patterning (Vincent et al., 2004), and playing an essential role as transcription factor, as it modulates several genes (Vorbrodt et al., 2008).

1.2.1.2 Basement membrane

The basement membrane is mainly responsible for anchoring ECs and pericytes, and to connect these cells with other brain neighbor cells (Carvey et al., 2009). It is composed by structural proteins (collagen IV and elastin), specialized proteins (laminin and fibronectin), heparin sulfate proteoglycans and other extracellular matrix proteins (Cardoso et al., 2010; Serlin et al., 2015). The basement membrane also contains cell adhesion molecules, as well as signaling proteins, which form a complex and extensive matrix (Carvey et al., 2009). Disruption of the basement membrane can promote changes in the ECs cytoskeleton, which in turn impacts TJs and barrier properties (Cardoso et al., 2010). In fact, matrix metalloproteinases (MMPs) are strongly associated with the degradation of the basement membrane, leading to the infiltration of leukocytes and other peripheral cells in the brain parenchyma as observed in several pathological conditions (Daneman & Prat, 2015).

1.2.1.3 Pericytes

Pericytes are vascular mural cells directly found on the abluminal side of the endothelium and that are embedded in the basement membrane (Daneman & Prat, 2015; Knox et al., 2022). These cells cover between 22% and 32% of brain capillaries (Fisher, 2009; Kim et al., 2006), and have contractile proteins that allow them to regulate the

capillary diameter and the cerebral blood flow (Cardoso et al., 2010; Hall et al., 2014). Moreover, pericytes are able to communicate with ECs by peg-socket junctions, gap junctions, and adhesion plaque junctions (Armulik et al., 2011). Additionally, pericytes are responsible to produce multiple components of the basement membrane, such as proteoglycans, that are thought to be an essential step in BBB differentiation (Cardoso et al., 2010; Dore-Duffy et al., 2006). Importantly, the absence of pericytes results in endothelial hyperplasia and abnormal vascular morphogenesis in the brain, which emphasize the crucial role of these cells in BBB structure (Persidsky et al., 2006).

1.2.1.4 Astrocytes

Astrocytes are glial cells that contribute to the maintenance of BBB integrity and function (Ballabh et al., 2004; Knox et al., 2022). These cells interact with ECs and pericytes by their highly specialized processes, called astrocytic endfeet (Herndon et al., 2017), and control metabolite levels, cerebral blood flow and vasodilation (Zlokovic, 2008). Moreover, astrocytes play an important role in the regulation of water and ion levels in brain parenchyma, since their endfeet express water channels named aquaporins, and potassium channels (Abbott et al., 2006). Astrocytes are also involved in the synthesis of basement membrane proteins such as proteoglycans, leading to an increase of charge selectivity in ECs (Cardoso et al., 2010). Curiously, there is some evidence that astrocytes can have a dual role in BBB properties. For instance, astrocytes can release multiple soluble factors that induce TJs formation (Alvarez et al., 2013) that are essential to barrier function (Abbott et al., 2006). On the other hand, upon insult, astrocytes can release MMPs (Rosenberg, 2002) and proinflammatory cytokines, such as tumor necrosis factor-alpha, that promote BBB breakdown (Abbott et al., 2006).

1.2.1.5 Microglia

Microglia are the resident immune cells in the brain, playing a crucial role in the regulation of brain development and homeostasis (Tay et al., 2017). Additionally, these highly ramified cells survey the local microenvironment for blood-borne substances and potential inflammatory agents, acting as the gatekeepers of the CNS (Cardoso et al., 2010; Dudvarski Stankovic et al., 2016), and influence the formation of neural circuits by

controlling synapse formation (Cristovão et al., 2014) and synapse elimination (Paolicelli et al., 2011). At the BBB, microglia are primarily found in the perivascular space and contribute to barrier properties (Choi & Kim, 2008; Thurgur & Pinteaux, 2019), however the mechanisms on how this occurs remain to be further elucidated (Keaney & Campbell, 2015; Ronaldson & Davis, 2020). Importantly, upon insult, microglia release inflammatory mediators as nitric oxide and interleukin-1β, that can lead to an increase in BBB permeability (Obermeier et al., 2013).

1.2.1.6 Neurons

Neurons are the fundamental units of the brain and CNS, however not much is known about the role of these cells in BBB structure (Cardoso et al., 2010). Neurons are not directly connected to brain ECs, and astrocytes modulate communication neurons and ECs (Kim et al., 2006; Liebner et al., 2018). In fact, ECs and astrocytes are innervated by noradrenergic, serotonergic, cholinergic, and GABAergic neurons, among others (Hawkins & Davis, 2005). Furthermore, neurons can regulate cerebral blood flow in response to metabolic demands, through the expression of enzymes unique for ECs (Persidsky et al., 2006).

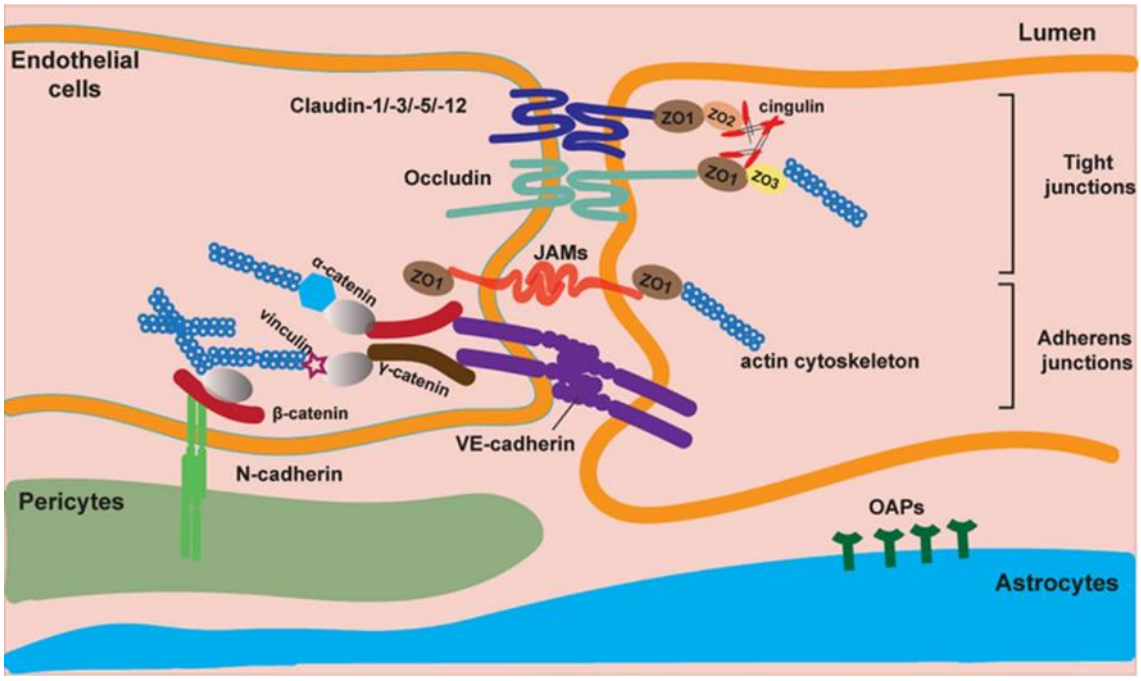

Figure 1.3. Molecular organization of intercellular junctions at the blood-brain barrier (BBB). The intercellular junctions are formed by tight (TJs) and adherens junctions (AJs). The TJs are composed by transmembrane proteins, including claudins, occludin and junctional adhesion molecules

(JAMs), and cytoplasmic accessory proteins, such as zonula occludens (ZO), cingulin, among others, that are linked to the actin cytoskeleton within the cell. Adherens junctions are composed mainly by vascular endothelial (VE)–cadherin, which interacts with catenins that attach the adherens junctions to the actin cytoskeleton, and N-cadherin, the most abundant cadherin in the brain tissue. Astrocytes contain a higher density of orthogonal arrays of particles (OAPs), that are formed by water channels named aquaporins (adapted from Kadry et al., 2020).

1.2.2 Transport across the blood-brain barrier

ECs are crucial elements to regulate the transport through the BBB (Figure 1.4). This transport across the BBB is ensured by two main routes, the paracellular pathway that takes place between adjacent ECs, and the transcellular pathway that occur through the ECs (Pardridge, 1999). The paracellular pathway consists of a passive diffusion of ions and low molecular weight solutes according to their concentration gradient (Haseloff et al., 2015; Petty & Lo, 2002). On the other hand, the transcellular pathway is mediated by different processes, including passive diffusion of small lipophilic substances (O_2, CO_2 and ethanol) (Abbott et al., 2006; Ballabh et al., 2004) or through specific transport systems (Obermeier et al., 2016; Stamatovic et al., 2008). There are two main types of transporters expressed by brain ECs: transporters that promote the movement of specific molecules down their concentration gradient, and efflux transporters that require ATP consumption to transport substances from brain to blood (Daneman & Prat, 2015; Knox et al., 2022; Stamatovic et al., 2008). For instance, the BBB is enriched in the glucose transporter-1 (GLUT1), particularly in the abluminal membrane (Cardoso et al., 2010). In opposition, efflux transporters as the ABC transporters act as efflux pumps for a variety of lipid-soluble compounds.

In addition, there are some macromolecules (peptides and proteins) with a particular role within the CNS that can cross the BBB via endocytotic mechanisms in a process called transcytosis (Abbott et al., 2010; Serlin et al., 2015). Transcytosis can be divided in receptor-mediated transcytosis or adsorptive-mediated transcytosis (Abbott et al., 2010).

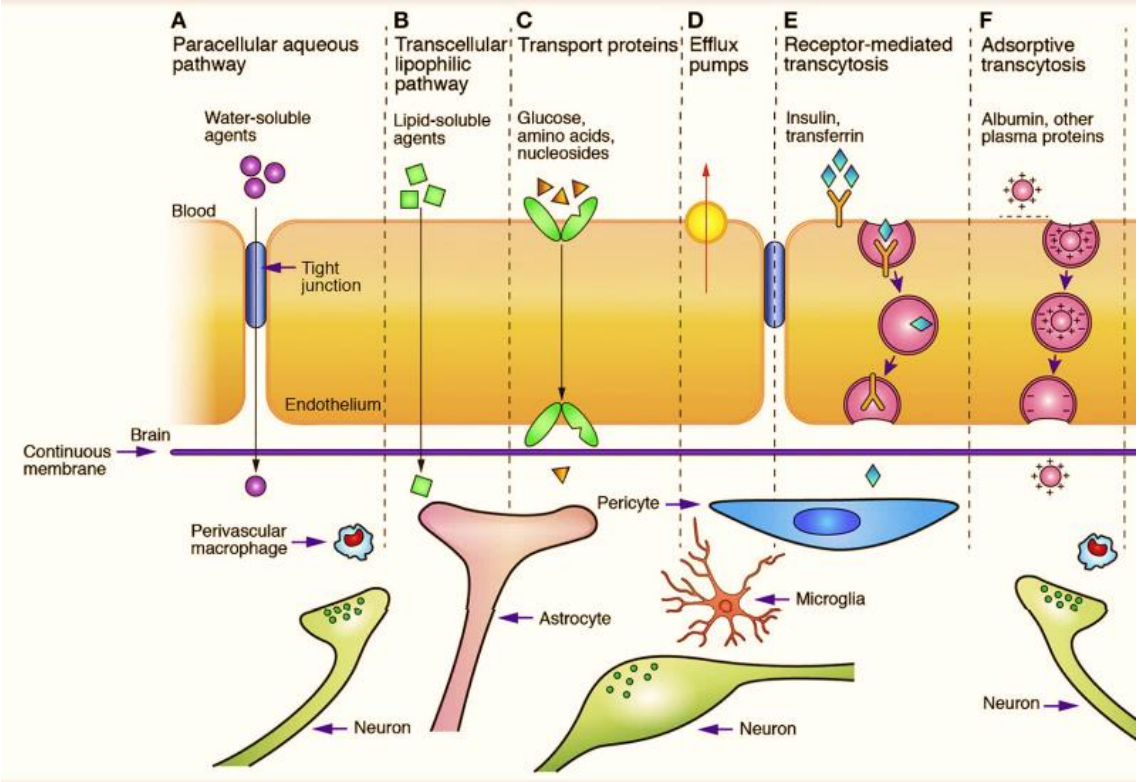

Figure 1.4. Transport across the blood-brain barrier (BBB). There are multiples routes for molecular traffic across the BBB, according to the characteristics of each molecule. (A) The paracellular pathway is responsible for the passage of small water-soluble agents to the brain parenchyma. (B) Lipid-soluble agents can diffuse through the endothelium. (C) Carrier-mediated transporters are required to transport crucial substances, including amino acids, glucose, and nucleosides into the brain. (D) Active efflux carriers may pump some lipid-soluble compounds out of the ECs. (E) Receptor-mediated transcytosis (involves the binding of a ligand to its specific receptors on the cell surface to transport macromolecules such as peptides and proteins across the endothelium. (F) Adsorptive-mediated transcytosis is induced when an excessively charged molecule interacts with cell surface binding sites and promote endocytosis and subsequent transcytosis (adapted from Chen & Liu, 2012).

1.2.3 BBB regulates immune cell trafficking into the CNS

The BBB is a highly specialized structure that regulates the entry of peripheral immune cells into the brain parenchyma (Cecchelli et al., 2007). For instance, the trafficking of leukocytes across the vascular endothelium is highly restricted, mainly due ECs low levels of adhesion molecules and inflammatory mediators (Alvarez et al., 2011). However, in neuroinflammatory diseases, leukocyte migration into the CNS is significantly upregulated, which contribute to BBB disruption (Engelhardt, 2006; Lucchinetti et al., 2000).

Leukocyte recruitment into the brain parenchyma is a complex and dynamic process that involves different steps (Carman & Martinelli, 2015). Briefly, it consists in the attachment of leukocytes to the vessel wall, followed by the diapedesis of immune

cells across the vascular endothelium and migration into the brain parenchyma (Langen et al., 2019). Adhesion molecules mediate the contact between leukocytes and ECs, playing a decisive role in the transmigration of leukocytes across the microvasculature (Greenwood et al., 2011; Takeshita & Ransohoff, 2012). Interestingly, an increase in the expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-selectin represents a key feature of peripheral inflammation and BBB dysfunction in multiple pathological conditions (Huang et al., 2021; Rossi et al., 2011).

1.2.4 Stress and the BBB

Chronic stress is considered an important risk factor for several disorders, including anxiety and depression. More recently, stress has been associated with cardio and cerebrovascular diseases (Everson-Rose et al., 2014). In fact, the impact of chronic stress in the BBB is a research topic that has been overlooked, despite its essential role in brain protection and hemostasis (Welcome & Mastorakis, 2020). Nevertheless, Menard and collaborators (2017) have already demonstrated that chronic stress promotes BBB permeability with the passage of interleukine-6 leading to depressive-like behavior. Moreover, other studies have shown that chronic stress leads to downregulation of TJs, (Sántha et al., 2015; Xu et al., 2019) and AJs (Xu et al., 2019) and subsequently behavioral and cognitive alterations. Importantly, others studies did not found an effect on BBB permeability caused by stress (Northrop & Yamamoto, 2012; Roszkowski & Bohacek, 2016). Therefore, it remains unclear how stress interferes with the cellular and molecular components of the BBB and if these alterations play a critical role on stress-associated behaviors.

1.3 The complement system

1.3.1 An overview

The complement system plays a critical role in innate immunity regulation, acting as the first line of defense against pathogens (Carpanini et al., 2019). In addition, this system also serves other pivotal functions in the CNS, including the elimination of

apoptotic cells and cellular debris, and in mediating synapse pruning during brain development (Alexander, 2018; Stephan et al., 2012; Veerhuis et al., 2011). Interestingly, although the liver is the main source of complement proteins, multiple resident brain cells (neurons, glial cells, and ECs) are able to express these proteins (Veerhuis et al., 2011; Wu et al., 2016). The complement system is composed by a large family of more than 30 proteins, present either as soluble proteins in the blood or as membrane-associated proteins, that are inactive and classified as zymogens (Hammad et al., 2018; Sarma & Ward, 2011). Complement activation occurs through three distinct pathways (classical, lectin and alternative), resulting in a cascade of proteolytic steps that cleave and activate zymogens (Figure 1.5) (Stephan et al., 2012). Importantly, all the pathways lead to the formation of C3 convertases (C4b2a or C3bBb), that cleave C3 into C3a and C3b (Brennan et al., 2012; Veerhuis et al., 2011). C3b helps to amplify the complement cascade and connects to C3 convertases, which results in the formation of C5 convertases (C4b2a3b or C3bBbC3b), and subsequent cleavage of C5 into C5a and C5b (Sarma & Ward, 2011). C5b recruits C6, C7, C8 and 10–16 C9 molecules to initiate the formation of the membrane attack complex (MAC) (Stephan et al., 2012).

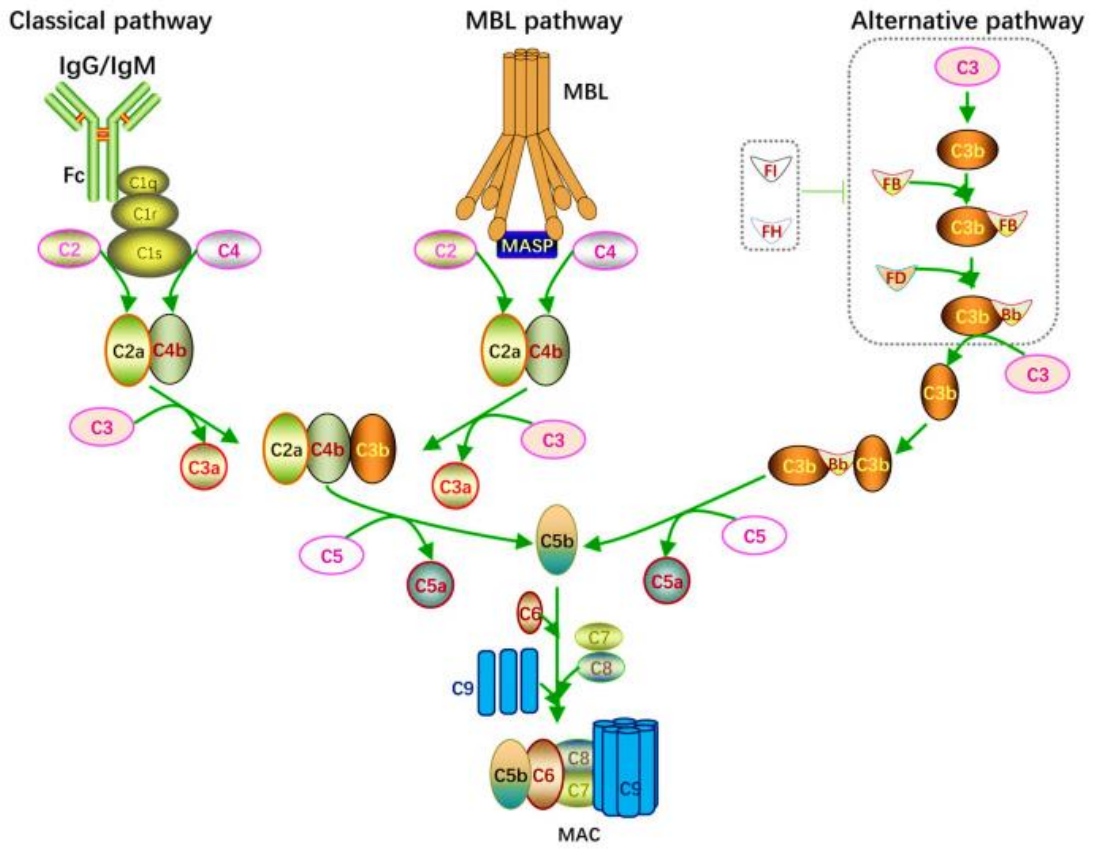

Figure 1.5. Schematic representation of the complement pathway. The complement cascade plays an important role in innate immunity regulation. Complement activation can occur via three pathways: classical, mannose-binding lectin (MBL) and alternative pathways. Regardless of their trigger, all pathways converge in a central molecule, C3, which is cleaved by C3 convertases (C4b2a or C3bBb) into C3a and C3b. Amplification of the cascade lead to the formation of the membrane attack complex (MAC) (adapted from Zhu et al., 2021).

1.3.2 The classical pathway

The classical pathway acts as an interface between innate immunity and adaptive immune response (Nayak et al., 2010). The classical pathway is initiated by the binding of C1q to the fragment crystallizable region of IgG and/or IgM antibodies (Hammad et al., 2018; Mathern & Heeger, 2015). The binding of C1q induces a conformational change in this protein, leading to the activation of C1r (Merle et al., 2015; Stephan et al., 2012). Consequently, C1r cleaves and generates C1s (Merle et al., 2015). Once activated, C1s cleaves C4 into C4a and C4b, and C2 into C2a and C2b, allowing the formation of C3 convertase, or C4b2a complex (Riihilä et al., 2019; Sarma & Ward, 2011). Then, C3 convertase cleave C3 into two molecules, the anaphylatoxin C3a and the opsonin C3b (Brennan et al., 2012). Anaphylatoxins are involved in histamine release from mast cells, smooth muscle contraction and can increase vascular permeability, whereas opsonins

increases phagocytosis of apoptotic cells and pathogens (Hammad et al., 2018; Sarma & Ward, 2011).

1.3.3 The mannose-binding lectin (MBL) pathway

The lectin pathway is triggered when MBL and ficolins bind to mannan and other carbohydrate moieties or acetylated moieties present on the surface of pathogens (Veerhuis et al., 2011). Both MBL and ficolins are found in the serum as complexes with MBL-associated serine proteases (MASPs), which are functionally homologous to C1r and C1s (Mathern & Heeger, 2015; Sørensen et al., 2005). There are four types of MASPs (1,2,3 and 19), however only MASP2 cleave both C4 and C2 to generate C3 convertase (Ricklin et al., 2010; Sarma & Ward, 2011).

1.3.4 The alternative pathway

The alternative pathway consists in the spontaneously and continuously hydrolysis of C3 at low level ("tickover"), leading to the formation of $C3_{H2O}$ (Bexborn et al., 2008; Ricklin et al., 2010). Factor B (fB) and factor D (fD) are later recruited, with fB being cleaved by fD to form C3 convertase, or C3bBb complex (Hammad et al., 2018). Importantly, the levels of C3bBb are regulated by properdin, a plasma protein activated by neutrophils and that prevents its cleavage by Factors H and I (Kemper et al., 2010).

1.3.5 Complement pathway and the BBB

The complement pathway is normally protective, playing a critical role in the regulation of brain homeostasis and development (Carpanini et al., 2019). However, complement dysregulation due to its abnormal activation, has been related with multiple CNS pathologies and neurodegenerative conditions (Hong et al., 2016). Particularly, C3a is considered a pro-inflammatory mediator that binds to its G-protein-coupled C3aR receptor and induces leukocyte activation, vascular permeability, and chemotaxis (Mastellos et al., 2016; Wang et al., 2019). C3 also regulates vascular endothelium activation and contributes to neutrophil recruitment into the brain parenchyma, suggesting that complement components from the circulation may play a significant role

in endothelial activation (Wu et al., 2016). Furthermore, increased levels of C3 were associated with BBB breakdown in mice with cerebral malaria (Lackner et al., 2008). Therefore, BBB dysfunction can occur through complement dysregulation or be exacerbated by complement activation (Alexander, 2018).

1.4 Objectives

Chronic stress is a major precipitant factor for anxiety- and depressive-like behavior, as well as cognitive deficits. Additionally, sex differences in brain structure, function, clinical manifestation, and prevalence of neuropsychiatric disorders are well documented. Nevertheless, it remains to be elucidated if chronic stress induces BBB alterations and if changes associated with stress are sex-specific. Moreover, dysfunction of the complement pathway has been associated with BBB leakage and several pathological conditions, but the impact of chronic stress in cerebrovascular function and C3 complement pathway remains elusive.

Thus, this work aims to unravel BBB alterations induced by chronic stress, with a particular focus on the C3 complement pathway and also to unveil if changes induced by stress exposure are sex-dependent.

Chapter II

Materials and Methods

2. Materials and Methods

2.1 Animals

Adult (2 months old) male (200-225g) and female (135-175g) Wistar rats were acquired from Charles River Laboratories (L'Arbresle, France). Rats of the same sex were paired-housed and maintained in standard environmental conditions (12h light/dark cycle, from 08:00 a.m. to 08:00 p.m.; 22°C, relative humidity of 55%) with food and water *ad libitum*. Animals were divided randomly into four different experimental groups: a group of males not submitted to unpredictable chronic mild stress (uCMS) protocol (Control male); a group of females not exposed to uCMS (Control female); a group of males submitted to uCMS (uCMS male); a group of females exposed to uCMS (uCMS female). All experiments were conducted by certified researchers in accordance with European (2010/63/EU) and Portuguese (DL no. 113/2013) laws. The present study was approved by Portuguese National Authority for Animal Health "DGAV". All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Unpredictable chronic mild stress (uCMS)

A validated unpredictable chronic mild stress (uCMS) protocol previously described (Alves et al., 2017; Gaspar et al., 2021; Mateus-Pinheiro et al., 2013; Willner, 2005) was applied during 6 weeks. This protocol is one of the most widely used and consists in the continuous and unpredictable exposure to a wide range of different mild stressors during 6 consecutive weeks (Nollet et al., 2013; Willner et al., 1987; Willner, 2017) (Table 1). The application of distinct stressors is an essential factor to prevent the habituation of animals to the protocol.

Table I. Schedule of chronic mild stressors administered for a 7-day period and repeated for 6 weeks.

Week I	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Confinement	Cage Replacement	Empty Bottle	Tilted Cage	Cage Replacement		
10h - 11h		Inacc. Food	1h Water Intake		Confinement		
11h - 12h	Strobe Lights	Overcrowding	Confinement	White Noise			
12h - 13h					Tilted Cage		
13h - 14h			Tilted Cage				
14h - 15h	Overcrowding	White Noise		Overcrowding	Strobe Lights		
15h - 16h							
16h - 17h							
17h - 18h							
O/N	Wet Bed + Food Deprivation	Water Dep. + O/N Illum.	Strobe Lights (LF)	Wet Bed	Rev. Light + Tilted Cage		

Week 2	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Cage Switch	Cage Replacement	Inacc. Food	White Noise	Cage Replacement		
10h - 11h		Empty Bottle	Tilted Cage		Confinement		
11h - 12h		White Noise			Cage Switch		
12h - 13h							
13h - 14h	Confinement						
14h - 15h	Strobe Lights	Overcrowding		Overcrowding	Strobe Lights		
15h - 16h							
16h - 17h			Confinement				
17h - 18h							
O/N	Water Dep. + Wet Bed	Food Deprivation+ ON Illumination	Strobe Lights (LF)	Wet Bed	Inv. Light + Tilted Cage		

Week 3	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Tilted Cage	Cage Replacement	Inacc. Food	Overcrowding	Cage Replacement		
10h - 11h		Empty Bottle	Confinement		Confinement		
11h - 12h		White Noise			Strobe Lights		
12h - 13h			Tilted Cage				
13h - 14h	Confinement			Cage Switch			
14h - 15h	Strobe Lights	Overcrowding			Tilted Cage		
15h - 16h				White Noise			
16h - 17h							
17h - 18h							
O/N	Water Dep. + Wet Bed	Food Dep.+O/N Illumination	Strobe Lights (LF)	Wet Bed	Rev. Light + Tilted Cage		

Week 4	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Confinement	Cage Replacement	Empty Bottle	Cage Switch	Cage Replacement		
10h - 11h		Inacc. Food	Overcrowding		White Noise		
11h - 12h	White Noise	Strobe Lights					
12h - 13h					Confinement		
13h - 14h		Tilted Cage	Confinement				
14h - 15h	Overcrowding		White Noise	Tilted Cage	Overcrowding		
15h - 16h							
16h - 17h							
17h - 18h							
O/N	Food Dep. + Wet Bed	Water Dep. + O/N Illum.	Strobe Lights (LF)	Wet Bed	Rev. Light		

Week 5	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Cage Switch	Cage Replacement	Empty Bottle	Overcrowding	Cage Replacement		
10h - 11h		Inacc. Food	Strobe Lights		Confinement		
11h - 12h		Tilted cage			White Noise		
12h - 13h							
13h - 14h	Strobe Lights		White Noise	Tilted Cage			
14h - 15h					Strobe Lights		
15h - 16h		Overcrowding		Cage Switch			
16h - 17h	Confinement		Confinement				
17h - 18h							
O/N	Food Dep. + Wet Bed	Water Dep. + O/N Illum.	Strobe lights (LF)	Wet Bed	Rev. Light + Tilted Cage		

Week 6	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Strobe Lights	Cage Replacement	Empty Bottle	Tilted Cage	Cage Replacement		
10h - 11h		Inacc. Food	Confinement		Confinement		
11h - 12h		Cage Switch		White Noise			
12h - 13h			Strobe Lights		Overcrowding		
13h - 14h	Confinement						
14h - 15h	Overcrowding	White Noise		Cage Switch			
15h - 16h			Overcrowding		Strobe Lights		
16h - 17h		Tilted Cage					
17h - 18h							
O/N	Food Dep. + Wet Bed	Water Dep. + O/N Illum.	Strobe lights (LF)	Wet Bed	Rev. Light + Tilted Cage		

The uCMS protocol used in the present study includes multiple stressors as confinement to a restricted space (1-2h, Figure 2.1.A), stroboscopic lighting (2-4h), overcrowding (3-4h, Figure 2.1.B), wet bedding for 8h, cage switch (3-4h), food or water deprivation overnight followed by exposure to inaccessible food/water for 1h, exposure to white noise (3-5h), placement in a tilted cage (30°, Figure 2.1.C), overnight illumination and reversed light/dark cycle for 48 h every 7 days. The uCMS animals were randomly exposed to 3-6 stressors every day for 6 weeks. In opposition, control rats were pair-housed, daily handled, and given access to water and standard chow diet *ad libitum*. Body weight was monitored weekly.

Figure 2.1. Photographs representing three of the stressors used in the uCMS protocol. (A) Photographic representation of confinement to a restricted space. Rats were confined to a plastic ventilated box, and without access to food and water. (B) Photographic representation of overcrowding. Rats were restrained to their homecage, with access to food and water. (C) Photographic representation of tilted cage. Cages were placed at the floor and tilted at a 30° angle.

2.3 Behavioral tests

After the conclusion of the uCMS protocol, behavior was monitored for anxious, anhedonic- and depressive-like behavior, as well as cognition. All behavioral studies were performed during the dark period of the light/dark cycle. The behavioral tests were conducted according to the following order: Elevated Plus Maze Test (EPM); Sucrose Preference Test (SPT); Novel Object Recognition (NOR); Forced Swimming Test (FST).

2.3.1 Elevated Plus Maze (EPM) Test

The Elevated Plus Maze Test was conducted to assess anxiety-like behavior. The EPM is based on the innate aversion that rodents have for open areas and preference for darker and close spaces. The behavioral apparatus (LE840; Panlab; Barcelona, Spain) consisted in two opposite open arms (45 cm × 10 cm) and two closed arms (45 cm × 10 cm × 35 cm) (Figure 2.2) elevated 65 cm above the floor and dimly illuminated by red light. Rats were individually placed in the center (neutral zone) of the maze and allowed to freely explore it during 5 min. Moreover, each trial was video recorded. The level of anxiety-like behavior was measured by the analysis of the time and number of entries in the open arms.

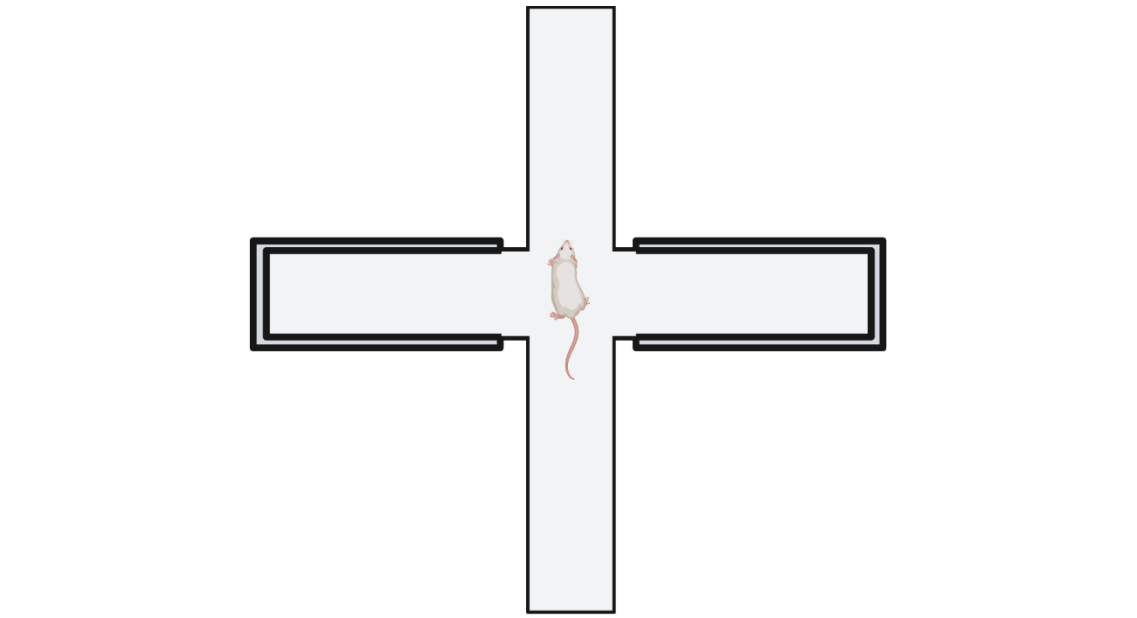

Figure 2.2. Schematic representation of the Elevated Plus Maze (EPM) Test. Anxiety-like behavior was evaluated on the EPM test, a four-armed maze that consists of two open arms (thin lines) and two closed arms (thick lines), elevated above the floor. Image created with BioRender.com

2.3.2 Sucrose Preference Test (SPT)

The Sucrose Preference Test was performed to evaluate anhedonia, a core component of depressive disorders, and characterized as the inability to feel pleasure and motivation. Baseline sucrose preference was measured before starting the uCMS protocol (three independent trials). Before each assay, animals were food and water deprived for 12h. For testing, two pre-weighed bottles containing water or a 2% (m/v) sucrose solution were presented to individually housed animals for 1h (Figure 2.3). After the end of uCMS protocol, animals were tested again for sucrose preference (SP) (one independent trial) and SP was calculated through the following formula: SP = (sucrose intake / (sucrose intake + water intake)) x 100, as previously described by Bekris et al., (2005). Anhedonia is defined as a reduction in SP relative to baseline levels.

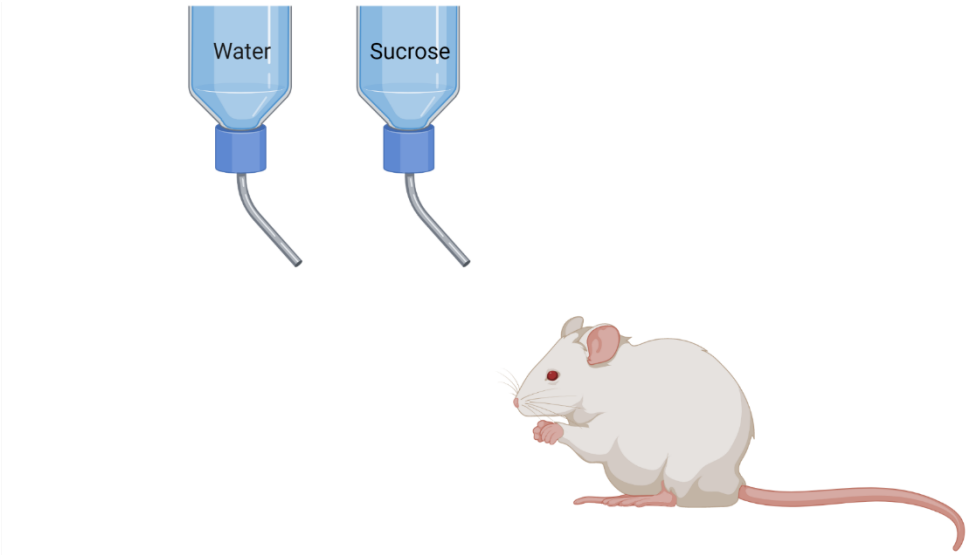

Figure 2.3. Schematic representation of Sucrose Preference Test (SPT). The SPT is a reward-based test used to measure anhedonia, a core symptom of depression. To evaluate anhedonia, two pre-weighed bottles containing water or a 2% (m/v) sucrose solution were presented to individually housed animals for 1h after the end of the uCMS protocol. Baseline sucrose preference value was assessed before the start of the uCMS protocol. Anhedonia was defined as a reduction in sucrose preference relative to baseline levels. Image created with BioRender.com

2.3.3 Novel Object Recognition (NOR)

The Novel Object Recognition test was used to evaluate cognition, particularly recognition memory. On the first day, rats were familiarized to the testing arena consisting of an acrylic box (60 (W) x 70 (D) x 40 (H) cm), for 10 min and with no object presentation. On the second day, animals were allowed to freely explore two identical

objects for 10 min and 1h later they returned to the arena for 3 min, with one the objects being replaced by a novel one. The familiar and novel objects were similar on size, but with different shape, texture and color (Figure 2.4). The NOR arena was cleaned with 10% ethanol between each trial to avoid odor cues. Each assay was video recorded and the time spent exploring each object was measured. The percentage of time spent exploring the novel object / (time exploring the familiar object + time exploring the novel object) was calculated as a measure of recognition memory (recognition index). The exploration of an object was considered when the animal 's nose is pointed towards and no more than 2 cm away from the object.

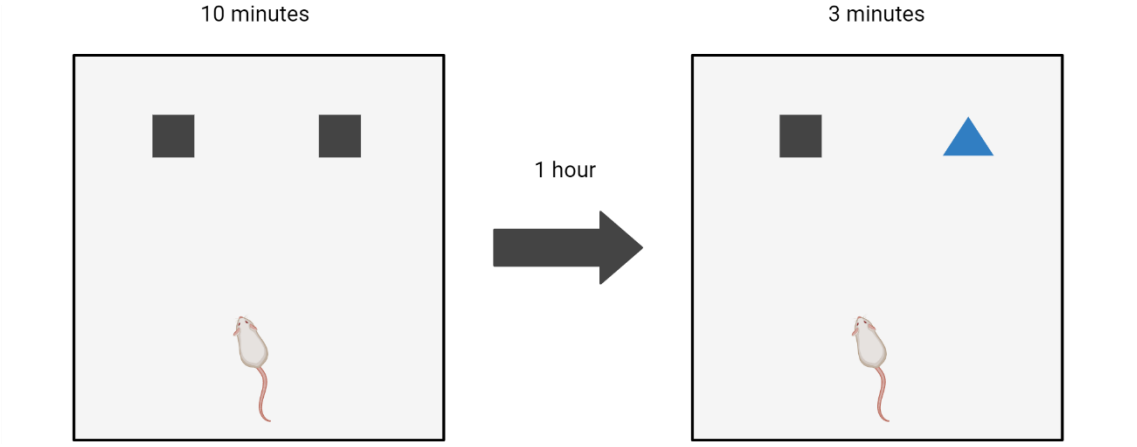

Figure 2.4. Schematic representation of Novel Object Recognition (NOR). The NOR Test was performed to evaluate cognition, particularly recognition memory. The image on the left illustrates the familiarization phase (with two copies of the same object) and the image on the right shows the test phase (with a familiar object and with a novel object). Image created with BioRender.com

2.3.4 Forced Swimming Test (FST)

The FST was used to specifically evaluate depressive-like behavior. This test was conducted on two different days. On the first day, rats were individually placed in transparent cylinders (62 cm height and 25.4 cm diameter) filled with water (25°C; 50 cm depth) for a 5 min pre-test session. On the following day, animals were subjected to the 5 min test session (Figure 2.5). Experiments were video recorded and the total time animals spend immobile (immobility time) was measured. Immobility was defined as an absence of movement or a floating state, without any activity other than that needed to keep the head above water. Behavioral despair was defined as an increase in the immobility time.

Figure 2.5. Schematic representation of Forced Swimming Test (FST). Immobility time, which is a marker of a passive coping strategy (one of the components of depressive-like behavior), was evaluated by FST. Rats were placed in a transparent cylinder filled with water and their behavior was determined. Image created with BioRender.com

2.4 Western Blot Analysis

Rats were anaesthetized with intraperitoneal injection of ketamine (50 mg/kg)/xylazine (10 mg/kg) and transcardially perfused with 0.01 M phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4). After brain removal, PFC and HIP were dissected on ice and stored at -80°C. Total protein of each brain region was obtained after homogenizing and lysing the tissue using Radio-Immunoprecipitation Assay (RIPA) lysis buffer [(150 mM NaCl; 50 mM Tris; 5 mM ethylene glycol tetraacetic acid (EGTA) pH 7.5; 1% Triton X-100; 0.5% deoxycholate (DOC); 0.1% sodium dodecyl sulfate (SDS)], supplemented with a protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany), phosphatase inhibitor (PhosSTOPTM, Roche Applied Science) and 1 mM dithiothreitol (DTT) (Bioron, Porto, Portugal). Thereafter, the lysates were sonicated and centrifuged at 16100 ×*g* for 15 min at 4°C. The supernatant was collected and stored at -80°C until protein quantification.

Protein concentration in each sample was determined by bicinchoninic acid (BCA) method (Pierce, Rockford, USA) with bovine serum albumin (BSA) as standard and stored at -20°C until further use. Protein samples were prepared by adding sample buffer (0.5 M Tris-HCl pH 6.8; 30% glycerol; 10% SDS; 0.6 M DTT; 0.01% bromophenol blue), and then heated at 95°C for 5 min. After samples denaturation, proteins of interest (Table 11) were separated by electrophoresis on 8%, 10% or 12% SDS-polyacrylamide

gel (depending on the target protein) at 110V for 5 min and at 150V until the end of the run. Proteins were then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Madrid Spain) in Tris-glycine buffer at (0.25A for 1h and 0.3A for 30 min), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) at (110V for 1h30) or CAPS + SDS 0.1% buffer at (110V for 1h30) (according to the target protein). Membranes were blocked with 5% (w/v) nonfat milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris; pH 7.6;) containing 0.1% (v/v) Tween-20 (TBS-T) for 1h at room temperature (RT). Afterwards, the membranes were incubated overnight at 4°C and 30 min at RT in the following day, with the primary antibodies (Table III) diluted in TBS-T containing nonfat milk. Membranes were washed 3 times in TBS-T and then incubated 1h at RT with respective alkaline phosphatase-conjugated secondary antibody (Table IV) diluted in TBS-T. Then, membranes were washed again 3 times in TBS-T and proteins were visualized using enhanced chemofluorescent (ECF) reagent (GE Healthcare) on Typhoon FLA 9000 (GE Healthcare Bioscience AB, Uppsala, Sweden), or with enhanced chemiluminescence (ECL) method kit (GE Healthcare) on ImageQuant LAS 500 (GE Healthcare Bioscience AB, Uppsala, Sweden). Immunoblots were re-probed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to ensure equal sample loading. Analysis and quantification of band density was performed using Image Studio 5.2 software (LI-COR Biosciences, NE, USA) and results were expressed in percentage of control.

Table II. List of proteins identified by western blot analysis.

Protein	Molecular Weight (kDa)	Amount of protein (µg)
C3aR	65	25
Claudin-5	18-23	50
Occludin	65-82	25
VE-Cadherin	130	50
VCAM-1	110	25
Albumin	67	50
GFAP	55	25
GAPDH	37	-

Table III. List of primary antibodies used in western blot analysis.

Primary Antibody	Dilution	Reference	Company
Mouse anti-C3aR	1:500	sc-133172	Santa Cruz Biotechnology
Mouse anti-Claudin 5	1:250	35-2500	Invitrogen
Rabbit anti-Occludin	1:200	71-1500	Invitrogen
Mouse anti-VE-Cadherin	1:200	sc-9989	Santa Cruz Biotechnology
Rabbit anti-VCAM-1	1:200	sc-1504	Santa Cruz Biotechnology
Goat anti-Albumin	1:20000	A90-134	Bethyl Laboratories, Montogomery, TX, USA
Rabbit anti-GFAP	1:5000	G9269	Sigma-Aldrich, St. Louis, MO, USA
Mouse anti-GAPDH	1:5000	MA5-15738	Thermo Fisher Scientific, Waltham, MA, USA

Table IV. List of secondary antibodies used in western blot analysis.

Secondary Antibody	Dilution	Company
Anti-mouse IgG+IgM alkaline phosphatase conjugated	1:5000	GE Healthcare
Anti-rabbit IgG alkaline phosphatase conjugated	1:10000	GE Healthcare
Anti-goat IgG alkaline phosphatase conjugated	1:10000	Invitrogen
Anti-rabbit IgG horseradish peroxidase conjugated	1:10000	GE Healthcare

2.5 Immunohistochemistry

For immunohistochemistry analysis, rats were deeply anesthetized intraperitoneal injection of ketamine (50 mg/kg)/xylazine (10 mg/kg), and transcardially perfused with 0.01 M PBS, pH 7.4. Then, brains were removed, fixed in 4% paraformaldehyde (PFA) for 24h at RT and transferred to 30% sucrose in 0.01 M PBS, pH 7.4, for 24 h at 4°C. After fixation, brains were cryopreserved at −80°C until further processing.

Coronal sections (50 μm) were cut on a cryostat (Leica CM3050S, Nussloch, Germany), placed in a 24-well plate with a crioprotectant solution (0.1 M phosphate buffer, 30% sucrose and 30% ethylene glycol) and stored at 4°C until further use. Afterwards, sections were washed 3 times with 0.01 M PBS and then, blocked with 5% BSA (Sigma-Aldrich) and 0.25% Triton X-100 in PBS (2h at RT). Thereafter, sections were incubated with the antibody for GFAP (mouse anti-GFAP conjugated toCy3 reactive dye; 1:1000, Sigma-Aldrich) and with 4′,6-diamidino-2-phenylindole (DAPI) (1:5000, Invitrogen), for nuclei staining, 2h at RT. Sections were washed 3 times with 0.01 M PBS and mounted on gelatinized slides, using glycergel (DAKO mounting medium). Images were recorded using a LSM 710 Meta Confocal microscope (Carl Zeiss, Oberkochen, Germany). GFAP immunoreactivity analysis was performed using Fiji (Image J software).

2.6 Quantification of corticosterone levels by ELISA Assay

Corticosterone levels in blood serum were measured using an ELISA kit (ab108821, Abcam, Cambridge, UK), according to the manufacturer's instructions. Blood samples were collected through puncture of the tail vein, between the diurnal nadir (8:00 a.m. - 9:00 a.m.) and diurnal zenith (8:00 p.m. - 9:00 p.m.). Blood samples and standard concentrations of corticosterone were added to a 96-well plate and incubated with biotinylated corticosterone during 2h (RT). Then, the plate wells were washed 5 times with wash solution, followed by incubation for 30 min at RT with the streptavidin-peroxidase conjugate. After another 5 washes, the chromogen substrate was added and incubated for 20 min or until obtain the optimal blue color density. Finally, the reaction

was stopped with stop solution, changing the color from blue to yellow and the plate was read in a microplate reader (Biotek Synergy HT) at 450 nm, with correction reading for 570 nm. Corticosterone concentration (ng/mL) was extrapolated from a standard curve.

2.7 Quantification of complement C3 levels by ELISA Assay

Complement C3 levels were measured in the PFC, HIP and blood serum of animal models using an enzyme-linked immunosorbent assay (ELISA) kit (ab157731, Abcam, Cambridge, UK), according to the manufacturer's instructions. Briefly, rat PFC and HIP samples were collected immediately following decapitation under anesthesia and lysed using an ELISA specific lysis buffer (pH 8.0; 150 mM NaCl; 10 mM Tris-HCl; 10% Triton X-100; 1mM ethylenediaminetetraacetic acid (EDTA), complemented with a protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany) and a phosphatase inhibitor (PhosSTOPTM, Roche Applied Science). For blood serum analysis, blood samples were collected directly from cardiac left ventricle from all animals. Before using, the lysates were centrifugated and the supernatants were quantified by BCA assay. Both samples and standard were added to a 96-well plate and incubated at RT for 20 min. Then, the plate wells were washed 4 times with wash solution and incubated at RT with horseradish peroxidase (HRP) during 20 min, in the dark. After another 4 washes, chromogen substrate solution was added and incubated at RT, in the dark. Ten minutes later, a stop solution was added, and the plate was immediately read at 450nm in a microplate reader (Biotek Synergy HT). A standard curve was used to calculate the C3 protein levels, expressed as ng/mL.

2.8 Estrous cycle analysis

Smears were obtained through vaginal cytology collected at the end of EPM test. Three cell types (nucleated epithelial cells, cornified epithelial cells and leukocytes) were noted to define the reproductive cycle (estrous), which is defined by the prevalence of each cell type: proestrus (nucleated), estrus (cornified), metestrus (all types in the same proportion) and diestrus (leukocytes) (Byers et al., 2012; Caligioni, 2009; Westwood,

2008). Images were acquired with a light microscope Leica DM 4000B (Leica, Wetzlar, Germany) with a 10x objective lens (Plan 10x/0.25PH1).

2.9 Statistical analysis

GraphPad Prism 8 (GraphPad Software, San Diego, CA) was used to perform statistical analysis. Outliers were identified using GraphPad Prism 8. Two-way Analysis of Variance (ANOVA) followed by a Bonferroni post hoc test was used to evaluate the effect of uCMS (Control *vs* uCMS) and the effect of sex (Male *vs* Female). Moreover, t-test was used to evaluate the effect of uCMS (Control vs uCMS). Results are presented as mean ± standard error of the mean (mean ± SEM). Statistical significance was indicated when $p<0.05$.

Chapter III

Results

3. Results

3.1 Effect of uCMS on the circadian corticosterone levels in both sexes

Previous studies have reported that chronic stress is associated with the disruption of the HPA axis, leading to a hypersecretion of corticosteroids (Bao & Swaab, 2019; Willner et al., 2013).

In the present study, a validated uCMS protocol (Alves et al., 2017; Gaspar et al., 2021; Mateus-Pinheiro et al., 2013) was applied during 6 weeks. After the end of the uCMS protocol, corticosterone levels were measured in the serum. No significant alterations were observed in the corticosterone levels of both males (Figure 3.1 A) and females exposed to uCMS (Figure 3.1 B), which suggest that chronic stress does not contribute to an imbalance in the HPA axis activity.

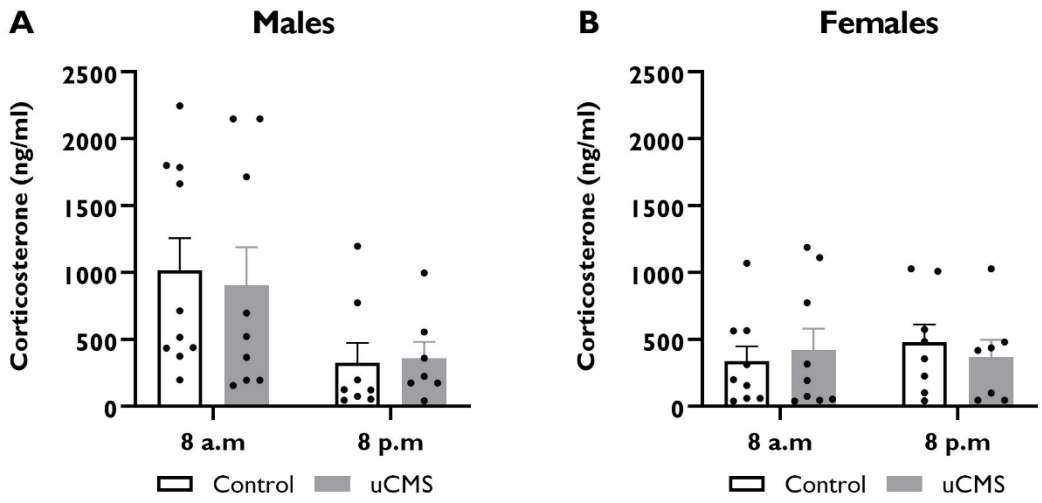

Figure 3.1. uCMS does not promote a dysregulation of the circadian corticosterone levels in both sexes. Corticosterone serum levels were measured at 8:00 a.m. and 8:00 p.m. by ELISA in male (A) and female (B) rats. Results are presented as the mean ± SEM of 7-10 animals, calculated using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

3.2 uCMS impairs body weight gain in males, but not in females

Body weight was determined weekly until the end of the uCMS protocol. Male rats exposed to uCMS presented an impaired weight gain (**$p < 0.01$, ***$p < 0.001$) comparing with control males particularly after 2 and 4 weeks of the uCMS procedure (Figure 3.2 A). By contrast, the uCMS protocol did not significantly affect body weight

gain in female rats (Figure 3.2 B). These data suggest that chronic stress impact on weight gain is sex dependent.

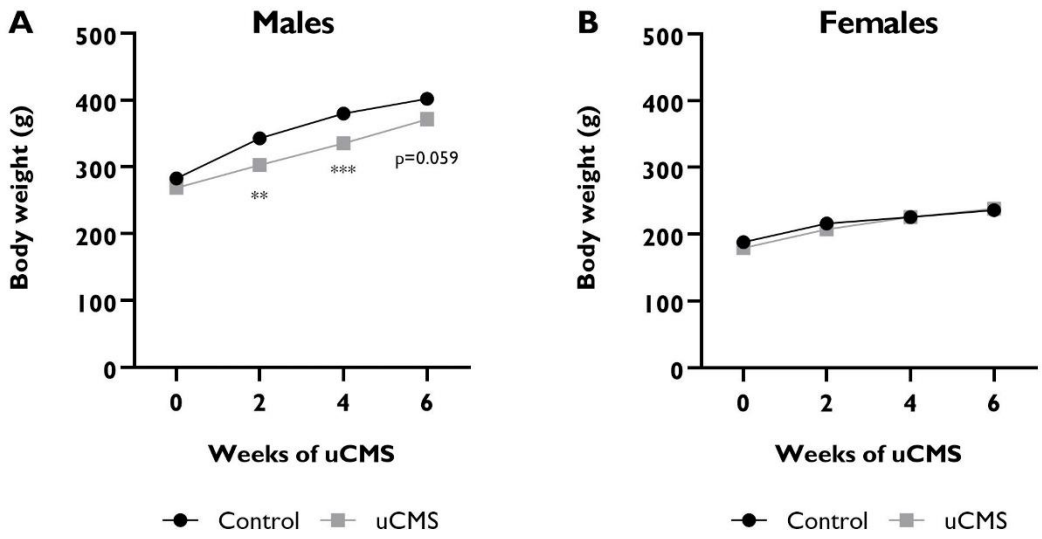

Figure 3.2. uCMS impairs body weight gain in males, but not in females. Body weight of male (A) and female (B) rats after the 6 weeks of uCMS protocol. Results are presented as the mean ± SEM of 9–10 animals. **p < 0.01, ***p < 0.001, comparing with control males using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

3.3 Behavioral alterations induced by uCMS

In order to evaluate behavior domains typically affected by chronic stress, namely anxious, anhedonic- and depressive-like behavior, as well as cognition, behavioral tests were conducted after the end of the uCMS protocol.

Anxiety-like behavior was assessed by EPM test. No significant differences were found in the time and number of entries in the open arms, in both males and females exposed to uCMS, comparing with respective controls (Figure 3.3 A, B).

In the SPT, that evaluates anhedonia, no differences were found in sucrose consumption of both males and females after the uCMS comparing with control animals (Figure 3.3 C).

To measure recognition memory both in males and females, NOR test was performed. The analysis of the recognition index, as an index of memory retention, showed that chronic stress did not impair recognition memory in both males and females (Figure 3.3 D).

In the FST, behavioral despair was calculated as time of immobility. uCMS induced a depressive-like behavior in male rats (**p < 0.01), as demonstrated by the increased

immobility time in the FST, compared with control animals. In females, no significant differences were observed between groups in immobility time. Moreover, sex-specific differences in the duration of immobility time were noted. Comparing only control groups, males present a significantly lower immobility time than females (##$p < 0.01$), suggesting a sex-specific performance of the animals in this test.

The estrous cycle analysis was performed in females to evaluate if the stage of the cycle could interfere with the behavioral results. According to these findings, females were distributed by all phases of the estrous cycle, suggesting that the phase of the estrous cycles did not influence the behavioral outcomes. (Table V).

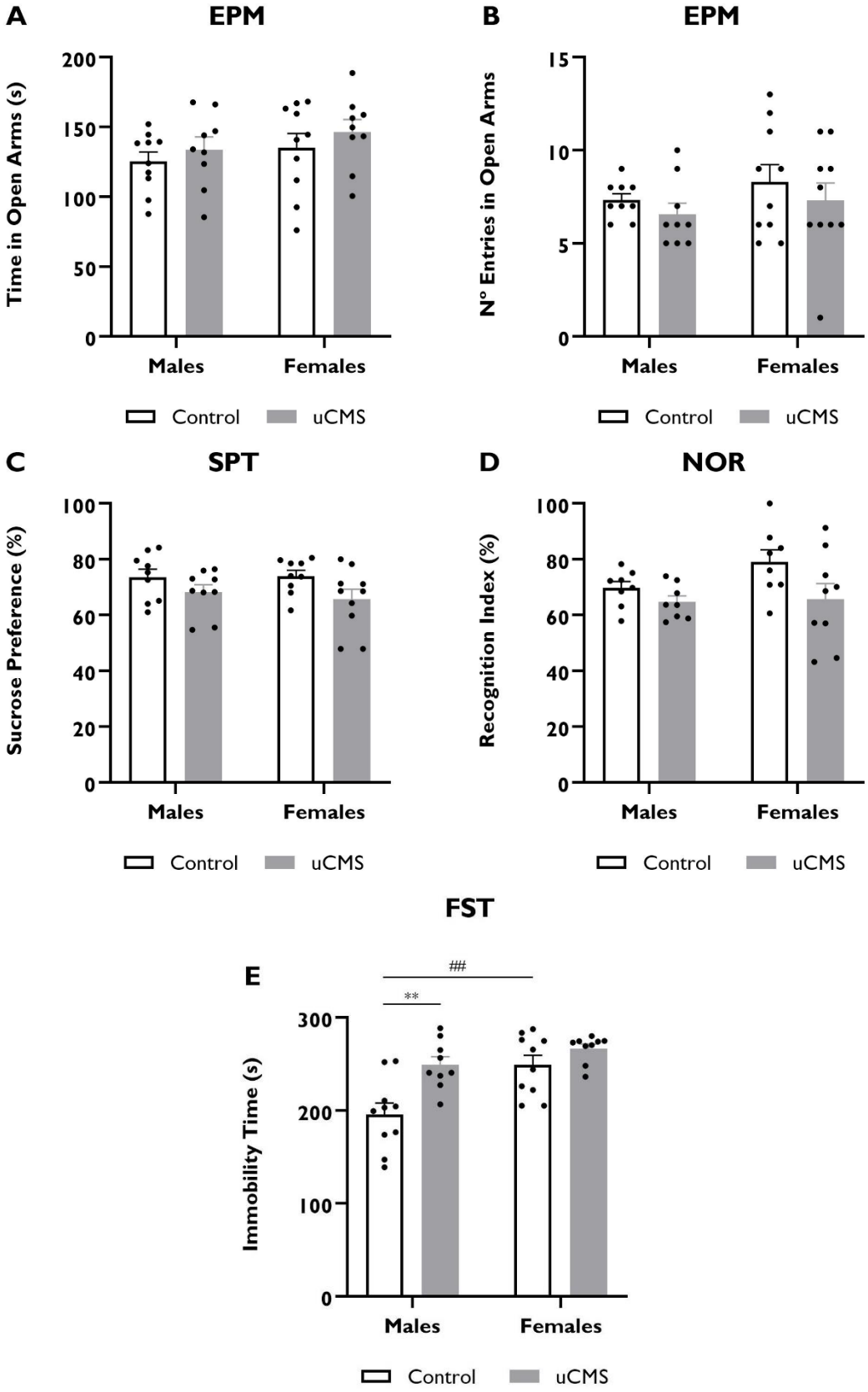

Figure 3.3. uCMS induces depressive-like behavior in males. (A) Time spent and (B) number of entries in open arms of the EPM test, used to evaluate anxiety-like behavior in males and females. (C) Anhedonic-like behavior in males and females was measured by the preference for sucrose solution in the SPT. (D) The recognition index was calculated using the NOR test, to evaluate recognition memory deficits in males and females. (E) Depressive-like behavior was assessed by the total time of immobility in

the FST in males and females. Results are presented as the mean ± SEM of 8-10 animals. **p < 0.01: control males compared with uCMS males; ##p < 0.01: control males compared with control females, calculated using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

Table V. Female rats estrous cycle determined at the end of EPM test.

	Proestrus	**Estrus**	**Metestrus**	**Diestrus**
Control	5	1	3	1
uCMS	1	2	4	3

3.4 Analysis of C3 and C3aR levels in the prefrontal cortex, hippocampus, and serum

Abnormal activation of the complement system has been related to multiple consequences in CNS disorders, including neuroinflammation (Carpanini et al., 2019). Therefore, C3 protein levels were determined in the PFC, HIP, and serum by ELISA, since C3 is positioned at the intersection point of all complement pathways. No significant differences in the C3 protein levels were detected in the PFC (Figure 3.4 A), HIP (Figure 3.4 B) and serum (Figure 3.4 C) following uCMS exposure of both males and females.

The cleavage of C3 into C3a and C3b is a critical step in all three complement pathways. Moreover, C3a is considered a pro-inflammatory mediator that acts through its receptor, C3aR, which has been implicated in leukocyte activation, vascular permeability, and chemotaxis (Mastellos et al., 2016; Y. Wang et al., 2019). To further clarify the role of C3/C3aR axis, the protein levels of C3aR in the PFC and in the HIP were assessed by western blot analysis. In the PFC, no significant differences were detected in both sexes, although a tendency to a decrease of C3aR protein levels was identified in males exposed to uCMS, compared with controls ($p = 0.09$) (Figure 3.4 D). Importantly, more experiments are needed to confirm this tendency. In the HIP, no significant alterations were observed in the C3aR protein levels in both sexes after the uCMS protocol (Figure 3.4 E). Overall, these findings suggest that chronic stress does not lead to alterations of C3 and C3aR protein levels.

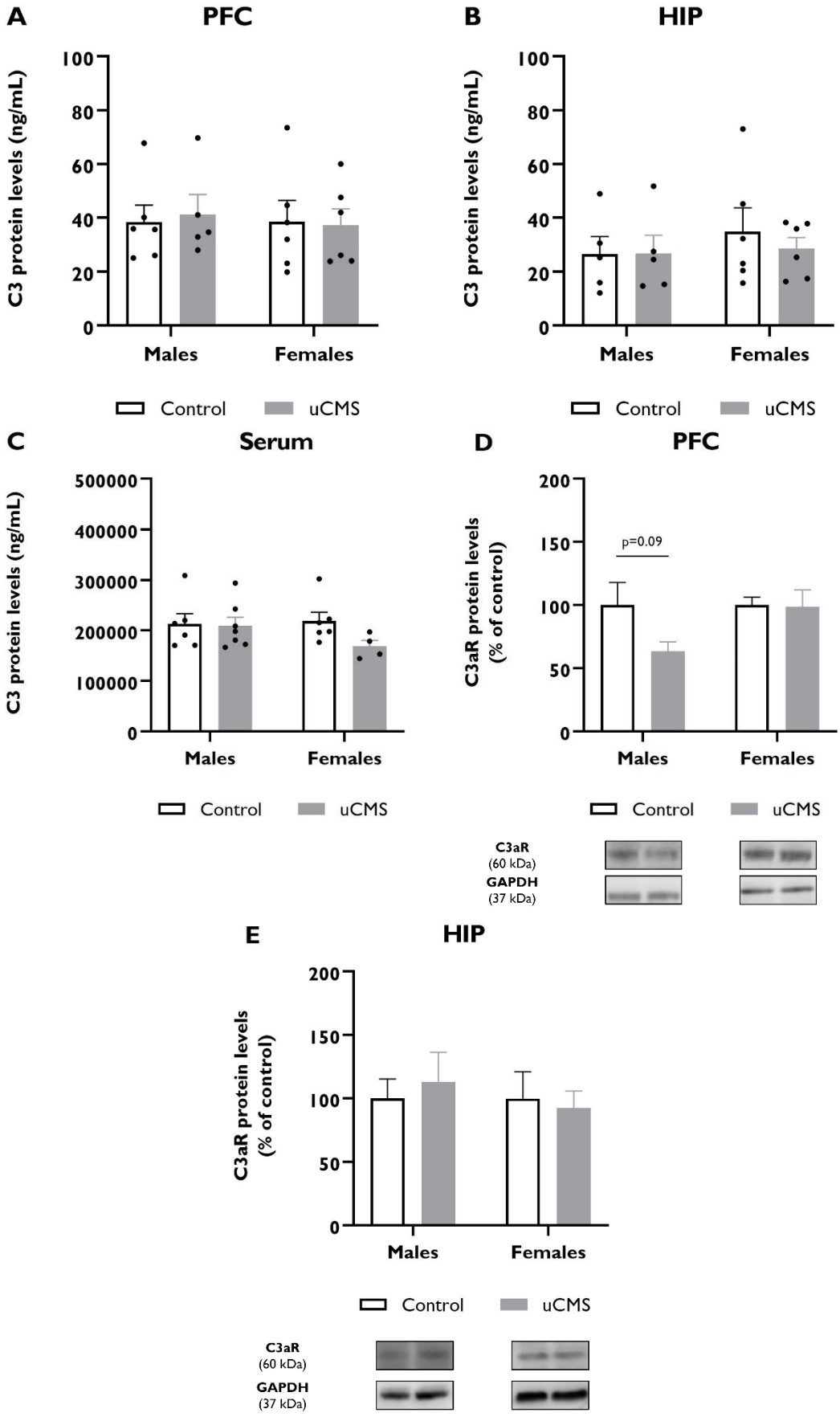

Figure 3.4. uCMS does not alter C3 and C3aR protein levels in the prefrontal cortex, hippocampus, and serum. Complement C3 levels were measured by ELISA in the prefrontal cortex

(A), hippocampus (B) and serum (C) in male and female rats. C3aR protein levels were evaluated by western blot analysis in the prefrontal cortex (D) and hippocampus (E) of male and female rats. Results are presented as the mean ± SEM of 4-7 animals, calculated using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

3.5 The impact of uCMS on BBB properties

The BBB is considered the gate-keeper of the CNS, regulating brain homeostasis and protecting the CNS against peripheral insults (Cardoso et al., 2010; Welcome & Mastorakis, 2020). To evaluate possible alterations in BBB integrity after chronic stress exposure, albumin protein levels in the PFC were evaluated by western blot analysis. Albumin is a blood serum protein with high molecular weight that under normal conditions does not cross BBB. According to the results obtained, uCMS did not promote the leakage of albumin into the PFC (Figure 3.5 A).

As previously mentioned, the identification of albumin in the brain parenchyma involves significant alterations in BBB permeability. Thus, although no significant changes were visualized in the albumin protein levels in the PFC after uCMS, other subtill alterations may occur. To better clarify possible BBB alterations, intercellular junctions that regulate the paracellular transport between ECs, namely TJ (claudin-5 and occludin) and AJ (VE-cadherin) proteins, were evaluated by western blot analysis in the PFC. No significant alterations were observed in the claudin-5 protein levels between control and uCMS groups in male rats. In contrast, a significant upregulation in the claudin-5 protein levels were observed in females exposed to uCMS (&p < 0.05) (Figure 3.5 B). Regarding occludin and VE-cadherin protein levels, no significant alterations were detected in males and females exposed to uCMS (Figure 3.5 C and D, respectively).

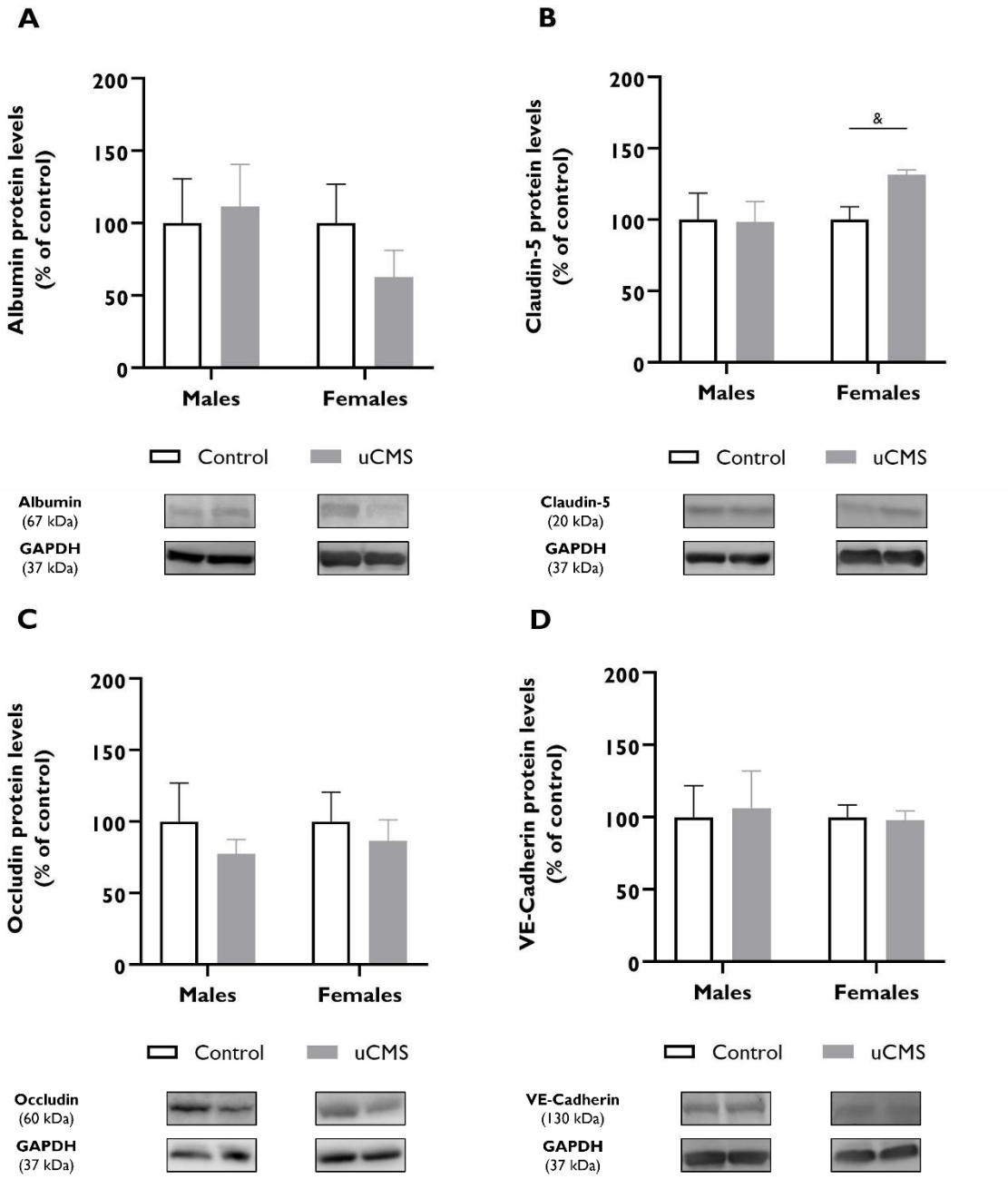

Figure 3.5. uCMS leads to an increase in the claudin-5 protein levels of females exposed to uCMS. Protein levels of albumin (A), claudin-5 (B), occludin (C) and VE-cadherin (D) were measured by western blot analysis in the prefrontal cortex in male and female rats. Results are presented as the mean ± SEM of 3-6 animals. &$p < 0.05$, control females compared with uCMS females, calculated using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

3.6 Effect of uCMS in brain leukocyte recruitment

The BBB is a dynamic structure that restrict most of the circulating cells from entering the brain (Russo & McGavern, 2015). However, under pathological conditions leukocyte extravasation can occur into CNS, a process that is mediated by adhesion

molecules (Cayrol et al., 2008). Therefore, protein levels of VCAM-1 in the PFC were measured by western blot analysis. Herein, no significant differences were visualized in VCAM-1 protein levels, suggesting that chronic stress exposure does not lead to leukocyte recruitment into to the PFC of male and female rats (Figure 3.6).

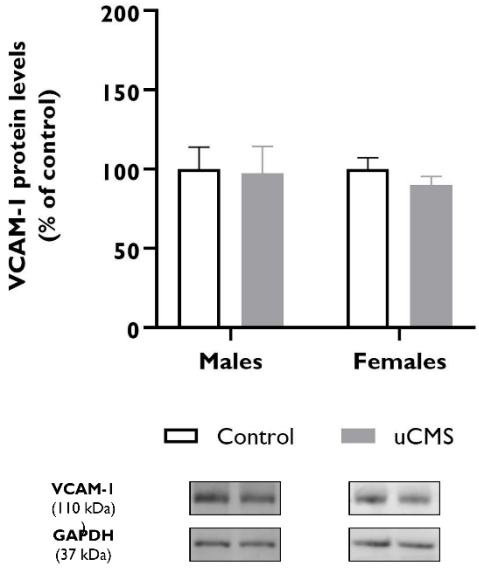

Figure 3.6. uCMS does not promote leukocyte recruitment into the brain. Protein levels of VCAM-1 were measured by western blot analysis in the prefrontal cortex in male and female rats. Results are presented as the mean ± SEM of 5-6 animals, calculated using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

3.7 Astrocytic response triggered by uCMS

To examine possible astrocytic reactivity induced by uCMS, coronal sections of the PFC were stained with GFAP and the respective immunoreactivity was analyzed (Figure 3.7 A). No significant alterations were found between control and uCMS male groups (Figure 3.7 B). Importantly, protein levels of GFAP in males and females were also evaluated by western blot analysis, and no significant differences were observed (Figure 3.7 C). These data suggest that uCMS does not trigger a significant response of astrocytes, at least in the PFC.

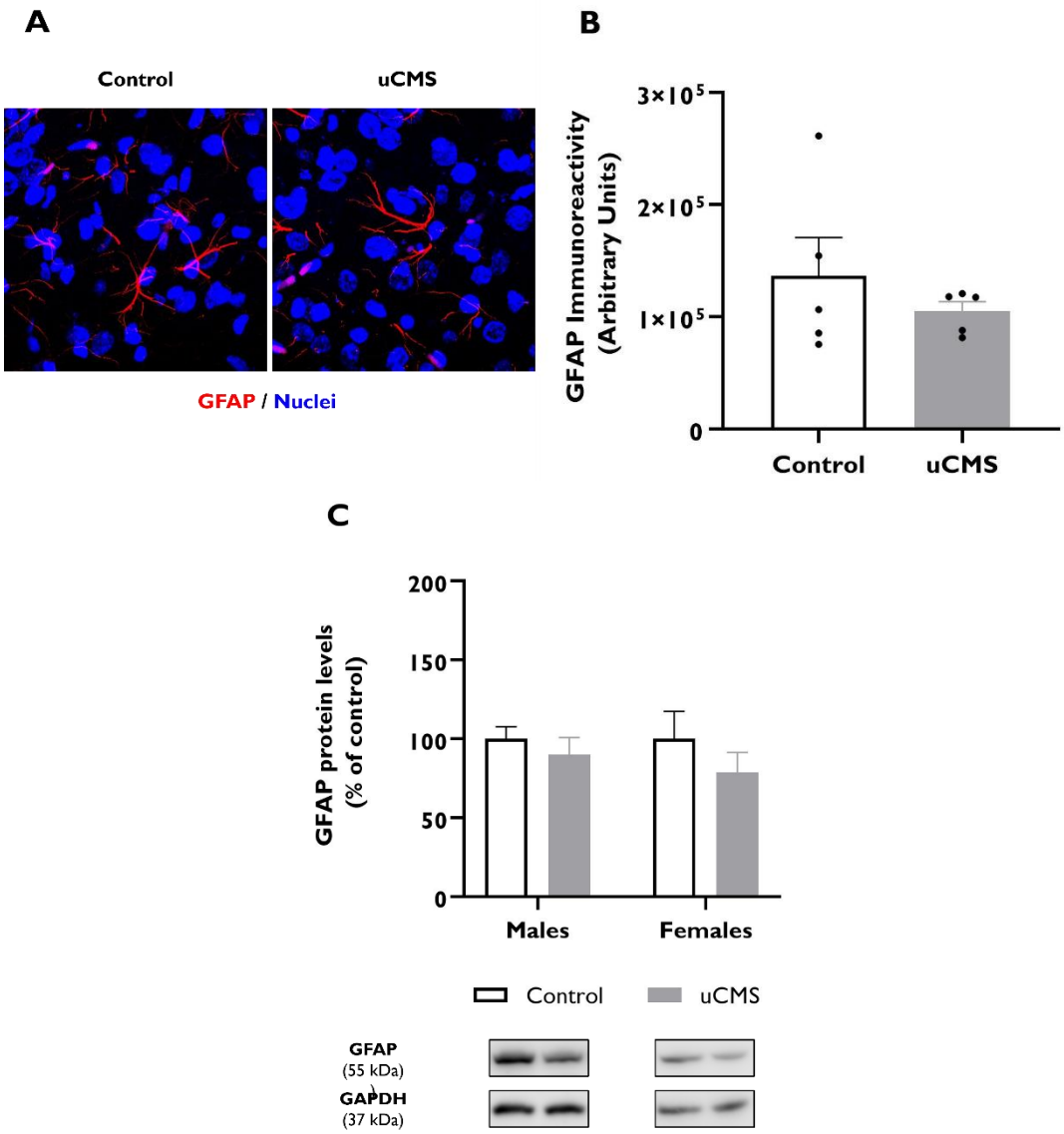

Figure 3.7. uCMS does not alter astrocytic response. (A) Representative images and (B) quantification of GFAP (red) showing that uCMS does not change GFAP immunoreactivity in male rats. Cell nuclei were also stained with DAPI (blue). Results are presented as the mean ± SEM, n=1 animal per condition, calculated using a t-test. Scale bar = 20 µm. (C) Protein levels of GFAP were measured by western blot analysis in the prefrontal cortex of male and female rats. Results are presented as the mean ± SEM of 5-6 animals, calculated using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

Chapter IV

Discussion

4. Discussion

Chronic stress is a contributing factor for several mood disorders, including depression, anxiety and post-traumatic stress disorder (Westfall et al., 2021), that are often associated with changes in behavioral and cellular domains (Franklin et al., 2012; Pittenger & Duman, 2008). Additionally, sex differences in the prevalence of these neuropsychiatric disorders are well established (Brivio et al., 2020; Seney & Sibille, 2014). Interestingly, recent data have suggested that stress is linked with cerebrovascular diseases (Everson-Rose et al., 2014). However, very little attention has been given to the effect of chronic stress on BBB structure and function. In fact, only a few studies examined the impact of stress on brain vasculature, and the main attention has been focused on the role of oxidative stress in vascular hemodynamics and angiogenesis (Burrage et al., 2018). Moreover, the complement pathway emerges as a new player underlying BBB alterations, since when dysregulated it may lead to the loss of BBB integrity and subsequently to CNS disorders (Alexander, 2018). Therefore, the present study aimed to explore the effect of chronic stress in BBB integrity and C3 complement pathway in both sexes, and to clarify if neurovascular alterations underlie stress-induced behavioral deficits.

To achieve these goals, a validated uCMS protocol (Alves et al., 2017; Gaspar et al., 2021; Mateus-Pinheiro et al., 2013) was applied for 6 weeks to male and female rats. The uCMS is one of the most widely used models to evaluate stress-induced alterations in behavior, however it is important to consider that it has been mostly described in male rodents (Frisbee et al., 2015). Additionally, it is demanding in time and space (Lopez & Bagot, 2021) and has been criticized for a lack of reliability (Antoniuk et al., 2019; Willner, 2017). For instance, some studies rarely detail the precise stressor schedule and timing, like if stressors are applied once or twice daily through a (variable) period of weeks, and also not detailing how stressors are used (e.g., white noise without specifying dB or duration) or how variably stressors are applied (e.g., cage tilt for 1–24 hours) (Lopez & Bagot, 2021). Additionally, sex differences in the susceptibility and resilience to stress depend on multiple characteristics of the stressor, including type, timing and severity (Hodes & Epperson, 2019). Despite some fragilities identified in published data, the uCMS is still considered a robust animal model to study the effect of stress exposure in behavioral outcomes.

Chronic stress is associated with impairments in HPA axis activity, which may lead to significant alterations in the production of corticosteroids (Pariante & Lightman, 2008). To analyze whether our stress protocol could lead to HPA axis hyperactivity, circulating corticosterone levels were measured after 6 weeks of uCMS exposure. No significant differences were observed in serum corticosterone levels in both sexes after uCMS, similarly to previous results obtained by others (Gaspar et al., 2022; Marco et al., 2017). On other hand, it has been described that uCMS exposure for 2 weeks induces an increase in serum corticosterone levels in both sexes, resulting in a disruption of the circadian corticosterone secretion levels (Gaspar et al., 2021). Moreover, it seems that males present increased basal corticosterone levels when compared to females, which contrasts to what has been described in rodents that report higher basal corticosterone levels in females than males (Oyola & Handa, 2017). Nonetheless, this depends on gonadal hormones, strain, age, time of sampling, housing conditions and diet (Kokras et al., 2019).

Previous studies have shown a decrease in body weight gain following exposure to uCMS protocol, particularly in males (Gaspar et al., 2022; Konkle et al., 2003; Xing et al., 2013). Consistent with these data, the present results showed that uCMS reduced body weight in males but not in females, suggesting that males may be more susceptible to the metabolic effects of chronic stress. In opposition, Kott et al., (2016) used an different stress protocol, which consisted in the chronic administration of corticosterone for 23 days, resulting in a decrease of body weight in females. Thus, these contrasting results in females may be explained by the type of stress paradigm used. Nevertheless, the intensity of the stressor can also influence these data.

In our study, a uCMS protocol was used in order to evaluate possible behavioral alterations induced by chronic stress in male and female rats. The present data revealed that uCMS did not induce anxiety-like behavior in both males and females, which is not in accordance with other studies that demonstrated that animals exposed to chronic stress spent less time in the open arms of the EPM test (Gaspar et al., 2022; Wang et al., 2018; Zhu et al., 2014). Moreover, 6 weeks of uCMS did not affect sucrose consumption of the animals of both sexes subjected to uCMS in the SPT, as previously described by Marco et al., (2017). On the contrary, other studies reported that 7 weeks (Bekris et al., 2005) or 6 weeks (Gaspar et al., 2022; Patrício et al., 2015) of uCMS lead to anhedonia in male rats, a core symptom of depression, while in females a decrease in

sucrose preference (Lu et al., 2015) or no alterations (Gaspar et al., 2022) have been described. These discrepancies might result from differences in sucrose concentration, number of hours of food and water deprivation and the day/night period of testing. For instance, we performed the behavioral tests during the night (high activity period) whereas others (Bekris et al., 2005; Gaspar et al., 2022) evaluated behavioral changes during the day (low activity period). Furthermore, stress-induced changes in sucrose preference are different between sexes as a result of intrinsic differences in taste and/or ingestion response (Curtis et al., 2004) or in reactivity to reward (Michaels & Holtzman, 2007). Regarding depressive-like behavior, uCMS exposure significantly increased immobility time of males in the FST, whereas in females no alterations were found as reported by other studies (Bielajew et al., 2003; Gaspar et al., 2022). These results are particularly intriguing because in humans, depression is twice more prevalent in women than men (Kokras & Dalla, 2017). Moreover, a significant increase in immobility time was also observed in control females compared to control males, which suggest that under physiological conditions there are also behavioral sex differences. Fluctuations in gonadal hormones have been shown to regulate the way how males and females cope with stress (Oyola & Handa, 2017), although the present data does not suggest an influence of the estrous cycle on the behavior, since female were distributed through all phases of the cycle. Nevertheless, it should be mentioned that the outcomes of uCMS exposure could be influenced by multiple variables under experimental conditions, including the type and severity of the stressor, exposure parameters, as well as the strain/species used (Frisbee et al., 2015; Hill et al., 2012). In terms of recognition memory, no significant differences were observed in both males and females upon chronic stress exposure in the NOR test, which is not in line with other studies showing that male animals exposed to uCMS present cognitive deficits (Alves et al., 2017; Mohamed et al., 2020). Curiously, to date there are no available data exploring cognitive effects on females exposed to uCMS.

The complement cascade represents one of the major effector mechanisms of the innate immune system (Dunkelberger & Song, 2010) and more recently, it has been proposed that it could be an important mediator of inflammation-related changes in cellular and behavioral processes in neuropsychiatric disorders (Feng et al., 2020). Although the number of studies exploring the link between complement and stress-related disorders are scarce, recent evidence has indicated that C3 expression was significantly upregulated in the PFC of stress-related mice model and of postmortem

depressed suicide subjects (Crider et al., 2018). The present work did not reveal any significant changes in C3 protein levels in the PFC, HIP, and serum of both males and females rats after uCMS exposure. Furthermore, no differences were found in the C3aR protein levels of males and females rats exposed to uCMS in the PFC and HIP, however a tendency to a decrease in the protein levels of C3aR in the PFC of males exposed to uCMS was observed. In opposite to our observations, a study by Crider and collaborators (2018) showed increased levels of C3aR in the PFC of male mice which contributed to depressive-like behavior, as demonstrated by an increase in immobility time in tail suspension and forced swimming tests. Indeed, the duration of uCMS, type of stressors used and the stress-related animal model could explain the differences observed in the C3aR protein levels between the present work and the study published by (Crider et al., 2018). Also, knocking out C3 in mice prevented the development of depressive-like phenotype following chronic stress (Crider et al., 2018; Tripathi et al., 2021). Thus, future studies are necessary to elucidate if there is a link between depressive-like behavior and an upregulation of C3/C3aR protein levels after chronic stress.

The BBB is a dynamic structure required to maintain a stable brain microenvironment, restricting the transport of toxic substances from the periphery into the brain parenchyma (Campos-Bedolla et al., 2014; Sá-Pereira et al., 2012). In fact, dysfunction of brain vasculature has been often associated with neurodegenerative and neuropsychiatric disorders (Kealy et al., 2020; Najjar et al., 2013), although only a few studies have provided novel insights about the effect of chronic stress in the BBB. Here, it was demonstrated that uCMS does not contribute to significant alterations in BBB structure in the PFC, since no albumin extravasation was observed in both males and females. Accordingly, previous studies in different animal models of stress-induced depression did not found alterations in BBB structure with Evans Blue, a dye with high affinity for blood serum albumin, in the PFC (Menard et al., 2017) and with FITC-dextran (10 kDa) (Northrop & Yamamoto, 2012) or FITC-dextran (70 kDa) (Roszkowski & Bohacek, 2016) in the cortex of male rodents. On the contrary, a study published by Dion-Albert et al., (2022) revealed the infiltration of a fluorophore-tagged 10 kDa dextran in the PFC of female mice, confirming BBB leakiness as a result of chronic social defeat stress. In addition, other papers reported stress-induced BBB alterations, namely an increase in BBB permeability with Evans Blue in the *nucleus accumbens* (Menard et al.,

2017), in the AMY (Xu et al., 2019) and with FITC-dextran (40 kDa) into the perivascular area (Lee et al., 2018) in male rodents. Thus, these differences between multiple studies could result from the types of tracers used to evaluate BBB integrity, particularly the size. Moreover, the duration and magnitude of the stressor can also influence the impact of chronic stress on the BBB (Sharma & Sharma, 2010), and it would be of particular interest to investigate if chronic stress has different effects on the vasculature of other brain regions such as the HIP, which has not been examined in the current study. Since no significant changes in albumin protein levels in the PFC were noted, other possible alterations in BBB induced by uCMS were explored in more detail. The TJs and AJs are crucial to the maintenance of barrier properties (Xu et al., 2019), hence is thought that downregulation of TJs and AJs proteins as a result of chronic stress might contribute to BBB damage. Regarding the impact of chronic stress in claudin-5 protein levels, no significant differences were observed between control and uCMS groups in the PFC of male rats, which is consistent with a study conducted by Menard et al., (2017) in a stress-related mice model. Noteworthy, a significant increase in claudin-5 protein levels in the PFC of females exposed to uCMS was detected. This could represent a compensatory mechanism of the BBB in response to a prolonged situation of stress. Curiously, a recent study found that chronic social defeat stress leads to a decrease of claudin-5 expression in the PFC, coincident with anxiety and depressive-like behaviors, and contributing to stress vulnerability, whereas no alterations in claudin-5 expression in the PFC and behavior were correlated with stress resilience in female mice (Dion-Albert et al., 2022). Nevertheless, the authors also demonstrated that stress vulnerability could be related with transcriptomic changes in the endothelium, including for genes involved in oxidative damage. Moreover, transcriptomic adaptations and changes in the expression of endothelial genes related to omega/3-omega-6 fatty acid synthesis may be involved with a stress-resilient phenotype (Dion-Albert et al., 2022). Noteworthy, the use of different stress paradigms may explain the discrepancies between studies. For instance, the present study applied an uCMS protocol, whereas Dion-Albert et al., (2022) used a model of chronic social defeat stress. The effect of uCMS was also assessed in occludin and VE-cadherin protein levels, but no significant alterations were found in the PFC of males and females. Additionally, others studies have also demonstrated that stress exposure does not induce alterations in occludin protein levels in the PFC (Menard et al., 2017) and brain microvessels (Northrop & Yamamoto, 2012) of male rodents.

Despite these data, there are contradictory observations. Some studies showed that stress significantly reduced occludin and VE-cadherin protein levels in the PFC, HIP (Cheng et al., 2018; Sántha et al., 2015) and AMY (Xu et al., 2019) of male rodents. Importantly, the present work uncovers for the first time that uCMS does not affect occludin and VE-cadherin protein levels in the PFC of female rats.

The BBB plays a key role in preventing the infiltration of peripheral immune cells, mainly due ECs low basal expression of adhesion molecules (Alvarez et al., 2011). Since leukocytes infiltration has been implicated in the establishment of anxiety and depression (Hodes et al., 2015), it was considered relevant to evaluate the protein levels of VCAM-1. However, no significant alterations were detected in VCAM-1 proteins levels in the PFC of both males and females exposed to uCMS, which cannot contribute to the depressive-like behavior observed in male rats. Moreover, the intact BBB properties in both male and female animals after uCMS could explain the unchanged VCAM-1 protein levels. In contrast, male mice subjected to repeated social stress demonstrated an increase in ICAM-1 and VCAM-1 protein expression in the PFC, HIP and AMY (Sawicki et al., 2015), suggesting that the type of stress protocol used may influence endothelial dysfunction by upregulation of adhesion molecules. Additionally, several studies found a significant association between higher levels of plasma markers of endothelial dysfunction, such as sVCAM-1 and sICAM-1, and depression (Lopez-Vilchez et al., 2016; Müller, 2019; van Agtmaal et al., 2017). Nonetheless, the protein levels of ICAM-1 were not examined in this work and it would be interesting in further studies to unravel if uCMS could lead to an increment of this adhesion molecule. The present study also revealed for the first time that uCMS does not interfere with VCAM-1 protein levels in the PFC of female rats.

The impact of uCMS in astrocytic reactivity was also evaluated in this work, since astrocytes play an essential role in the integrity and normal function of the BBB (Zlokovic, 2008). In the present work, no significant alterations were observed in GFAP fluorescence intensity and protein levels in the PFC of male and female rats. Similar findings were reported by Northrop & Yamamoto (2012), who found no changes in GFAP immunoreactivity of isolated cortical microvessels from male rats exposed to uCMS. However, there are some discrepancies in the literature regarding the effect of stress in GFAP reactivity of different brain regions. For instance, an increase in GFAP immunoreactivity was observed in the DG of male mice submitted to uCMS followed

by social isolation (Du Preez et al., 2021). In contrast, chronic restraint stress induced a significant decrease in GFAP fluorescence intensity in the PFC (Sántha et al., 2015), and in GFAP protein levels in the periaqueductal gray matter (Imbe et al., 2012) in male rats. The differences observed among studies may result, at least in part, from the stress paradigms used, the animal model and the experimental methods applied. Therefore, further studies are necessary to understand whether chronic stress might contribute to alterations in astrocyte reactivity in the PFC.

Chapter V

Conclusion and Future Perspectives

5. Conclusion and Future Perspectives

Chronic stress is well documented to cause harmful effects on both physical and mental health, contributing to an increased risk for the development of neuropsychiatric disorders. Nevertheless, the main cellular target of the fundamental research in this field has been the neuronal population. Importantly, over the last few years, this idea is progressively shifting focus to the role of non-neuronal cells, such as the brain ECs. Given the critical role of the brain vasculature in the maintenance of a normal cerebrovascular function, it is reasonable to hypothesize a link between BBB alterations and mental health conditions. Therefore, the present study investigated the possible neurovascular changes induced by unpredictable chronic mild stress, with focus on the C3 complement pathway. Additionally, sex-dependent alterations were determined.

The results obtained in this study led to the following main conclusions:

- uCMS impaired body weight gain in males, but not in females.
- uCMS promoted a depressive-like behavior in males, with no significant changes in females behavior.
- C3 protein levels were not altered in the PFC, HIP, and serum of both sexes after uCMS.
- C3aR protein levels were not significantly different between males and females in the PFC and HIP, despite a tendency to decrease in the PFC of males exposed to uCMS.
- uCMS had no significant impact on BBB properties in males, including no alterations of VCAM-1 protein levels. On the other hand, an increase in claudin-5 protein levels was found in the PFC of females.
- Astrocytic reactivity was not altered by uCMS in both sexes.

Overall, the present study shows chronic mild stress promotes depressive-like behavior in males, but no alterations in their BBB structure. Moreover, despite a tendency to decrease of C3aR protein levels in the PFC of males, uCMS did not cause alterations in C3/C3aR signaling. In opposition, no significant changes were observed in females after uCMS and this behavior stability was accompanied by an increase in claudin-5 protein levels in the PFC. Thus, it is plausible that an upregulation in claudin-5 might be associated with a stress-resilient phenotype.

Importantly, the results obtained in this work suggest that uCMS has no major impact on the neurovasculature on both sexes, but further studies are necessary to

explore in more detail other possible brain alterations induced by uCMS. Several other brain regions associated with emotional-related disorders, such as the AMY and *nucleus accumbens* could be explored, as well as the use of a different stress paradigm.

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