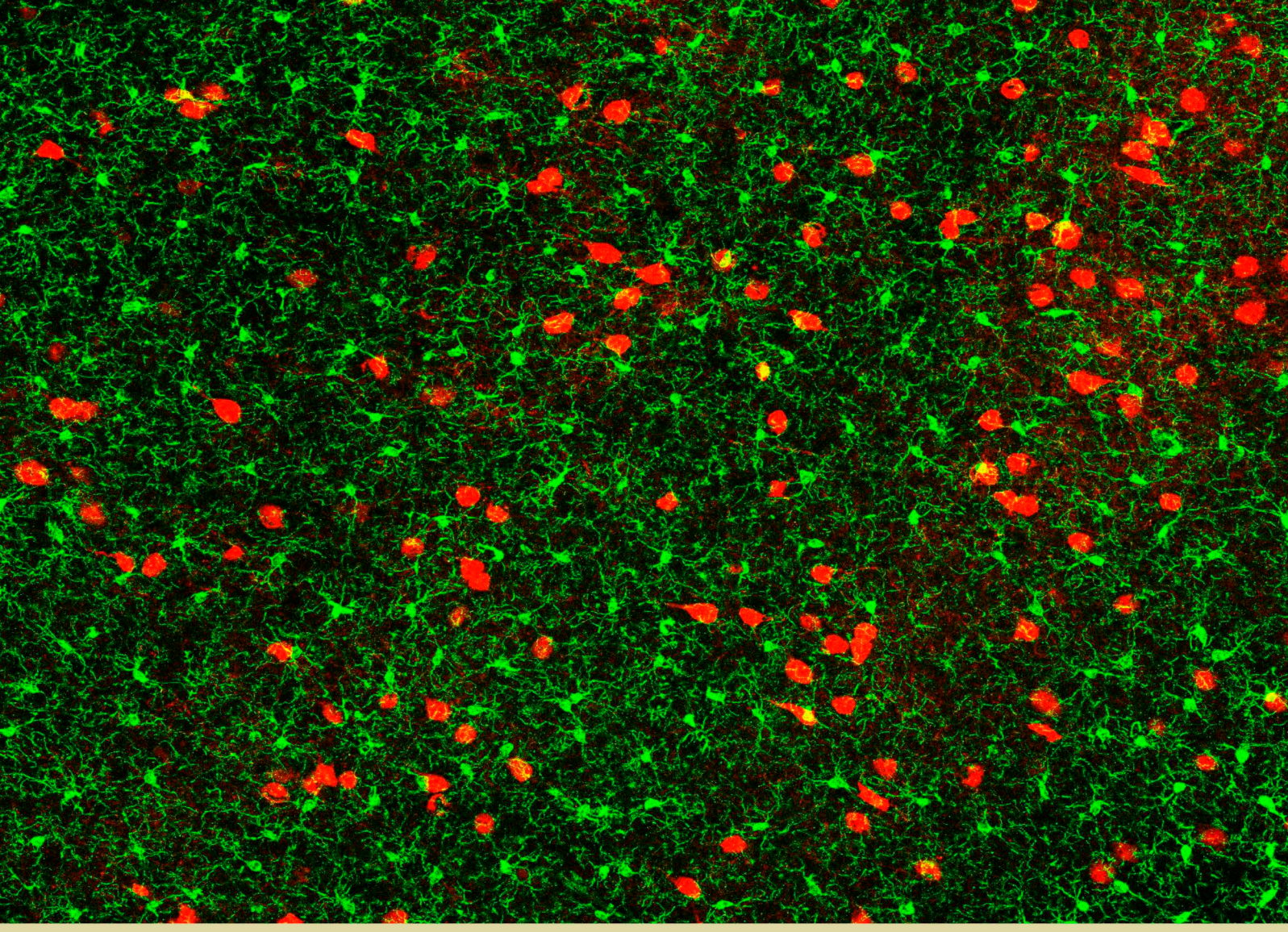

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THE IMPACT OF EARLY-LIFE STRESS TROUGHOUT LIFE: MATERNAL SEPARATIONINDUCED CHANGES IN BEHAVIOR AND MICROGLIA MORPHOLOGY

Dissertação de Mestrado em Investigação Biomédica,
orientada pela Doutora Ana Luísa Colaço Cardoso e pela Professora Doutora Guiomar Gonçalves Oliveira
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To my parents

To Fábio

“To live in a world without becoming ware if it’s meaning is like wandering about in a great library without touching the books”

Dan Brown

In “The Lost Symbol”

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ABSTRACT

Childhood exposure to stress in forms of abuse or neglect is common worldwide. Reports have demonstrated that early life stress is associated with overall volume loss, as well as with reduced neuronal density in several brain regions. Furthermore, an increasingly number of clinical studies have demonstrated a clear relationship between stressful events in the early-life period and neuropsychiatric/neurodevelopmental disorders such as depression, autism spectrum disorders, schizophrenia, psychosis and post-traumatic stress disorders.

The mechanisms underlying this relationship are not clear, but microglia cells have been suggested as mediators of the interaction between stress and alterations in neuronal circuits, which underly these pathologies. These cells, that until recently were believed to remain in a resting state until challenged, are now known to participate in several neurodevelopmental processes essential for correct formation of the brain network. Being a part of the immune system, microglia cells are very sensitive to environmental cues and pro-inflammatory stimulus such as stress in early-life stages. Moreover, alterations in these cells during the critical periods of development lead to impairments in microglia-mediated processes and these impairments have already been correlated with neuronal circuit alterations. However, most of these mechanisms have only been described in male animals, with little information available concerning the impact of early-life stress in the sexual dysmorphism that is present during brain development under physiological conditions.

The present study aimed to investigate the impact of early-life stress in microglia morphology, density and function in the medial prefrontal cortex, a brain region known to be particularly relevant in processes such as complex cognitive function, decision making and social cognition, as well as its impact in social and anxiety-like behaviors during adolescence, focusing in the differences between genders.

Our results revealed that early-life stress causes alterations in social behavior and triggers depressive and risk-taking behaviors in male adolescent animals but not in females. In addition, exposure to this type of stress led a general activation of microglia cells and to opposite changes in microglia density and morphology in both genders, highlighting the sexual dysmorphic characteristics of these cells. Although further investigation in this subject is still warranted, we hypothesize that the differences observed in microglia phenotype can contribute to the increase resilience observed in females following stress in early-life, a result that might help clarify the different incidence of several neuropsychiatric disorders between genders

RESUMO

A exposição ao stress, nas formas de abuso e negligência, durante a infância é muito comum mundialmente. Cada vez mais estudos têm vindo a demonstrar que a exposição a formas intensas de stress durante os primeiros períodos de vida está relacionada com uma perda de volume e de densidade neuronal em várias regiões do cérebro, bem como com várias doenças neuropsiquiátricas e do neurodesenvolvimento, como por exemplo depressão, doenças do espetro do autismo, esquizofrenia e stress pós-traumático.

Os mecanismos que estão na base desta corelação não são ainda conhecidos, mas cada vez mais tem sido sugerido que a microglia possa ter um papel importante na interação entre o stress e as alterações dos circuitos neuronais que estão na base destas patologias. Até recentemente acreditava-se que estas células permaneciam num estado de repouso até existir um estímulo que levasse á sua ativação. No entanto, hoje em dia já se sabe que durante o seu estado de "repouso", a microglia participa em vários processos do neurodesenvolvimento essenciais á correta formação das redes e circuitos neuronais. Fazendo parte do sistema imune, a microglia é extramente sensível ao ambiente que a rodeia, especialmente a estímulos pró-inflamatórios como o stress durante os primeiros períodos de vida, e alterações nestas células têm sido relacionadas com défices nos processos neuronais mediados pela microglia e consequentemente com alterações dos circuitos neuronais.

Presentemente a maioria destes processos só está estudada em machos, havendo pouca informação disponível acerca do impacto do stress durante os as fases precoces da vida no dimorfismo sexual que se sabe existir durante o desenvolvimento cerebral em condições fisiológicas.
Assim, o presente estudo pretendeu investigar o impacto do stress em fases precoces do desenvolvimento na morfologia, densidade e função da microglia do córtex pré-frontal medial, uma região particularmente relevante na função cognitiva complexa e em processos de decisão e cognição social, bem como o seu impacto em comportamentos sociais e de ansiedade durante a adolescência, focando especialmente as diferenças entre géneros.

Com este trabalho foi possível demonstrar que o stress durante as fases precoces do desenvolvimento pode levar a alterações do comportamento social na adolescência, despoletando comportamentos depressivos e de risco em machos, mas não em fêmeas. Para além disso, foi observado um aumento da ativação de microglia, assim como

alterações opostas na densidade e morfologia destas células em machos e fêmeas, o que evidencia o seu dimorfismo sexual. Apesar deste tema necessitar de mais investigação, este trabalho sugere que as diferenças observadas na microglia de machos e fêmeas possam estar relacionadas com o aumento da resiliência face ao stress observado nas fêmeas, um resultado que pode ajudar a explicar a diferente incidência de várias doenças neuropsiquiátricas entre os géneros.

ABBREVIATIONS

CNS	Central Nervous System
Ys	Yolk sac
HSC	Hematopoietic stem cells
EMP	Erythromyeloid progenitors
E	Embryonic day
P	Post-natal day
PCD	Programed cell death
BDNF	Brain-derived neurotrophic factor
ASD	Autism Spectrum Disorders
ELS	Early-life stress
MS	Maternal separation
MSUS	Maternal separation unpredictable stress
PGE2	Prostaglandin E2
GC	Glucocorticoids
LPS	Lipopolysaccharide
PFC	Pre-frontal Cortex
HPA	Hypothalamic-pituitary-adrenal
CRH	Corticotropin releasing hormone
AVP	Arginine vasopressin
GR	Glucocorticoid Receptor
MR	Mineralocorticoid Receptor
PTSD	Post-traumatic stress disorders
IL-1β	Interleukin 1β
mPFC	Medial pre-frontal cortex
MO	Medial orbital cortex
PrL	Pre-limbic cortex
CgL	Anterior cingulate cortex
IL	Infralimbic cortex
IL-6	Interleukin 6
EPM	Elevated plus maze
FST	Forced swimming test
Cg1	Anterior cingulated cortex region 1

CHAPTER 1 | INTRODUCTION

1. Microglia

Microglia are the resident immune cells of the Central Nervous System (CNS), representing 5-15% of total brain cells and are part of the non-neuronal cells that provide support and protection to neuronal functions. Microglia density varies according to the brain region, with higher densities observed in the hippocampus, basal ganglia and substantia nigra[1]. It has become increasingly clear throughout recent years that microglia constitute a distinct and specialized tissue-resident macrophage population, whose specific characteristics arise from exposure to microenvironmental cues unique to the CNS. Still, microglia profiles are incredibly diverse, varying with age, sex and developmental stages, which suggests that microglia functional roles are shaped by their environmental milieu

1.1 Microglia discovery and definition

In 1932, Pio del Rio-Hortega introduced the concept of microglia in the book "Cytology and Cellular Pathology of the Nervous System" as a distinct element of the CNS, distinguishing these cells from the non-neuronal, non-astrocytic elements and from the macroglia (constituted by oligodendroglia and oligodendrocytes). In the chapter "Microglia", del Rio-Hortega postulated 8 statements , some of them we can still consider valid today: 1) Microglia invade the brain during early stages of development; 2) the invading cells present a ameboid morphology; 3) Microglia enter all brain regions using blood vessels and white matter tracts as guiding structures for migration; 4) Once in the brain, and as it matures, microglia acquires a much more branched, ramified morphology; 5) In the mature brain, microglia cells are evenly scattered throughout all regions and exhibit little variations; 6) Each microglia cell appears to occupy a well-defined territory; 7) After an insult, these cells suffer profound morphological transformations retracting their processes and acquiring an ameboid-like morphology similar to the one observed in early development; 8) Microglia cells have the ability to migrate, proliferate and phagocyte. del Rio-Hortega named this class of cells as "microglia" and the individual cells as "microgliocyte". This name then evolved to microglial cells, a term which remains until the present day. In the years following del Rio-Hortega postulation, several investigators shadowed his hypothesis and further elaborated upon it. In 1939, Kershman studied the infiltration of microglia cells during the development of the embryonic brain and described hot spots of migration such as at the *plexus choroideus,* defining them as microglia fountains[2]. In the late 1960s, with the introduction of the facial nerve lesion model by Georg Kreutzberg[3], it became possible to study microglia's response to injury in tissue presenting an intact blood-brain barrier and to distinguish their response from the invading monocytes, allowing the conclusion that microglia plays an important role in the regeneration of the brain in case of injury.

1.2 Origin

For many years there were two main theories about microglia origin: Some argued that microglia cells had its origin in the neuroectoderm, while others believed in a mesodermal origin, considering the morphological and phenotypic similarities with macrophages[4,5]. However, the observation that microglia is present in the neuroepithelium at embryonic day 9.5 (E9.5) suggested that their precursors might originate in the yolk sac (YS) during the primitive haematopoiesis[6].

Recent studies confirmed this last hypothesis showing that, unlike all other brain cell types that share a neuroectodermal origin, microglia originate from primitive haematopoietic stem cells (HSCs) in the extraembryonic YS, more specifically from noncommitted $CD45^{-}$ $c\text{-}Kit^{+}$ erythro-myeloid progenitors (EMPs)[7,8,9,6]. One of the seminal works in this field was published by Ginhoux and colleagues in 2010 and consisted in lineage tracing studies performed in the offspring of the crossing of floxed Rosa26 eYFP animals with animals expressing the tamoxifen-activated MER-Cre-MER recombinase gene, under the control of one of the endogenous promoters of the runt-related transcription factor 1 (Runx1) locus. Recombination, leading to eYFP expression in $Runx1^{+}$ cells, can be induced in the knock-in embryos by a single injection of 4-hydroxytamoxifen into pregnant females at different gestational days, and occurs in a short time frame (less than 24h), allowing to follow the fate of YS cells and their progeny. Runx1 expression can be first identified at E6.5 and is strongly up-regulated around E7.5 in the proximal visceral yolk sac region. Importantly, until E8.0, $Runx1^{+}$ cells are restricted to the extra-embryonic yolk sac and are absent from the embryo proper or allantois[7]. Therefore, injection of 4-hydroxytamoxifen before this time point irreversibly tags YS progenitors and their progeny, but not fetal-liver derived progeny. On the contrary, injection of tamoxifen at E8.5 or after will allow the tagging of FL-derived progeny and not the YS progenitors. In their work, Ginhoux and colleagues hypothesised that if microglia predominantly derives from YS progenitors they should express eYFP in the adult CNS when the injection of 4-hydroxytamoxifen occurs at E7.5[10], and that was indeed what they observed. The relative number of tagged microglia in mice injected at E7.25 was much larger than the number of blood monocytes and other circulating leukocytes. On the other hand, the relative number of tagged microglia in mice injected from E8.0 forward was dramatically decreased. Undetectable levels were reached in mice injected at E8.5. These results strongly suggest that YS macrophages and microglia have the same origin[7] and that this origin is outside the embryo proper.

Microglia enter the brain rudiment via the leptomeninges and lateral ventricles at early developmental stages (by day E9.5 in mice embryos), distribute throughout the cortical wall from both directions, at different speeds and with varying rates of proliferation and maturation, seeding the whole developing brain[11]. The absence of microglial progenitors in the brain despite normal YS haematopoiesis in E9.5–10.5 Ncx-1$^{-/-}$ mouse embryos with defective blood circulation, supports the notion that brain recruitment of YS progenitors depends on a functional circulatory system. However, some microglia are present in the brain parenchyma prior to its vascularization, suggesting that at least some level of microglial dispersion in the brain occurs independently of the cerebral vasculature[7]. EMPs mature into CD45^{+} c-kit^{-} CX3CR1^{+} cells that proliferate and differentiate into microglia in a Pu.1 and Irf8-dependent manner[12]. The number of microglia in the brain increases during the first two postnatal weeks reaching a peak in density at post-natal day (P)14. In the third postnatal week, microglia density begins to decline due to concomitant increase in microglial apoptosis and decrease in proliferation, until postnatal week 6, when microglial numbers reach a level two times lower compared with the levels at P14. This new density is then maintained throughout adulthood[13].

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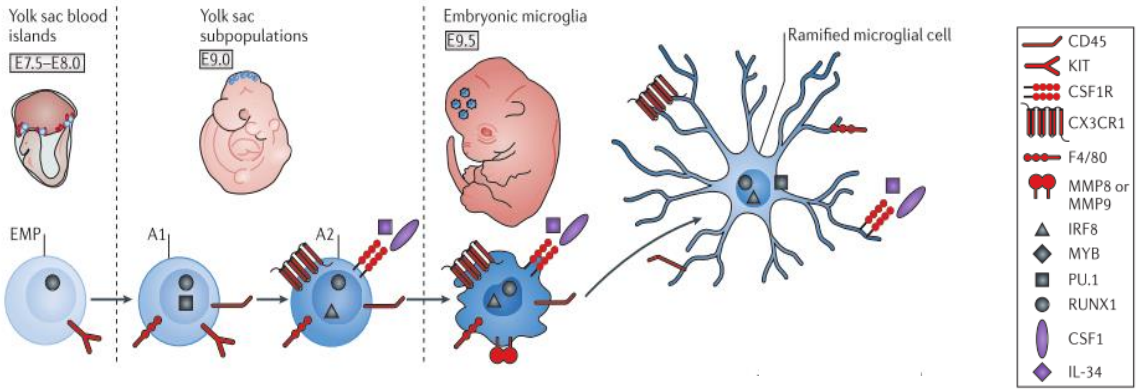

Figure 1| Representation of microglia origin and developmental stages. Microglia derive from hematopoietic stem cells in the extraembryonic YS, more specifically from noncommitted CD45^{-} c-Kit^{+} erythro-myeloid progenitors that are formed during primitive hematopoiesis, which begins at embryonic day 7.5(E7.5). Around E9 these cells start to upregulate the expression of the CD antigen CD45 and other myeloid cell markers such as the CX3CR1, a chemokine receptor, as well as the colony-stimulating factor 1 receptor (CSF1R). These cells then enter the brain rudiment via the leptomeninges and lateral ventricles around E9.5 and distribute through the brain parenchyma, seeding the developing brain. Adapted from: Prinz, M. *et al*, Nature Reviews Neuroscience (2014)

A similar pattern can be found in human foetuses. The first cells with macrophage-like characteristics appear in the YS and mesenchyme in the 4th week of gestation and in the pre-hematopoietic liver in the 5th week. Colonization of the spinal cord begins around the 9th week

and the major influx and distribution of microglia starts at about 16th week. Microglia take up to the 22nd week to become widely distributed within the intermediate zone. Two distinct populations of fetal microglia emerge throughout the developing CNS: 1) amoeboid and 2) progressively ramified cells displaying secondary and tertiary branches. Some studies have also shown that the distribution of microglia advances from the germinal layer via the intermediate zone and towards the cortical plate throughout this period, with ramified cells being found predominantly within the intermediate zone. This migratory response occurs in a ‘‘wave’’-like pattern and is stabilised by 20–22 weeks. Despite this, only close to term, at 35th week, that totally differentiated microglial populations can be detected within the developing human brain[5,14].

In the adult brain, nearly no cell exchange between blood and brain parenchyma is expected to occur in healthy conditions. This has been demonstrated by experiments where mouse monocytes were removed by bone-marrow irradiation, followed by bone-marrow transplantation with GFP-labelled monocytes. While early reports[15] employing this procedure indicated that GFP^+ monocytes also invaded the brain parenchyma and were, therefore, responsible for maintaining microglia numbers in the adult brain, recent studies, where the blood-brain barrier was protected from radiation, demonstrated that the presence of GFP^+ monocytes in the normal undisturbed brain is almost negligible[16]. This clearly indicates that, in the healthy brain, microglia cells exist as a stable and self-replicating population.

1.3 Identification and markers

Since del-Rio Hortega identification of microglia by cytomorphological criteria, the identification of microglia cells has been facilitated due to the development of staining procedures that take advantage of the expression of certain exclusive molecules by these cells. These methods allow the discrimination of microglia from others CNS-resident cells, such as neurons, astrocytes, oligodendrocytes and endothelial cells, as well as from infiltrating monocytes and other brain macrophages. In both human and animal tissue, microglia can be visualized thanks to a variety of cell surface-associated or intracellular molecules. In experimental research, immunoglobulin receptors (CD16, CD32, CD64), leucocyte common antigen CD45, F4/80 antigen, β-glucan receptor dectin-1, the glucose transporter 5 (GLUT5) or the mannose receptor CD206 are useful targets, even though some present overlapping recognition of other brain macrophages/macrophage-like cells. On the other hand, antibodies against IBA-1, a protein with a suggested role in calcium homeostasis, have proven to be helpful in visualizing microglia[17] in microscopy studies, providing a detailed view of their processes. IBA-1

expression is also useful as an indicator of the inflammatory microglia phenotype, since its expression increases upon microglial activation[18].

The recent generation of microglia-specific genetically modified animals has further facilitated microglia studies. Animals expressing fluorescent proteins under the control of microglia expressed receptors, such as the fraktaline receptor (CX3CR1)[19] or the receptor for macrophage colony-stimulating factor (CSF1R)[20], have been used as microglia-labelled mouse models.

1.4 Development

When they first invade the developing brain, microglia exhibit an amoeboid body and share similarities regarding surface antigens, functional proprieties and morphology, with other macrophages normally recruited only upon injury and disease states. At this stage, microglia are highly motile, phagocytic and proliferative cells. They are believed to play an important role in the elimination of cellular debris that result from naturally occurring cell death and to selectively eliminate certain axonal projections. Subsequently, these amoeboid cells down-regulate their antigen expression acquiring an intermediate morphology that later mature into ramified microglia, presenting small cell bodies with long processes[14]. Several studies have shown that an age-related decrease in the numbers of amoeboid microglia was correlated with an increase in the numbers of ramified microglia[21]. Even though not much is known about what converts microglia to its “surveillant”, ramified morphology, studies in cell cultures have provided some clues. Astrocyte conditioned medium was shown to increase ramification in blood monocytes in culture and combining conditioned medium with ATP or adenosine resulted in an even more extensive ramification, indicating that purines are among the factors that can induce microglia ramification. Further studies using antibodies against transforming growth factor-α (TGF-α), M-CSF and granulocyte/macrophage colony stimulating factor (GM-CSF), all growth factors released from astrocytes, have demonstrated that these molecules are necessary for ramification to occur.

1.5 Microglial Dynamics

Microglia may adopt several morphological states throughout their life-cycle, such as amoeboid, intermediate/bipolar, reactive/activated and ramified, displaying considerable phenotypic heterogeneity. Most often, microglia are classified into two typical morphologically distinguishable configurations: they can present a round cell body with long processes and

highly branched morphology or a round cell body with fewer processes presenting an amoeboid shape. Infection, trauma, ischemia, neurodegenerative diseases or other brain insults that cause loss of brain homeostasis and is indicative of real or potential danger to the CNS triggers phenotypic transformations in microglia, characterized by the shortening of their processes, acquisition of an amoeboid shape, upregulation of antigen presentation molecules and a proliferative and phagocyting phenotype. These changes are summarily defined as "microglial activation"[22,23]. During this process, microglia became highly motile and can move towards the lesion or infection site, attracted by chemotactic gradients. Microglia can also proliferate to increase their number, allowing a better defence against the insult, as well as providing protection and restoration of tissue homeostasis. Other elements present in the activation process are the induction or rearrangement of surface molecules necessary for cell-cell and cell-matrix interactions, alterations in intracellular enzymes, release of multiple factors with proinflammatory and immunoregulatory effects, production of neurotrophic factors and even physical association with endangered neurons. Activated microglia can also employ their phagocytic activities to clear tissue debris, damaged cells or microorganisms, similarly to the functions of peripheral macrophages.

For a very long time it was suggested that branched microglia were in a resting state, while amoeboid cells were active. However, several studies[22,23,24] have clarified the active nature of 'resting', branched microglia. *In vivo* two-photon microscopy studies[25,26] in the CX3CR1$^{+/GFP}$ mice showed that microglia processes are highly mobile and are continuously extending and retracting in the time scale of seconds. This, together with constant integration and interpretation of environmental cues, allows the otherwise stationary microglia to thoroughly scan their environment without disturbing the fine-wired neuronal structures that surround them, and change into executive states of activity upon appearance of danger signals that indicate an eminent threat to the CNS. Considering the speed at which microglia processes move, is possible to estimate that these cells can survey the complete mouse brain parenchyma within periods of only a few hours. This basal activity has led to a shift in nomenclature, with quiescent, non-ameboid microglia no longer called "resting" but instead called "surveying". Despite their importance, microglia-mediated surveillance and defence are not the only functions perpetrated by microglia in the healthy brain. Microglia also play other very important roles in the maintenance of CNS homeostasis that can easily be overlooked and that will be addressed below. Nevertheless, failure to protect neurons and induction of maladapted responses that cause excessive or chronic microglia activation can have serious implications and lead to several pathological states.

1.6 Functions in the developing brain

Two key functional features define microglia: immune defence and maintenance of CNS homeostasis. In context of disease and injury, microglia have a recognized role as phagocytes due to their ability to rapidly clear dead or dying neurons and other cellular debris and bridge the gap between innate and adaptive immune responses, by recruiting other immune cells to the place of injury. But microglia's functions are far wider in the healthy and developing brain. There is a growing body of evidence that shows that microglia play a physiological, non-inflammatory role that is crucial during CNS development and for the regulation of neuronal plasticity.

1.6.1 Formation and Maintenance of blood vessels

As already discussed, microglia are present in the brain before the complete formation of blood vessels in the CNS. Microglia are, therefore, in a unique position to influence the early development, migration and refinement of the growing CNS and retinal vascular systems. Recent data provided evidence that microglia facilitate angiogenic sprouting during development. Targeted deletion of PU.1, a transcription factor required for the development of microglia, results in a significant reduced vascular branching and complexity. Similar results are observed in mice lacking M-CSF, which is also important for microglial development [27]. In addition, time-lapse imaging in the developing zebrafish brain provided direct visual evidence for microglia promoting the fusion of nearby blood vessels.[28]. Another study tested, *in vitro*, the ability of microglia to promote angiogenesis using an aortic ring culture system. While aortic rings cultured without microglia failed to elaborate complex branching vessels, in the presence of microglia or microglia conditioned medium, complex vessels were formed, which indicates that microglia and microglia diffusible signals contribute to angiogenesis. Taken together, these studies suggest that microglia play a critical role in the development of CNS vascular system and in angiogenesis.

1.6.2 Induction of developmental neuronal apoptosis

During development, more neurons are formed than the ones which survive until adulthood and many die as a result of programmed cell death (PCD) or developmental apoptosis[29], allowing the refinement of neuronal circuits throughout the brain. Microglia, following their establishment in the CNS, regulate neuronal circuit development. Amoeboid microglia have long been known to phagocyte apoptotic neurons and cellular debris associated with PCD. However, more recent studies in the developing cerebellum[30], vertebrate spinal cord[31] and hippocampus[32], suggest that microglia could play an active role in inducing neuronal cell death.

Several mechanisms are involved in this process including microglia responding to neuron-released “eat-me” signals, microglia priming neurons to PCD and even microglia directly trigging PCD by releasing neurotoxic substances. Marín-Teva *et al.*[30] has demonstrated, using cerebellum organotypic slices, that microglia triggers Purkinje cell death. When microglia cells were depleted from the slices, Purkinje cells did not undergo PCD. This effect appears to be mediated by microglia realising of superoxide ions. Another study from Rigato and coworkers[33] shows a correlation between microglia arrival at the spinal cord of transgenic CX3CR1$^{+/GFP}$ mice during embryonic development and motor neuron cell death. In the rodent hippocampus, developmental apoptosis occurs during the first postnatal week. During this period, microglia are often found to be near cleaved caspase-3 positive neurons, a marker for apoptosis. Wakselman *et al.*[32] found that the loss of CD11b and DAP12 which, in the brain, are exclusively expressed by microglia, results in a significant reduction in cleaved caspase-3 positive neuronal staining, suggesting a reduction in PCD in these conditions. Similarly to what occurs in the cerebellum, apoptosis in the hippocampus was caused by microglial superoxide production [32]. Together, these studies suggest that microglial superoxide production controls, at least in part, PCD and that microglia could play an important role in regulating neuronal development by controlling neuronal numbers during embryonic and early post-natal development. Also, since this has been observed in several different regions of the CNS, it suggests that PCD regulation by microglia may be widespread in the developing CNS.

1.6.3 Neuronal survival

It is known that during development, several neurotrophic factors are responsible for the survival and differentiation of neurons and for facilitating the establishment of neuronal circuits. Some of these same factors are also responsible for promoting the health and survival of neurons in adult stages of life. In fact, microglial cells release several trophic factors that can contribute to the formation of neuronal circuits, as well as neuron survival. One key example is brain-derived neurotrophic factor (BDNF). BDNF is known to be necessary for neuronal survival and was thought to be mainly neuronal-derived [34]. However, recent studies have shown that non-activated microglia can also secret BDNF [35]. Another example is insulin-like growth factor-1 (IGF-1) which is released by surrounding microglia promoting survival of layer V cortical neurons during postnatal development[36]. Immunohistochemical studies have shown that microglia accumulates close to sub-cerebral and callosal projecting axons shortly after birth and depletion of microglia in the brain at this age promotes cell death of layer V cortical neurons.[36] Furthermore, is was shown that this supporting role was mediated by fractalkine (CX3CL1-CX3CR1) signalling and IGF-1 released by microglia, since CX3CR1 deficiency or IGF-1 inhibition also resulted in the death of layer V cortical neurons.[36] In addition to these

factors, microglia also secret other trophic factors such as epidermal growth factor, nerve growth factor and basic fibroblast growth factor, which are known to play important roles in neuronal development, maintenance and function throughout life[37]. Having this into account it is easy to understand how microglia plays a critical role in neuronal health and neuronal survival in the CNS.

1.6.4 Synaptic Pruning, Remodeling and Plasticity

As stated previously, even in the healthy, non-injured brain, microglia continuously extend and retract their processes in a way that allows them to monitor their surroundings. *In vivo* imaging experiments have demonstrated that microglia constantly establish brief (4-5 min) contacts with synaptic structures and that, in case of injury such as ischemia, these contacts lasted for a significant longer period often causing the disappearance of synapses.

Observations that microglia undergo morphological maturations that occur concomitantly with the period of synaptic maturation, together with studies pointing that these cells express several receptors for neuronal signalling that are upregulated during early post-natal development, pointed towards the hypothesis that microglia could play a role in the phagocytic elimination of synapses, being part of the mechanism behind the extended pruning of excessive synaptic connections during development[38]. This hypothesis has gained strength over the last few years, as several studies reported the selective engulfment of synaptic structures by microglia cells. Mice lacking either CX3CR1[39] or complement components[40] presented an excess of immature excitatory synapses as consequence of failure to eliminate immature synaptic connections during the second and third postnatal weeks, both in the hippocampus and the lateral geniculate nucleus, respectively. In fact, $CX3CR1^{-/-}$ mice show persistent deficits in synaptic multiplicity, reduced functional connectivity between brain regions, impaired social interaction and increased grooming behavior[41]. These alterations persist into adulthood, suggesting that failure to eliminate synapses at the correct developmental window prevents the normal strengthening of synaptic connections.

Microglia is also very likely to be required for activity-dependent synaptic plasticity, since studies in mice lacking the G-coupled purinergic receptor P2Y12, which is abundantly and selectively expressed in homeostatic microglia and rapidly downregulated following the initiation of inflammation[42], show deficits in early monocular deprivation-associated visual cortical plasticity[43]. On the other hand, it has been shown that microglia can also be an important players in synapse formation in the adult brain, as some studies pointed out that

these cells can elicit calcium transients and the formation of filipodia from dendritic branches[44,45] and that they are essential for learning-induced synapse formation[46].

1.7 Role in neurodevelopmental disease and other neurological disorders

1.7.1 Autism spectrum disorders

There is growing evidence from human studies that microglia are dysfunctional in developmental disorders such as autism-spectrum disorder (ASD). *Post-mortem* studies have revealed that individuals with autism present altered microglia number, morphology and neuronal interactions in several brain regions, as well as altered expression of microglia-specific genes including markers of inflammation[47,48,49]. Human-brain imaging studies using positron-emission tomography (PET) with resource to [11C](R)-PK11195, a well-known ligand of microglia expressed translocator protein, have revealed an increase in microglia number in several regions of the brain of adults with ASD[50], a discovery consistent with the previously discussed *post-mortem* studies. Even though there are few genetic human studies implicating genes expressed by microglia in ASD, aberrant responses of these cells to insult during critical developmental time points can possibly originate neuronal and circuitry disfunction, a finding frequently present in these neurodevelopmental disorders.

Several other environmental factors can interfere with microglia function, particularly during early post-natal development and influence normal brain development. Systemic or local inflammation, maternal immune challenge, early-life stress (ELS) among other factors can lead to an abnormal function of these cells and ultimately be implicated in several disorders as it will be discussed later.

1.7.2 Schizophrenia

The first study to report microglia alterations in patients with schizophrenia was performed by Fisman in 1975. Since then, several other studies have shown a correlation between schizophrenia, immune activation and microglia. Genome-wide studies[51] have hinted at a connection between schizophrenia and immune activation, showing particular correlations with markers of the MHC (major histocompatibility complex) coding region. In addition, an accumulating body of evidence also suggest that neuroinflammation, characterized by an

increase in the serum concentration of several pro-inflammatory cytokines, plays an important role in the pathology of this disease[52,53].

Microglial activation or increased microglia numbers in individuals with schizophrenia has also been observed in *post-mortem* studies[54]. One of these studies, by Steiner *et al*.[55] showed that schizophrenia patients present elevated microglial density in the anterior cingulate cortex and mediodorsal thalamus. *In vivo* studies, using PET and employing the [11C] (R)-PK11195 technique, have also reported increased microglial activation in the grey matter and hippocampus of patients with schizophrenia[56]. Other study from Doorduin *et al*., employing the same technique, also showed that microglia activation is present during the first 5 years of the disease[57].

Several studies have also indicated that maternal infections such as toxoplasmosis[58], influenza[59] and rubella[60], increase the prevalence of schizophrenia in patients with genetic predisposition. Since microglia, as stated above, plays an important role during development and is very sensitive to environmental cues, it has been suggested that microglia activation caused by maternal infection, can be an important player in the development of schizophrenia under these circumstances.

1.7.3 Neurodegenerative disorders

It has been known for a long time that microglia cells are highly sensitive to injury and there is plenty evidence that chronic overactive microglia cells can present neurotoxic features, producing an excessive amount of superoxide[61], nitric oxide (NO)[62], tumour necrosis factor-α (TNF-α)[63] and other pro-inflammatory mediators. The factors that can cause this excess of activation can range from environmental toxins to physical brain injury and neuronal cell death. Microglia has been shown to be present in large numbers in several neurodegenerative diseases, a condition termed microgliosis, while inflammation *per se* has been accepted as an important primary component of a wide range of neuropathologies. Taken together, these evidences strongly suggest an important role for microglia cells in the pathology of neurodegenerative disorders.

In fact, microglia overactivation has been reported in Alzheimer's disease, throughout the entire time-course of disease progression and even preceding neuropil destruction and the triggering of symptoms. Activated microglia clusters can be found at sites of Aβ aggregation and release several neurotoxic factors and inflammatory cytokines, leading to synaptic loss and vascular damage[64].

In Parkinson's disease, numerous activated microglia are present in the vicinity of damaged neurons in the substantia nigra, putamen, hippocampus, cingulate cortex and temporal cortex[65] and several *in vitro* studies reveal that damaged dopaminergic neurons release several factors that activate microglia, implicating these cells in neuronal degeneration associated with this disease[66,67].

Microglia overactivation has also been linked to the pathology of several others neurodegenerative disorders such as Huntington's disease[68], multiple sclerosis[69] and amyotrophic lateral sclerosis[70]. In summary, overactivated microglia play a role in neuronal loss and vascular damage in diverse neuropathological conditions

1.8 Microglia gender differences

In addition to regional and age heterogeneity[71], microglia also present several gender-related differences. Several studies have reported significant differences in the overall number and morphology of microglia from male and female subjects in several brain regions, such as the hippocampus, preoptic area, parietal cortex and amygdala, across the lifespan. These differences are observed in steady state conditions, but also in response to chronic stress[72]. Considering that microglia express both estrogen and progesterone receptors, these differences could be dependent on steroid hormones[73]. Treating female mice with estradiol in the first two days after birth leads to the full masculinization of microglia number. However, co-treating females with estradiol and the microglial inhibitor minocycline, prevented the estradiol-mediated masculinizing effect on overall microglia numbers[74]. Recent data suggests that additional inflammatory mediators may play a role in gender differentiation of the brain during development. Significant gender differences were found in the expression of inflammatory genes and in the levels of pro-inflammatory cytokines in several brain regions[75]. In the preoptic area (POA), a highly sexually dimorphic brain region necessary for the establishment of adult male sex behavior, estradiol upregulates the production of prostaglandin E2 (PGE2) to induce sex-specific brain development. This upregulation results in a higher density of dendritic spines and adult sexual behavior in males[76]. A study from Kathryn *et al*. has shown that males have twice as many microglia in an activated morphological state and that microglia inhibition during the critical period of sexual differentiation prevented sex-differences in microglia numbers and

inhibited the estradiol-induced upregulation of PGE2, as well as estradiol induced masculinization of dendritic spines and adult sexual behavior.[77]

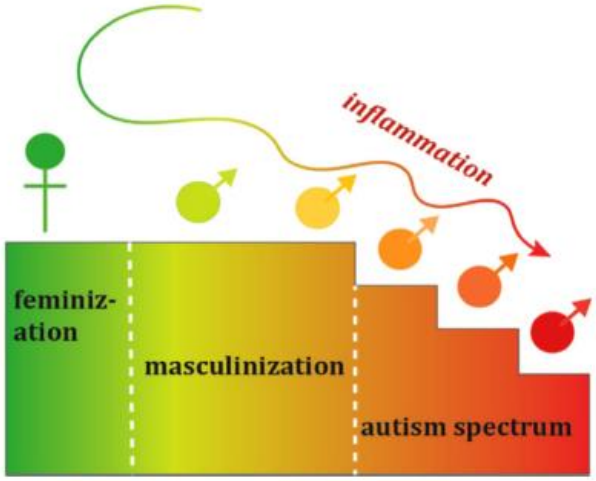

Figure 2| Brain masculinization involves inflammatory signaling molecules such as prostaglandins, which in great part are derived from microglia, increasing vulnerability to neurodevelopmental disorders such as ASD. In: McCarthy *et al.,* Biol. Psychiatry (2017)

Caetano et al. has also described that prenatal injections of dexamethasone, a synthetic glucocorticoid (GC), which mimics the effects of prenatal stress, in Wistar rats, induces long-term gender-specific changes in microglia morphology in the prefrontal cortex, with females presenting a general decrease whereas males displayed an increase in the number and length of microglia processes. A result that is justified by the different basal morphology of microglia cells in males and females, which is normally characterized by a higher complexity in females with microglia presenting higher number and longer processes than in males during development[78].

However, another study from Schwarz *et a*l, in Sprague–Dawley rats come to demonstrate that during adolescent, at approximately P30, a shift in this gender differences occurs. Around this age, females present significantly more microglia with an activated phenotype, characterized by longer and thicker processes within several brain regions (such as the hippocampus, amygdala and parietal cortex) than males. Together with this shift in morphology, they also reported that females had significantly higher levels of several cytokines at this age, specifically within the IL-1 and IL-10 families as well as the Toll-like receptor signaling protein, TOLLIP, suggesting that microglia within the female juvenile and adult brain may be functionally different or have a fundamentally different biochemistry than microglia within the male brain.[79]

This data strongly indicates that microglia interacts with the nervous and endocrine systems during critical phases of development, playing an essential role in the process through which estradiol upregulates prostaglandin production and, consequently, brain masculinization, with a shift occurring in their morphology and cytokine around the early adult age.

Ex-vivo studies performed in microglia derived from male and female brains also suggests that these cells present different inflammatory responses to treatment with lipopolysaccharide (LPS) and estradiol, which suggests that the differences between genders in microglia may be more extensive than just differences in number and morphology[80]. The recognition of these important differences, as well as the unravelling of the biological mechanisms behind them, is critical for our understanding of microglia's role in the healthy and diseased brain. Microglia-associated gender differences may help to explain, for example, the increased incidence of certain neurodevelopmental and neuropsychiatric disorders, such as depression and ASDs, in female or male patients, respectively.

2. Early-Life Stress

2.1. Stress

All living organisms survive by maintaining a complex dynamic equilibrium which is called homeostasis. This equilibrium is constantly threatened by physical or psychological events, known as stressors and, therefore, several physiological and behavioral responses are triggered to reinstate homeostasis. There are several types of stressors, including: 1) physiologic stress, which consists of external or internal conditions that may threaten the equilibrium of the biological systems and that activate circuits in the brainstem and hypothalamic regions[81] and 2) psychological stress, which is mostly associated with social behaviors and is known to trigger the activation of brain regions related to emotion and decision making, such as the amygdala and the pre-frontal cortex (PFC)[82]. Independently of their nature, stressors are most often classified as acute or chronic. Stress responses can be specific to the stressor or can be generalized and nonspecific, in cases where the magnitude of the threat to the system exceeds a certain threshold[83].

2.1.1. Stress Response

The typical stress response consists in two time-separated domains: a quick and a delayed response. The first consists in a “fight of flight” response, with activation of the autonomic nervous system, particularly the sympathetic nervous system, leading to the release of catecholamines: adrenaline from the adrenal medulla and noradrenaline from widely distributed synapses.[84] These hormones act by elevating blood pressure and respiration rates and increasing blood flow to vital organs, such as the heart and muscles. This response is short-term and is followed by the activation of the parasympathetic nervous system which takes the system back to normal, basal conditions.

After this first stage, the hypothalamic-pituitary–adrenal (HPA) axis is activated, releasing GCs, which act as transcriptional regulators of glucocorticoid responsive genes, triggering a slower and long-lasting response. HPA axis activation is triggered by the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the paraventricular nucleus. When CRH reaches the pituitary gland, it induces the release of adrenocorticotropic hormone (ACTH), which in turn leads to the releases of GCs from the adrenal gland to the bloodstream. The concentration of GCs in the blood reaches a peak approximately 15 to 30 min after the stressful event and these molecules bind to the glucocorticoid (GR) and mineralocorticoid (MR) receptors present in several organs, including the brain. One to 2 h after the end of the stressful

event, the levels of GCs start to induce a negative feedback on the system, returning GCs levels to baseline.[84] In this second response the gonadal and adipose axis, as well as the immune system, also play an important role, redirecting energy from less urgent functions, such as digestion, to the response to the stressful event.[85]

An effective stress response should be activated when needed and immediately terminated afterwards, since while acute and short-term stress can be considered beneficial and adaptative, an excessive, inadequate or extensively prolonged stress response, caused, for example, by chronic stress, can lead to dysregulation in the HPA axis, exposing the brain and other peripheral tissues to these powerful steroids and contributing to several pathological outcomes.

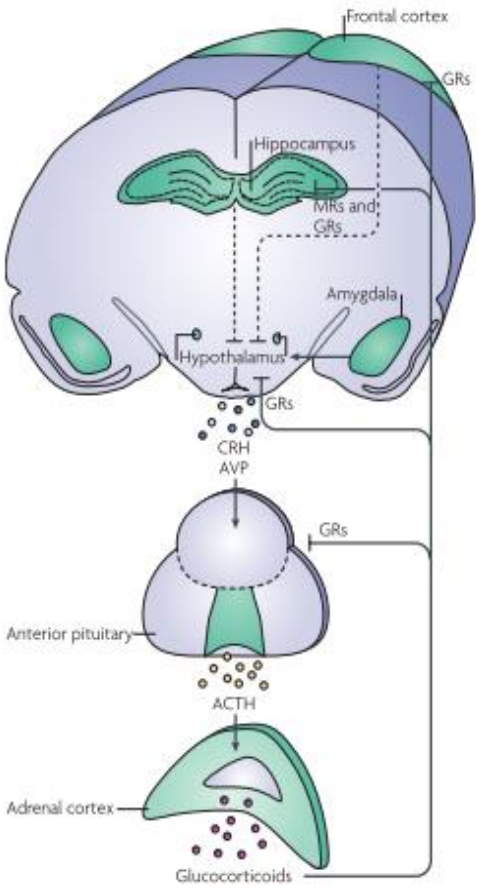

Figure 3 | Stress response mechanisms. Upon a stressful event, corticotropin-releasing hormone and arginine vasopressin are released in the paraventricular nucleus, inducing the production of adrenocorticotropic hormone which acts upon the adrenal gland leasing to the release of glucocorticoids to the bloodstream. In Lupien *et a*l., Nature Rev. Neuroscience (2009)

2.1.2. Impact of stress in the brain

The brain is one of the most susceptible organs to be modified by stressful events. The effects of stress on brain functions is highly dependent on the context and nature of the stressor. Mild and short-lasting stress can improve memory, by enhancing synaptic plasticity, reflecting the necessity of remembering circumstances that are perceived as threatening.[86] However, when these mechanisms are constant and intensively activated they can lead to harmful effects.

Two types of corticosteroid receptors are present in the brain. The MR which has high-affinity and is expressed mostly in the limbic areas and GR, which presents a 10-fold lower affinity for corticosterone, but is more ubiquitously distributed.[87] These receptors are part of the family of nuclear receptors and, in their inactivated form, they can be found in the cytoplasm. Once the ligand binds MRs or GRs, the receptor translocate to the nucleus and binds to response elements in the promotor region of certain responsive genes. On the other hand, activated receptor monomers can also interact with other transcription factors leading to the suppression of other specific genes. This results in a long-lasting and delayed impact on neuronal function[88]. As stated previously, stress also leads to release of CHR and AVP which, together with the described actions of GCs, induce changes in the electrical properties, proliferative capacity and shape of cells in the brain, ultimately giving rise to the behavioral and pathological aspects associated with chronic stress responses.

Several studies have provided strong evidence that chronic stress or stress in the early-life stage can result in long-term behavioral and health consequences. In particular, severe or prolonged stress is known to have a profound impact in human mental health, triggering several disorders such as post-traumatic stress disorder (PTSD)[89], depression[90], schizophrenia[91] or anxiety disorders[92] in susceptible individuals and may even trigger psychotic episodes[93].

Brain alterations following stress were also observed in rodent models. Cognitive brain networks, such as those in the hippocampus and PFC, as well as in regions involved in emotional processes, such as the amygdala and the nucleus accumbens, and other networks involved in social behaviours, appear to be particularly vulnerable to the effects of stress[94,95,96]. Chronic stress or chronic exogenous administration of glucocorticoids leads to dendritic atrophy of hippocampal CA3 pyramidal neurons[97]. Also, chronic stress in adult rats causes hippocampal volume loss[98] and inhibits neurogenesis in the dentate gyrus[99]. In the PFC, pyramidal neurons in the layer II/III have also been shown to present dendritic retraction and a reduction in spine number in response to chronic stress[100]. Glucocorticoid hypersecretion has also been associated with a reduction in the volume of the anterior cingulate cortex[101].

These alterations, however, do not seem to be permanent and are reversible in only a few weeks after the end of the stressor.

2.1.3. The impact of stress in microglia cells

It has been long known that stress has the ability to alter immune responses due to the effects of glucocorticoids on immune cells. Even though in the peripheral immune system glucocorticoids are known immunosupressors, recent *in vivo* studies have demonstrated that glucocorticoids can enhance the activity and proliferation of microglia cells in the CNS. Protocols of chronic unpredictable stress[102] and brief shock tail[103] stress were shown to potentiate the expression of interleukin-1β (IL-1β), a pro-inflammatory cytokine, in the hippocampus and PFC, following the injection of LPS 24 h after the stressor occurs. Several other studies have also shown that both acute and chronic stress can have profound impacts in the immunophenotype of microglial cells, triggering the upregulation of several activation markers, such as the F4/80 antigen[104], the major histocompatibility complex II (MHCII)[105-106], and the toll-like receptor 4 (TLR4)[107], as well as the de-ramification of microglia in the medial amygdala, PFC and hippocampus,[107] suggesting a shift towards a pro-inflammatory state. This activation process seems to be dependent of GC, since the blockage of these receptors using GC antagonists eliminates the activation of microglia cells and the potentiation of neuroinflammatory processes[102,105]. Acute stress was also shown to sensitize hippocampal microglia *ex vivo.* Microglia isolated 24 h post-stress and presented with an *ex vivo* challenge with LPS showed an increase in the pro-inflammatory response triggered by this immunogenic stimulus [106]. Stress alone has been shown to be able to induce microglia proliferation through a process mediated *in vivo* by corticosterone-induced NMDS receptor activation[104]. Altogether, these data support the notion that microglial cells are very susceptible to stress and that stress can lead to profound alterations in the number, morphology and gene expression of microglia, contributing to a pro-inflammatory environment within the CNS.

2.2. Early-Life stress

The early post-natal brain is far from being maturated, which makes childhood a critical period for neurodevelopment. The process of brain maturation is complex and is regulated by both genetic and environmental inputs. During the first stages of life outside the womb, the brain continues to experience critical developmental processes, such as selective neuronal apoptosis, axonal and dendritic growth, synaptic stabilization and synaptic pruning[108]. The complexity of these events renders the brain particularly susceptible to stress. In fact, when stress is experienced during this crucial period, the effects on brain function can be striking

and long-lasting or even permanent, when compared to the effects of stress in the adult brain[109,110]. ELS can lead to alterations in neuronal structure and in the wiring of neuronal circuits which, in adulthood, can translate into impaired behavior, cognition and changes in overall emotional responses[111,112]. Taking this into account, it is easy to understand that ELS is considered an important risk factor for several neuropsychiatric disorders, such as mood and anxiety disorders, depression, ASD, schizophrenia, psychosis and PTSD [113,96,114,115,116,117]. A recent World Mental Health survey has pointed that early-life adversity, such as that found in dysfunctional family clusters (related to violence, physical and sexual abuse, neglect, and substance abuse), is strongly correlated with 59.5%, 32.6% and 13.6% of childhood, adolescence and adulthood-onset mood and anxiety disorders[118].

Several studies have also shown that the outcome of ELS depends on several aspects of the stressful experience, such as time, quality, severity and duration of the event. Because of the immaturity of both the brain and the stress response in the early-life period, several factors that are a cause of stress in adulthood, may not be stressful in the perinatal period[119]. In humans, ELS presents both physical and emotional components, although the emotional aspect is dominant. Abnormal maternal care, including neglect and lack of compassion, is one of the main causes of ELS[120] and the complete absence of maternal care can lead to disastrous emotional and cognitive consequences later in life.[121] In rodents, as is the case in humans, maternal care plays a critical role in development, by providing not only nutrition, but also important environmental sensory cues to the pups[122,123]. As such and given the importance of impaired maternal care to the development of ELS, several mouse models of ELS have attempted to mimic the effects observed in humans by manipulating the maternal-pup interaction.

2.2.1. Models of early-life stress

There are three main developmental periods during which stress can have severe and permanent effects in offspring development and in behavior: 1) the prenatal period, which comprises the gestational time and during which stress mediators in the mother can reach the fetus, 2) the postnatal period, which corresponds to the period following birth and P23 in mice (8 years in humans) and in which the mother-pup integration is of crucial importance and 3) the juvenile/adolescence period, which goes from the P23 to P60 in mice (from 8 to 18 years in humans) and during which the brain is still undergoing important developmental changes. While stress in the prenatal period has programming effects on the development of several brain regions involved in the regulation of the HPA axis, such as the hippocampus, the amygdala and the PFC, post-natal stress has an enormous variety of consequences that vary

according to the type and timepoint of the stressors. During the post-natal period, all developing areas are sensitive to the effects of stress hormones, nevertheless, some areas undergo an important period of fast development and growth, during which they became particularly susceptible to the effects of stress. From birth to P7 (2 years in humans) the hippocampus is developing and, consequently, is the brain area most vulnerable at this time age. On the other hand, the amygdala continues to develop until adulthood (P78 in mice and 20 years in humans), meaning that exposure to stress from birth to late childhood can possibly provoke changes in this region's volume. During adolescence (8 to 12 years in humans and P21 to P40 in mice), the frontal cortex experiences a major increase in volume and, consequently, exposure to stress during this period has major effects in frontal cortex organization. Evidence also suggests that adolescence is a period of vulnerability to stress due to a prolonged GC response that can be observed at this stage and that can endure up to adulthood with potentiation/incubation effects. Taking this into account, stress experienced during this time periods can lead to long-lasting manifestations in brain development and consequently changes in behavior that can persist into adulthood [96,112,124].

2.2.1.1. Prenatal Models

Experimental investigations using animal models that mimic stress experiences during gestation in humans are key to understand the mechanisms at the base of the long-term consequences of prenatal stress experience. In rodents, prenatal stress can be induced by the exposure of the pregnant dams to repeated or single stressors during distinct gestational phases, with the objective of transferring the stress experience to the embryo in utero. There are several different types of stressors that can be induced to the dam such as: maternal immune activation induced by LPS or GC injection, malnutrition or unpredictable mental or physical stress. The long-term consequences of pre-natal stress in the offspring can vary according to the type of stressor and the gestational phase at which dams are exposed. These consequences vary from learning impairments, depressive and anxiety-like behaviors, increased sensitivity to drug abuse and altered immune response. For example, an immune challenge by LPS on E 9.5 increased IL-1β serum levels in the adult offspring, following a second LPS stimulus, revealing that prenatal LPS exposure modified the immune response to a LPS challenge in adulthood[125] and induced autistic-like behavioral and immune disturbances in childhood and adulthood[126,127]. Other studies have also shown that prenatal corticosterone injections result in an impairment in both the acquisition and recall of cue-conditioned fear extinction in males, as well as decreased levels of GR proteins in the medial PFC (mPFC), hippocampus and hypothalamus[128]. Studies have also shown that the dopaminergic system, which is involved in reward and drug-seeking behaviors, is also altered by the interaction

between prenatal stress, higher levels of GC and alterations in dopaminergic neurons[129]. In addition to this, prenatal maternal stress and the consequent exposure of the fetus to higher levels of GC during the gestational period, has been linked to a delay in the maturation of neurons and glia, as well as to significant alterations in the process of myelinization and vascularization, which leads to altered neurogenesis, neuronal structure and synapse formation[130,131].

2.2.1.2. Postnatal Models

After birth and during the first weeks of life, rodent pups stay in the maternal nest. During this time period, it is known that mother-pup and pup-pup interactions play a crucial role in the development of the brain and in the acquisition of social skills.[132] Several studies have shown that alterations in these interactions can have a major impact in the offspring development, inducing profound and long-lasting effects on emotionality and stress response. In particular, the responsiveness of the HPA axis can be severely deteriorated by interrupting usual mother-pup interactions, which may induce persistent changes in the neurobiology, physiology, and emotional behavior of adult animals[133,134,135,136]. Even though the initial work on the mechanisms of stress response indicated that during the first two weeks of the rodent's life (P1 to P14) the HPA system is relatively unresponsive to stress, presenting low corticosterone levels and a lack of response to a variety of typical stressors, which seems to protect the immature brain from the deleterious effects of GCs, it is also known that immature pups have an increase in plasma corticosterone levels in response to age-appropriate stressors[137,138].
One of the most well characterized models of postnatal ELS is the maternal separation model (MS), which consists in daily separation of the pups from their mother for a determined period of time. This model is considered one of the most potent stressors to the pups, leading to alterations in HPA axis and, consequently, to changes in both basal and stress-induced corticosterone levels, not only in the postnatal period but also persisting into adulthood[112,133]. However, the alterations caused by maternal separation are much deeper than the HPA axis and include changes in the CRH levels in several brain regions[139], which can be related with alterations in neuronal architecture and anxiety and depressive-like behaviors[140]. Intriguingly, depending of the MS protocol applied, especially in what concerns the duration of the protocol (short-term or prolonged separation), the HPA axis can be hypo or hyper activated, respectively[141,142,143]. The alterations in behavior also vary according to the stress protocol[133]. A deeper examination of the MS protocols will be described below.

Other postnatal stress models are focused on promoting changes in maternal behavior, causing fragmented or erratic maternal care, to mimic depressed and severely stress mothers,

or mimicking a poverty and low-resource environment, by providing limited nest material. This impoverished environment prevents the dam to build a satisfactory nest, which results in chronic stress and erratic maternal behavior. The disrupted maternal care leads to chronic stress in the pups and results in hypertrophied adrenal glands at the end of the stress period, which, in adulthood, is traduced by progressive loss of cognitive function[144].

2.2.1.3. Adolescence

The adolescence period in rodents has been described to have three stages: an early adolescence period, from P21 to 34, a mid-adolescence period from P34 to 46 and a late adolescence period from P46 to 59[112]. Although the early and mid-adolescence periods are times of significant brain development, especially in the PFC, there is little research on the effects of stress in this time period. However, it is known that HPA function in adolescent rodents is characterized by a long-lasting activation in response to stress and that, during puberty, the rise of GCs is delayed and prolonged in response to several types of stressors, when compared to adult animals[145], due to the incomplete maturation of the negative-feedback systems[146].

There are several different paradigms for inducing stress in the adolescence period that lead to different consequences. Social isolation paradigms are often used, with the most common paradigm being the social isolation rearing paradigm where pups are weaned from the dam and single-housed from P28 to 55. Animals subjected to this paradigm present a variety of alterations in behavior and in HPA axis activity[147,148].

Other paradigms also commonly used are the social defeat and chronic variable stress paradigms. The social defeat paradigm consists in the exposure of a subordinate juvenile male or female rodent to a dominant adult conspecific. Juvenile rodents subjected to this paradigm have been show to present depressive-like behavior and social subjugations[149]. The chronic variable stress paradigm includes exposure, twice a day, to different stressors including: social isolation, crowding, forced swimming and restrain[150]. Like the MS model, this paradigm varies in the duration of exposure and in the number and type of stressors employed, which leads to different results according to the chosen protocol (decreased or increased HPA reactivity)[151].

Overall, acute and chronic stress experienced during adolescence have the potential to significantly alter the HPA axis activity and, consequently, cause anxiety-like and avoidance

behaviors, as well as altered spatial learning and increased susceptibility to drugs of abuse[152,153].

2.3. Maternal Separation

2.3.1. Maternal bonding

Freud was the first to discuss the relevance of mother-infant interaction over 100 years ago. Later in the 1950s, Bolwby's attachment theory postulated that the ability of the child to form a specific bond with the caregiver and the nature of this attachment, lays the foundation for the child's future model of social relationships, affecting their social, emotional and cognitive development throughout life. This theory likewise suggests that many forms of emotional distress and personality disturbance in adulthood can be explained by the disruption of the bonding process in early childhood.[154] This theory gained significant relevance in the 1970s with the work of Rutter, thanks to the wide acceptance that alterations in familiar relationships during childhood could have long-lasting or even permanent effects in the child's development[155]. The central principals of these theories (that the relationship between the parents and the chid is the critical foundation for the emotional health of the infant) continues to influence the conceptual bases for work in paediatric mental health disorders until today[156].

In fact, there is substantial evidence from several epidemiologic studies pointing to the existence of a strong link between early-life adversity and mental disorders later in life. Meta-analysis studies have shown that maladaptive families can have an important impact in the adult-onset of several mental health disorders[157,158]. For instance, improper maternal care and maternal neglect in the first years of life induce alterations in the social brain networks leading to enhanced processing of angry emotions and reduced processing of happy emotions[159]. Childhood maltreatment has also been linked to cognitive and emotional alterations, such as an increased risk for major depression, and can have a significant impact in hippocampal and PFC volume[160]. Parental loss during childhood has also been linked to alcohol and substance dependency and psychosis[161, 162].

But not only the mental health is affected by ELS, stressful events experienced in the first years of life produce harmful effects also in processes related to growth, metabolism, reproduction and immune responses [96,113,163,]. For instance, exposure to ELS has been linked to higher levels of inflammatory cytokines such as C-reactive protein[164] and interleukin-6[165] later in life.

Taken together, this evidence suggests that ELS and maternal separation can have profound impact in several biological systems particularly in brain development, being considered an important cause of major health problems due to the persistence of their deleterious effects throughout life and the possibility of transmission to the next generations.

2.3.2. Rodent protocols of maternal separation

Since Bowlby postulated the attachment theory in the early 50s several researchers developed a variety of maternal separation protocols (as shown in Table 1) to evaluate the influence of early experiences on behavioral phenotypes and neuroendocrine responses. The types of protocols used can be classified in 6 different groups: Non-Handled (NH), Animal Standard Facility Rearing (AFR) and Early Handling (EH) which are normally used as control groups and Brief Maternal Separation (BMS), Prolonged Maternal Separation (MS), Maternal Separation and Unexpected Stress (MSUS) and Maternal Deprivation (MD) used as separation protocols.[166]

In addition to this classification, it is also possible to classify the methodological details that often vary among different protocols, called methodological options Maternal separation protocols can differ in: 1) the chronicity of stressors, using single or repeated episodes of separation, 2) the pup's contact with each other during the separation period, meaning that pups can remain together as a litter or have no contact with each other (pup isolation), 3) the presence or absence of temperature control, 4) the time period during which the protocol is applied and 5) whether the pups remain in the home-cage during the separation or are placed in a new cage[167].

As reviewed in Tractenberg *et al.,* most of the studies (67.70% from the 94 studies evaluated) applied the MS protocol, while only 31.85% used the BMS protocol. A very small percentage of studies (11.45%) opted for MD exposure. In relation to the control groups, 85.41% of the studies used AFR as a control group, 9.37% used NH and only 5.20% used EH. Despite protocol differences all models were able to reproduce adverse early-life experiences which included feeding deprivation, altered maternal care and neglect, as well as to induce behavioral and phycological alterations such as depressive and anxiety-like behaviors.

Table 1| Maternal Separation protocols Adapted from Nylander and Roman, Psychopharmacology (2013) and Tractenberg *et al,* Neuroscience and Biobehavioral Reviews (2016)

Type of post-natal stress procedure	Abbreviation	Definition	Purpose	Others
Non-handled	NH	Pups remain undisturbed and in contact with the dam. No manipulation of the litter and no cage change or cleaning until weaning.	Controls for repeated handling and brief or prolonged separation.	Not possible to distinguish between handling and separation effects.
Animal standard facility Rearing	AFR	Pups remain constantly together and in contact with the dam except for cage changing or cleaning.		
Early-handling	EH	Brief manipulation of the pups by the investigator by a short period of time (<5min).	Control for prolonged separations. Simulate wild- life conditions.	Controls exposed to the same handling. Associated with beneficial effects later in life.
Brief Maternal separation	BMS	Pups and dam are separated for a brief period (≥ 10min and <60 min).		
Prolonged maternal separation	MS	Pups are separated from the dam for a prolonged period (≥ 60 min to <480 min).	Disrupt social mother- pup interactions. Simulate early- life social stress and risk environment.	Associated with negative consequences later in live.
Maternal Separation and unpredictable stress	MSUS	Pups are separated from the dam for 3h and the dams are subjected to unpredictable stress.		
Maternal deprivation	MD	Pups are deprived from maternal care for 24h. Pups can return to the dam for short periods of time for feeding.		

2.3.3. Impact of MS in behavior

In humans, adverse early-life experiences such as childhood abuse or traumatic events have been shown to contribute for the development of psychiatric conditions like depression and PTSD later in life. Young children with a history of institutionalization often show poor attention, hyperactivity, difficulty with emotion regulation, elevated levels of anxiety, increased rates of attachment disorders, and indiscriminate friendliness[168,169,170,171,172]. Despite this evidence, conducting studies in children with history of psychological trauma and/or abuse history is very difficult and, therefore, animal models such as described above are of great value. Previous animal studies in rodents and monkeys have revealed that ELS induced by MS has negative effects on behavioral and neurobiological phenotypes in adulthood.[173] The importance of the maternal bonding in pups development was first approached by Harlow in the 1970's with studies in maternal separated rhesus monkeys[174] and its work was rapidly replicated in rodents. A study by Lippmann and co-workers have shown that maternal separation in rats produced long-term behavioral deficits, such as decreased locomotor activity, increased number of rearing counts as well as increased time spent grooming compared to control animals, behavioral alterations that resemble the ones underlie mood disorders[175]. Other study by Leussis *et al.*, in 2012, demonstrate that MS increases depressive-like behaviors in mice during adolescence in a sex-dependent way[176] and more recent studies, such as the one performed by Portero *et al.* were able to demonstrate that MS induces deficits in social interaction similar to the ones displayed in ASD being associated with higher vulnerability to develop alcohol drinking behaviors, due to a decreased sensitivity to alcohol-induced rewarding effects[177].

2.3.4. Impact of MS in mPFC circuit formation

The mPFC is one of the most susceptible brain regions to the effects of MS due to the fact that is one of the last brain regions to mature[178] and because it presents a significant density of GC receptors[179]. The rodent mPFC includes the medial orbital cortex (MO), the ventral part of the prelimbic (PrL) cortex, the dorsal anterior cingulate (CgL) cortex and the infralimbic (IL) cortex. In terms of function, the mPFC has been implicated in early-social cognition[180], emotional processing[181] and emotional learning[182], in working memory and decision making[183], and in inmate fear regulation[184]. As reviewed in McEwen *et al.* the amygdala-hippocampus-mPFC circuit has also been implicated in anxiety and depressive-like behaviors[185]. Moreover, the mPFC has been shown to exert inhibitory effects on the main monoamine projections to subcortical regions that control the stress response, acting as an inhibitory feedback control over the HPA axis activity[186]. Taking this into account it is easy to understand that ELS, and

especially MS, can have a profound impact in the development of the mPFC circuits leading to long-lasting structural and functional consequences.

In fact, several human studies have been showing that adults who have experienced adverse early-life conditions present reductions in hippocampus and frontal cortex grey-mater volume[187]. Preclinical studies in mouse models also find similar changes[188,189]. In mice, PMS was shown to significantly increase the dendritic complexity and dendritic spine number in the hippocampal CA1 and CA3[190], as well in the mPFC[191]. Other studies using a precocious rodent, the degu (Octodon degus), a diurnal South American rodent that lives in complex social family structures and that are born with relatively mature sensory systems, was able to show that MS was accompanied by a significant decrease in brain activity in a number of brain areas, including the PFC[192] and that animals exposed to repeated MS presented higher dendritic spine densities in the prefrontal anterior cingulated cortex and in the IL cortex when compared to non-stressed controls[193], which may indicate a delay or impairment in synaptic pruning during development. Changes in spine density were also observed in the amygdala and in the hipoccampus[194]. Moreover, changes in inhibitory system were also reported with MS in this model, leading to a decrease of presumably inhibitory shaft synapses[193] and alterations in inhibitory GABAergic interneurons[195,196]. Furthermore, alterations in the dopaminergic and serotonergic fibre innervation where reported in the mPFC, hippocampus and amygdala[197,198].

This alterations on spine density result in an increased excitatory input on pyramidal neurons that is amplified by the reduction in parvalbumin-positive GABAergic interneurons resulting in an excitatory/inhibitory imbalance in the modulation of pyramidal neurons activity, which is amplified by the alterations in the modulatory function of the dopaminergic and serotoninergic systems, presumably leading to alterations in the regulation of the hippocampus-amygdala-PFC circuits and the HPA axis and resulting in the behavioral outcomes above mentioned[199]..

2.3.5. Impact of MS in gene expression

Several groups have been focusing in the genetic and epigenetic alterations caused by MS in several rodent's brain regions, such as the mPFC, hippocampus and amygdala over the past few years. The epigenetic signature of MS includes alterations in DNA methylation of genes involved in neuronal signalling, transcription and translation, in histone modifications and alterations in miRNA expression levels.

In the mPFC of adult mice, the MS and early-weening protocol lead to the downregulation of several translation initiation factors such as *Eif2a* and *Eif5b* and to the upregulation of certain

translation-related genes such as *Eif2c1*, *Eif2c2*, *Eif4ebp2* and *Rplp1*, *Rps2*, *Rps6ka2*, and *Rps6kc1* which suggests a dysregulation of translation. Downregulation of several myelin related genes, including *Cldn11*, *Cnp*, *Ermn*, *Mag*, *Mal*, *Mbp*, *Mog*, *Plp1*, and *Ugt8a* and DNA methylation in the promoter region of some dysregulated genes (*Mbp*, *Cdc42se1*, *Drd1*, *Gad1*, *Gnai*, *Gng2*, *Kcnab2*, *Kcnh1*, *Ppp2r1a*, *Prkaca*, *Prkar1b* and *Prkcc*) were also reported[200]. Another study from Benekareddy *et al.* also reported an upregulation of cellular developmental and neuronal plasticity-related genes such as the *Grin2d*, *Nlgn1*, *Camk1g*, *Ppp3r2* and *Ppp3ca* and an enrichment in genes involved in G-protein signalling (*Kcna3*, *Kcnh2*, *Kcnh7*) and in signalling transduction (*Art5*, *Gnb1* and *Impa1*)[201].

Alterations in hippocampal epigenome were also reported in animals subjected to poor maternal care. Weaver *et al.* reported alterations in DNA methylation at a specific sequence upstream of the glucocorticoid receptor gene (*Nr3c1*) in the hippocampus of the offspring, with direct impact in transcription[202].

ELS was also linked to epigenetic alterations in the arginine vasopressin gene (*Avp*)[202], in the hypothalamic paraventricular nucleus and in other genes such as *Bdnf*[203], *Crh*[202], *Dlgap2*[204], *Mecp2*, *Cnr1* and *Crhr2*[205].

Together, these findings strongly suggest that epigenetic changes can occur in multiple neurobiological pathways in response to early-life stress.

2.3.6. Impact of MS in immune response and microglia activation

Both animal[206,207,208,209] and clinical[210,211,212] studies have presented evidence that ELS can induce changes in the immune system. Studies in infant rhesus monkeys have shown that MS enhances expression of genes involved in inflammation and T-lymphocyte activation and reduces expression of genes involved in innate immune response functions, including antigen presentation and antimicrobial responses to bacteria, fungi and viruses[213]. Another study from Hennessy *et al*. showed that administration of anti-inflammatory agents, such as alpha-melanocyte stimulating hormone or IL-10, or a prostaglandin synthesis inhibitor to guinea-pigs undergoing MS resulted in a decrease in passive behaviors[214]. Moreover, Heerden and colleges found that mice subjected to MS presented altered expression of several genes whose products are important modulators of the immune system, such as Foxp3, IL-17, and Ccl5[215]. Furthermore, another study, from Dimatelis *et al.* presented evidence that MS induced a down-regulation of several cytokine expression levels such CCL7, CCR4, IL-10, IL-1β, IL-5R-α and CD11b[216]. Alterations in the number of CD11b-positive cells in the blood immediately after the MS protocol have also been reported[217].

Nevertheless, the peripheric immune system is not the only immune hub altered by ELS. Several studies have shown that chronic ELS leads to neuroinflammation and alterations in microglia number, morphology and motility in several brain regions involved in stress response, such as the mPFC, hippocampus and hypothalamus[217,218,219,220,221]. Gracia-Rubio *et al*. has shown that MS leads to microglia activation in female MSEW mice in the mPFC and DG, CA1 and CA3 areas of the hippocampus[219]. Another study from Roque and co-workers presented evidence that MS induces microglia activation in the hippocampus, with increased expression of IL-1 and diminished expression TNF-α, and in the hypothalamus, leading to increased expression of IL-6 and TNF-α. These results indicate that MS induces a differential activation of glial cells, depending on the brain structure involved[221]. Microglia motility and ramification in adulthood has also been shown to be altered in the somatosensory cortex of mice subjected to MS, with microglia from MS mice presenting a more ramified profile and higher motility when compared to controls[218]. Furthermore, MS have also been shown to prime microglia and enhance microglia response to an LPS stimulus later in life (two-hit model) [222].

3. Final considerations

Microglia has been shown to play a critical role in neurodevelopment, by supporting neuronal function and survival, refining neuronal circuits through synaptic pruning and synaptic remodelling and by participating in synaptic plasticity mechanisms. However, as an integral part of the immune system and the only type of immune cells in the healthy brain parenchyma, microglia cells are also very sensitive to environmental cues and pro-inflammatory stimulus. In fact, several neuropsychiatric and neurodevelopmental disorders, such as ASD and schizophrenia, are now starting to reveal an important neuroinflammatory component that includes major microglia dysfunctions. On the other hand, the young brain is particularly susceptible to stress and the occurrence of ELS has been directly linked to a higher risk for developing neuropsychiatric disorders, such as major depression and PTSD, as well as to alterations in microglia number, morphology and cytokine expression. Taken together, this evidence suggests that ELS-mediated neuroinflammation and microglia dysfunction can contribute to alterations in neuronal circuits that are implicated in several neuropsychiatric disorders throughout life.

4. Objectives and framework

Microglia disfunction has been suggested to play an important role in several neuropsychiatric/neurodevelopmental disorders such as ASD, schizophrenia and major depression. ELS is a known risk factor for the development of these same pathologies. Even though the underlying mechanisms of the relation between ELS and microglia-mediated neuroinflammation are not known, a correlation between stress in the early-life periods and alterations in microglia activation, with consequences to microglia-mediated physiological processes, which are crucial for the correct formation of neuronal circuits, has already been recognized. However, most of the studies performed so far concerning the relation between ELS, inflammation and neuropsychiatric/neurodevelopmental disorders have been focused on male animals, with little information available about the impact of ELS in the female brain

Therefore, the core aims of this study are:

1. To evaluate the differences in microglia density, morphology and function in the mPFC of male and female adolescent mice;
2. To determine the impact of ELS in microglia activation, morphology and function;
3. To assess the impact of ELS on social behaviors and investigate the contribution of this type of stress to the onset of depressive and anxiety-like behaviors.

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CHAPTER 2 | MATERIALS AND METHODS

1. Animals and housing conditions

Adult C57Bl6/J females and males (~12 weeks old) were used for mating. Mattings were performed by a male with two females over 1 week. Afterwards the male was removed from the breeding cage and each pregnant female was single housed.

Experiments were performed in adolescent (P40) and adult (P90) male and female mice. Tested animals were maintained in controlled conditions: temperature (22°C) and humidity (60%), on a 12-hours light/dark cycle (lights on at 07:00 am) with water and food *ad libitum.* Cage changes were performed twice per week.

Animal maintenance, treatments and experimental procedures were conducted according with Animals Use and Care Guidelines issued by FELASA (Federation for Laboratory Animal Science Associations) and the guidelines and regulations approved by Portuguese–ORBEA (Local agency responsible for Animal Welfare of the University of Coimbra/CNC) and DGAV (Portuguese Regulatory Agency) – and European directives on animal welfare.

2. Maternal Separation and Unexpected Stress

The maternal separation and unpredictable maternal stress (MSUS) model constitutes a well described neonatal stress model for rodents. I this work, the MSUS was employed to reproduce the effects of childhood neglect and abuse, aiming at identifying the possible effects of ELS on microglia and microglia-mediated immune responses.

In preparation for the MSUS protocol, pregnant females were individually housed and inspected daily for delivery. Delivery date was assigned as day 0. Maternal separation of 3 hours per day was performed during the first 2 weeks (from P2 to P14) during the dark cycle. Mothers and pups were placed in separated clean cages with bedding. Food and water was provided to the dams and the pups remained together during the separation period without temperature control.

Cages were placed side-by-side to allow visual and olfactory contact. The timing of the separation was unpredictable and comprised a 3h period between 06:00 pm and 03:00 am. Maternal unpredictable stress consisted of 20 min of restraint in a Plexiglas tube or 5 minutes of forced swim in cold water (18°C). A MSUS schedule example is presented bellow in Table 2.

Control litters were left undisturbed except for cage changes. MSUS and control cages were changed twice per week and pups were weighted on PND2, 7, 14, and 21. At PND21 pups were marked with paw tattoos (subcutaneous ink injection) to allow the identification of each animal throughout the experimental period. Litters were weaned at PND21 and males and females were reared in social groups of 4 to 5 mice from the same treatment and from different mothers per cage (MSUS and controls were allocated in separate cages). Animals were left undisturbed until PND30, except for cage maintenance twice per week. From PND30 to PND40, male and female mice were tested in several behavior paradigms, which will be described in the next sections. The MSUS protocol was performed in 3 temporally independent cohorts, each one composed by at least two litters of MSUS and control animals. One of the cohorts was maintained until PND90 for another set of behavioral experiments, in order to assess if alterations in behavior at PND40 were sustained until adulthood. Between PND40 and PND90 animals were left undisturbed except for cage maintenance twice per week.

Table 2 | Example of a MSUS schedule

Day	PND	MS timing	Maternal tress	Time of maternal stress
1	PND2	21:00-00:00	Forced swimming	22:00-22:05
2	PND3	18:00-21:00	Restrain	19:40-20:00
3	PND4	20:00-23:00	Forced swimming	22:30-22:35
4	PND5	23:00-02:00	Restrain	01:40-02:00
5	PND6	18:45-21:45	Forced swimming	21:40-21:45
6	PND7	00:00-03:00	Restrain	00:00-00:20
7	PND8	20:00-23:00	Forced swimming	21:00-21:05
8	PND9	20:00-23:00	Forced swimming	21:00-21:05
9	PND10	00:00-03:00	Restrain	00:00-00:20
10	PND11	18:45-21:45	Forced swimming	21:40-21:45
11	PND12	23:00-02:00	Restrain	01:40-02:00
12	PND13	20:00-23:00	Forced swimming	22:30-22:35
13	PND14	18:00-21:00	Restrain	19:40-20:00

3. Behavior assessment

Adolescent and adult MSUS and control, male and female mice were tested in a range of behavioral tests in order to validate the MSUS protocol and assess how ELS alters social behavior. The EPM and FST were performed to assess anxiety and depressive like-behaviors, respectively, and sociability was assed in the three-chamber test. Behavior was assessed between P30 and P40 (adolescence period) and P90 and P100 (adulthood). Behavior tests were performed with at least one day interval between different paradigms and test order was the same between cohorts. Tests were performed in the same behavior room during the non-active light cycle. Mice were transferred to the behavior room the day before the test in order to acclimatize. All tests were conducted between 09:00 am and 06:00pm. All behavior quantification and analysis were performed by trained experimenters blinded to the treatment, and behaviors were monitored either by direct observation or using automated video tracking (Ethovision or Observer XT, Noldus Information Technology).

4.1 Elevated Plus Maze

Anxiety-like behaviors were assessed using the elevated plus maze (EPM).

Juvenile (P30-P40) MSUS and control mice were tested in the EPM. The maze consisted of a plus shape apparatus with two open (without walls) and two closed (with walls) arms, each one with 30cm x 5cm and elevated 50cm above the floor. A ledge of 5mm in the height was built around the open arms to prevent excess falling of the animals. The illumination was provided by two white LED lamps positioned over the maze open arms so that the open arms surface was illuminated at approximately 300 lux. Each mouse was placed in the center of the apparatus facing one of the closed arms. Movements were recorded with a video camera for a total of 10 minutes and total time spent in the open arms, as well as latency to enter the open arm were quantified automatically using Ethovision (Noldus Information Technology). The arena was cleaned with 70% ethanol at the end of each trial, whipped with paper towels to remove residues and odors and allowed to completely dry for 10 minutes before the next trial. Trials were always performed first in males and second with females always alternating between controls and MSUS.

4.2 Forced swimming test

The forced swimming test (FST) was performed to assess depressive-like behaviors defined by increased immobility and decrease latency to immobility. Juvenile (P30-P40) and MSUS and control mice were tested in the FST. The surrounding light was indirect and homogeneously adjusted with white LED lamps to approximately 20 lux. Males were tested first and females second. Two mice from different treatments and from the same sex were tested simultaneously, side by side. Mice were placed in a 2L glass beaker (19,3 cm high and 13,1 cm diameter) filled up to 1,5L (12cm) with water at 18 ± 1°C and behaviors were videotaped for 6 minutes. Animals were then removed and allowed to dry in a clean cage before returning to their home cage. Latency to stop swimming and total time spent immobile were scored manually using Observer XT (Noldus Information Technology). Immobility was defined as the lack of motion of the entire body except small movements to keep the mouse's head above water. The water was changed, and the beaker was cleaned with 70% ethanol and distilled water between trials.

4.3 Three-Chamber Social Test

The three-chamber social test was performed in order to assess social behaviors. The arena consisted of a rectangular apparatus divided in three chambers by transparent partitions each bearing an opening to allow communication between adjacent chambers. Juvenile (P30-P40) and adult (P90-P100) MSUS and control mice were tested in the three-chamber social test to evaluate social interaction and social memory. Males were tested first and females second.

The apparatus was illuminated with indirect and homogeneous white LED lamps adjusted approximately to 20 lux. Each mouse performed three sequential sessions. In the first session (acclimatization) the tested mouse was allowed to freely explore the three chambers for 20 minutes in order to familiarize itself to the arena. Next, in the second session, the tested mouse was limited to the middle chamber while an empty small wire cage (empty) and a small wire cage with an age and sex matched unfamiliar wildtype C57Bl6/J mouse (Stranger 1) were introduced in one corner of each of the side chambers, aiming at testing social preference. The mouse was left to freely explore the arena for 20 minutes. In the third and final session, the tested mouse was again gently constrained to the middle chamber while the empty wire cage was replaced by other age and sex matched unfamiliar wild type C57Bl6/J mouse (Stranger 2). The mouse was tested for social memory by being allowed to explore the three chambers for 10 minutes. The stranger mice were previously habituated to be trapped in the small wire cages and were alternatively used as Stranger 1 and 2 to ensure that both animals were used

in the same number of trials between the two experimental groups. The positions of the Empty and Stranger 1 cages were also alternated between trials. Tested mice were video-tracked during the total 50 minutes. Time spent in close proximity (considered whenever the tested animal was inside the surrounding area, up to approximately 10 cm away) with the small wire cages was calculated using Ethovision (Noldus Information Technology). The arena was cleaned with ethanol 70% between trials and wiped with paper towels to remove residues and odors and allowed to completely dry before the next trial. Males were tested first and females second and trials were alternated between control and MSUS mice.

5. Brain dissection and tissue processing for microglia morphology and immunohistochemistry analysis

Adolescent (P40) and adult (P100) MSUS and control mice were sacrificed, and brains were dissected and processed for brain slice imaging. Briefly, animals were anesthetized with isoflurane (Abbott, USA) and perfused transcardially with approximately 40 mL of PBS 1%. Whole brain was dissected and post-fixed overnight in 4%PFA, afterwards transferred to a solution of 30% sucrose in PBS for cryopreservation and frozen at -80ºC until further processing. Coronal sections of 50 µm were serially cut using a cryostat (Thermo scientific CryoStar NX50) and all slices encompassing the mPFC were collected into 48 multi well plates with Azide 0,02% PBS 1% and stored at 4ºC until further processing.

6. Immunohistochemistry (Transmission)

Microglia cell density was quantified by immunohistochemistry staining, in order to investigate possible effects of ELS on microglia number. Coronal brain slices (50µm), containing the mPFC, were stained for IBA-1. Before the immunohistochemistry protocol, sections were washed 5 times for 5 minutes with PBS 1x. The immunohistochemistry staining was performed in free-floating sections incubated in 24 multi-well plates (10 sections per well). Briefly, sections were incubated for 30 minutes at 37ºC with phenylhidrazine to quench peroxidase activity. Following washing with PBS, sections were blocked and permeabilized with 10% normal goat serum (Thermo Scientific, USA) 0,1%Triton in PBS for 1h at room temperature (RT) and then incubated with the primary rabbit anti-IBA-1 antibody (Wako, USA) 1:1000 in the same blocking solution, at 4ºC over-night under constant agitation. On the next day, sections were washed with PBS and incubated with the secondary biotinylated antibody anti-rabbit IG (Vector Labs, USA)1:200 for 2h at RT, also under constant agitation. Following another washing step with

PBS, bound antibodies were visualized using the VECTASTAIN® ABC kit (Vector), according to the instructions of the manufacturer, and 3,3'-diaminobenzidine tetrahydrochloride (DAB metal concentrate - Vector) was used as substrate. After washing with PBS, brain sections were mounted on microscope slides and dried at RT, followed by dehydration with an ethanol gradient and xylene. Slides were then covered with Eukitt® (PanReac AppiChem, USA) mounting medium and a microscope coverslip. Brain slices where visualized in an Axio Imager 2 microscope (Carl Zeiss, Germany), equipped with ×5, and ×20 objectives, employing the commercial software Stereo Investigator (MBF Bioscience) for unbiased stereology analysis.

7. Stereology

For each animal, 10 random sections with a fixed distance of 150 μm between each other, were analyzed using stereology, following a random choice of brain slices. A number code was attributed to each animal to ensure blinding of the investigator performing the stereology analysis. Unbiased stereology estimation of microglia cells was performed using the commercial software Stereo Investigator (MBF Bioscience). Following IBA-1 staining, sections were visualized under an Axio Imager 2 (Carl Zeiss, Germany) and the regions of interest (ROIs) were identified in each section: Medial Orbital Cortex (MO), Pre-Limbic Cortex (PrL), anterior Cingulate Cortex1 (CG1) and Infralimbic Cortex (IL). Microglia cells were counted using a sampling grid of 160 x 160 μm and a counting frame of 80 x 80. The same sampling and stereology parameters were used for all ROIs and in all animals and an area sampling fraction of 0,19 and an error coefficient bellow 0,05 was achieved.

8. Immunohistochemistry (Fluorescence)

Microglia cell morphology was assed by immunohistochemistry staining in order to investigate possible effects of ELS.

For IBA-1 fluorescence labelling, rabbit anti-Iba1 antibody was once again used. Briefly, sections were washed with PBS, permeabilized with 10% normal goat serum 0,1%Triton in PBS for 1h at RT and then incubated with primary rabbit anti-IBA-1 antibody (Vector) 1:1000 in the same blocking solution, at 4ºC over-night under constant agitation. On the next day, sections were washed and incubated with the necessary secondary antibody: anti-rabbit Alexa Fluor® 488 (Invitrogen, USA) for 2h at RT, also under agitation. Following washing, brain slices were mounted onto microscope slides with Vectashield mounting medium (Vector) and

visualized in a 710 LSM confocal microscope (Zeiss), equipped with x40 objective, employing the Zen Black software.

9. Morphological Analysis

Microglia morphology analysis was performed in order to assess if ELS could induce alterations in morphological parameters. Z-stacks from microglia cells from the MO, PrL, CG1 and IL regions of the mPFC were acquired in a 710 LSM confocal microscope (Zeiss) with a Pln Apo 40x/1,4 DIC lens. Microglia cells were randomly chosen and at least 10 cells were acquired per animal. Each image was a compilation of images taken across the Z plane of the sections with a step of 0,4 and a medium of 30 planes per cell. Confocal microscope settings were kept the same for all scans. Microglia cells were traced and reconstructed using the surface and filament tools from the Imaris software (Bitplane). While surface analysis allowed to calculate the total area, volume and sphericity of each cell, filament analysis allowed to perform a 3D reconstruction of microglia branches in order to perform sholl analysis and calculate the total length and number of branches.

10. Brain tissue collection, RNA extraction and quantitative real-time PCR

RNA brain samples from adult (P100) control and MSUS mice were extracted to identify possible genetic correlates of ELS effects on microglia and behavior. Animals were anesthetized with isoflurane (Abbott) and sacrificed by transcardial perfusion with PBS. Brains were dissected and the mPFC was collected and immediately frozen and stored at -80ºC until processing for RNA extraction. Total RNA was purified from the mPFC using NucleoSpin® RNA (Macherey Nagel, USA), according to the manufacturer's protocol. During the procedure, a step of elimination of genomic DNA was performed in the silica columns using the DNase provided with the kit. Samples were eluted in 30 µL of RNase free water and concentrations were measured in a Nanodrop 2000 spectrometer (Thermo Scientific). Samples were stored at -80ºC until gene expression analysis.

For quantification of gene expression, 1 µg of total RNA was transcribed using the NZYtech First-Strand cDNA Synthesis Kit (NZYTech, Portugal). cDNA was diluted 20 x before preparation of the qRT-PCR reactions. The relative expression of each gene of interest was quantified by qRT-PCR using the NZYSpeedy qPCR Green Master Mix (NZYTech) and pre-

designed primers (see table 3). Briefly, the mixture of cDNA sample and master mix (per primer) was heated at 95ºC for 2 minutes, followed by 40 cycles of 5 seconds for the annealing of primers at 58º or 60º (depending on the melting temperature of each primer set) and 30 seconds for amplification at 72ºC. After amplification, a melting curve protocol was performed with 1-minute heating at 55ºC followed by 80 steps of 10 seconds with a 0.5ºC increment until reaching 90ºC. All reactions were performed in duplicate in a StepOne™ Plus device (Thermo Fisher). A standard curve was prepared for each gene, using 10x, 50x and 100x dilutions from a control sample, in order to determine the efficiency of each primer set. Two negative controls (no reverse transcription – NRT and no target control – NTC) were run for each gene. The gene HPRT was used as reference gene throughout this study. Calculation of mRNA fold change for each gene were performed according to the Pfaffl Method.

Table 3 | Primer sequences used for qRT-PCR reactions

Name	Forward Primmer	Reverse Primmer	Expected amplification size
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT	89
IL-6	TCTATACCACTTCACAAGTCGGA	GAATTGCCATTGCACAACTCTTT	88
COX-1	AAGGAGTCTCTCGCTCTGGT	ACAGGGATTGACTGGTGAGG	106
COX-2	CTCCACTCATGAGCAGTCCC	AACCCTGGTCGGTTTGATGT	95

CHAPTER 3 | RESULTS

To investigate the impact of ELS on social behaviors and in the number and morphology of microglia cells, a set of experiments were performed according to the time line shown in Figure 4. All pups in this study were weighted at P2, P7, P14, P21 and P40 (for some studies, also at P90). During the MSUS protocol, pups from MSUS designated litters were separated from the dam for 3h/day from P2 to P14. Control litters were kept with the damns, undisturbed at all times, except for weighting and cage changing. After induction of ELS, a general behavior characterization was performed on adolescent (P40) male and female mice to assess behavior impairments. In some MSUS and control litters, another behavior analysis was performed at P90, in order to access if behavior alterations persisted in adulthood. All experiments and analysis were performed in parallel for animals from both treatment groups – MSUS and Control, and in both male and female mice.

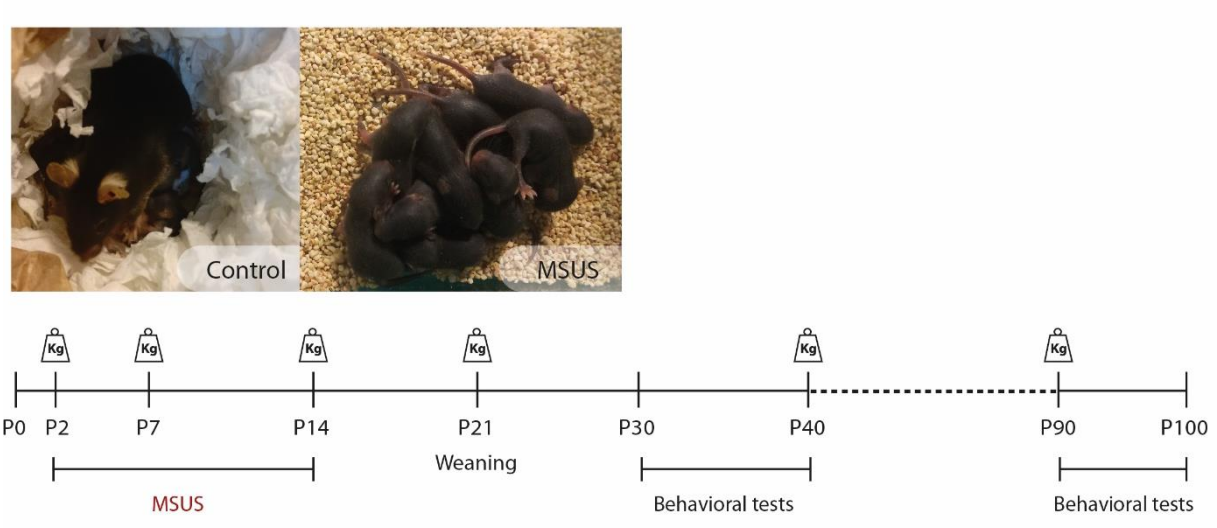


Figure 4 | Experimental time points

1. Changes in body weight in adolescent and adult mice exposed to MSUS

Before the induction of ELS, pups from both treatment groups did not display body weight differences (Figure 5). However, throughout the two weeks of the MSUS protocol it was possible to observe that both male (Figure 5a) and female (Figure 5b) MSUS pups showed a decrease in total body weight that persisted until adolescence (P40) but that was no longer present in adulthood (P90). This temporary loss of weight is probably related with less nursing time, since MSUS dams were absent for 3h/day and, even when they returned to the cage, spent less time in the nest, with respect to control dams. Overall, since adult mice do not present differences in body weight between MSUS and Control groups, these results indicate

a normal development of ELS adult mice. In addition, no differences were observed between Control and MSUS groups regarding overall physical health.

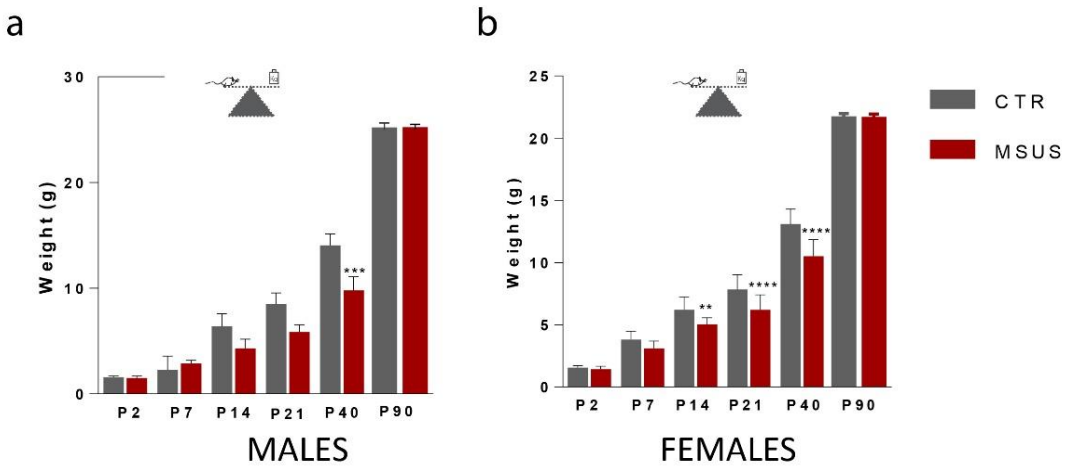


Figure 5 | Exposure to MSUS protocol significantly affects body weight independent of gender in adolescence but not in adulthood. **(**a) Weight gain of MSUS and control male mice during the first 90 days of life (controls n=10 MSUS n=7 **(**b) Weight gain of MSUS and control female mice during the first 90 days of life (controls n=11 MSUS n=13 P ≤ 0.05, **p<0.01, ***p<0.001 and ****p<0.0001, according to two-way ANOVA test. Data are presented as mean ± SEM

2. Increased depressive like and risk-taking behavior in adolescent male mice subjected to MSUS

Several studies have shown that ELS induces alterations in anxiety and mood related behaviors[223]. In this study, we employed the FST and the EPM paradigms to evaluate the anxiogenic impact of the MSUS protocol. In the FST, we observed that male MSUS mice showed a slightly more depressive-like behavior, since despite not showing significant differences in the immobilization time (data not shown), these animals presented a tendency to exhibit lower latencies to stop swimming during the test (Figure 6a). Interestingly, and contrary to males, MSUS females did not display differences with respect to age-matched controls (Figure 6a), which indicates that exposure to ELS seems to trigger an early depressive phenotype in male but not in female mice. Regarding the EPM test, no significant differences were observed in the time spent in the open arms of the maze nor in the latency to open arms (Figure 6b-c). However, we observed that female and especially male MSUS mice had a tendency to present a lower latency to enter the open arms (Figure 6c) and a slight increase in the time spent (Figure 6b) in the open arms of the maze, which may indicate a more exploratory and risk-taking behavior, with respect to control animals, with no overt changes in anxiety-like behaviors.

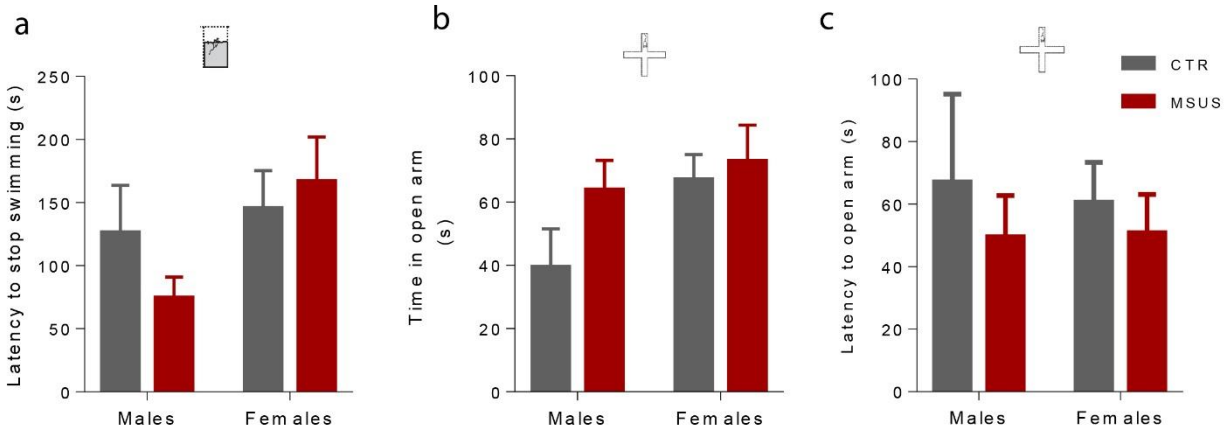


Figure 6 | Adolescent male mice subjected to the MSUS protocol display a tendency for depressive-like and risk-taking behaviors. (a) Effects of MSUS on depressive like-behaviors assessed by the forced swimming test. Latency to first stop swimming of MSUS male and female mice compared to controls. (b-c) Effects of MSUS on anxiety-like behaviors assed by the elevated plus maze test. (b) Time spent in the open arms by adolescent male and female MSUS mice compared to controls. (c) Latency to first entrance in the open arms by adolescent male and female MSUS mice compared to controls. Results from 3 separated cohorts. For all tests control males n= 11, control females n=12, MSUS males n=11 and MSUS females n=13. Data are presented as mean ± SEM

3. Gender and age specific alterations in sociability in male and female mice subjected to MSUS

ELS has also been shown to cause alterations in social preference and social memory[224,225]. In this study, social behaviors were accessed at two different time points (P40 and P90) by two different evaluations (Figure 7a) employing the three-chamber social test: 1) the social preference test, which explores the social nature of mice by exposing the animal to an empty cage versus a cage with a stranger mouse, and 2) the social novelty test, which allows evaluation of social memory, by exposing the test mouse to the previous stranger mouse and to a new stranger mouse.

3.1 In adolescence

At P40, and as expected, both control male (Figure 7b) and female (Figure 7d) mice spent significant more time in proximity with the cage containing an unfamiliar social stimulus (stranger 1) than with the empty cage (empty). MSUS male mice also spent more time with the stranger but, contrarily to control animals, the difference was not statistically significant (Figure 7b), which indicates that, at this age male mice subjected to ELS are slightly less social than age and sex-matched controls. Female MSUS mice on the other hand, do not appear to display

any changes in social preference when compared to controls (Figure 7d). This indicates that maternal separation affects sociability in males during the adolescence phase but not in females

On the second part of the test, both male and female Control and MSUS mice presented the expected behavior (Figures 7c and 7e respectively), spending significant more time interacting with the novel mouse (stranger 2), with respect to the familiar mouse (stranger 1). No differences were observed between Control and MSUS mice (Figures 7c and 7d), which leads us to conclude that ELS does not seem to have an impact in social memory at P40, regardless of the gender.

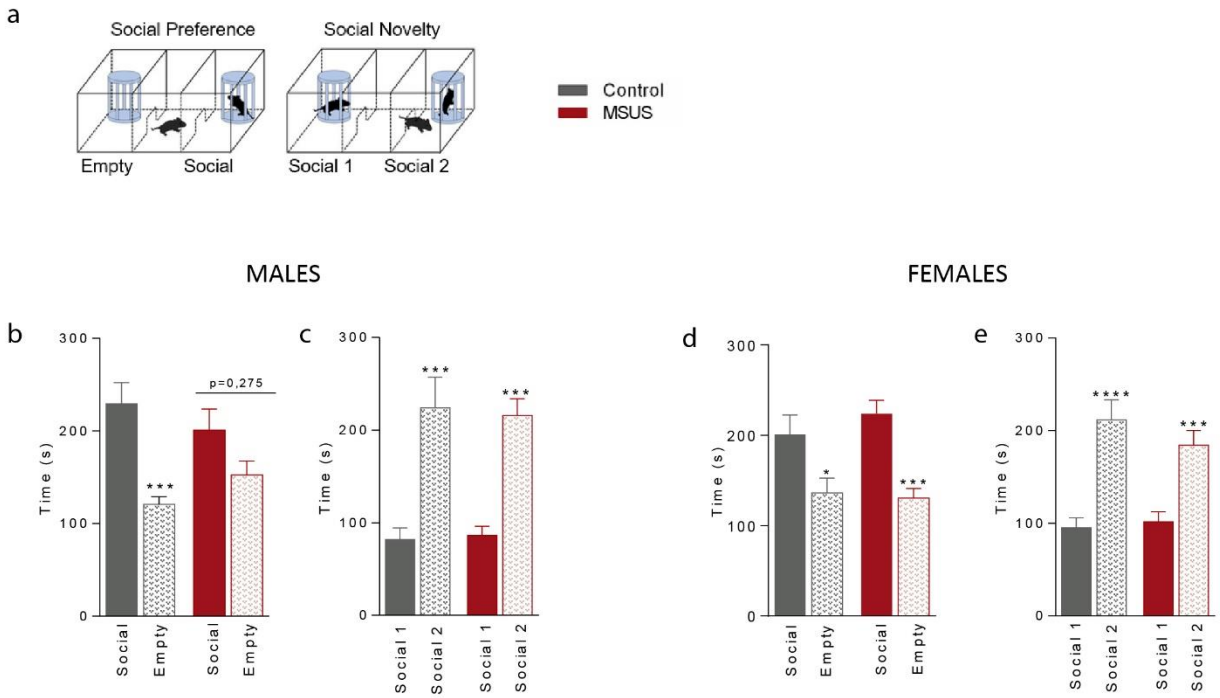


Figure 7 | Maternal separated adolescent male mice display a tendency to be less social than age-matched male controls. (a) Representation of the 3-chamber apparatus and trial display. Total time spent by adolescent MSUS male (b) and female mice (d) in close proximity to a social partner in a cage (Social 1) versus an empty cage (Empty) compared to control animals (males n=11 and females n=10 for each group). Total time spent by adolescent MSUS male (c) and female mice (e) in close proximity to a new social partner (Social 2) versus a familiar social partner (Social 1), compared to control animals (males n=11 and females n=10 for each group). *P ≤ 0.05, **p<0.01, ***p<0.001 and ****p<0.0001, according to ordinary one-way ANOVA test. Data are presented as mean ± SEM

3.2 In adulthood

In order to evaluate if the observed changes in social behavior were reversed, persisted or were aggravated in adulthood, one cohort of both MSUS and control mice was re-tested at P90 in the three-chamber test. During first part of the trial, both adult male (Figure 8a) and

female (Figure 8d) control mice presented the expected behavior, spending more time with the social stimulus than with the empty cage. Female MSUS mice presented a similar behavior to their age and sex-matched controls. However, male MSUS mice tended to spend more time with the social stimulus than conspecific controls. In the second part of the trial, both male (Figure 8b) and female (Figure 8d) control mice spent more time with the unknown stimulus than with the familiar one. Although male MSUS mice also preferred the new stimulus, they spent, overall, more time socializing than controls (Figure 8f), a result that is in opposition to the tendency observed during adolescence.

At this age, it is also possible to observe that female mice spent significantly more time socializing than males (Figure 8f).

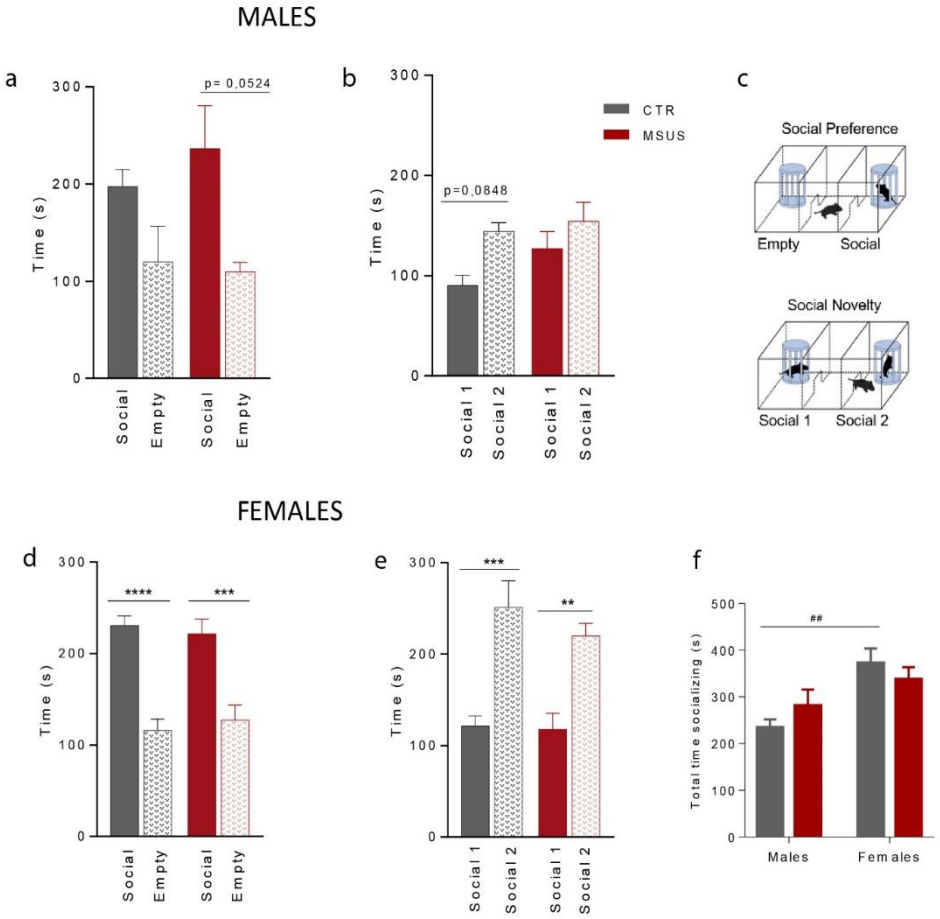


Figure 8 | Maternal separated adult male mice display a tendency to socialize more than sex matched controls. Total time spent by adult MSUS male (a) and female mice (d) in close proximity to a social partner in a cage (Social 1) versus an empty cage (Empty) compared to control animals (males n=4 and females n=6 for each group) Total time spent by adult MSUS male (b) and female mice (e) in close proximity to a new social partner (Social 2) versus a familiar social partner (Social 1) compared to control animals. (c) Representation of the 3-chamber apparatus and trial display. (f) Total time spent by adult MSUS male and female mice in close proximity to a social partner (Social 1 + Social 2) compared to controls. (males n=4 and females n=6 for each group). **p<0.01, ***p<0.001 and ****p<0.0001, $^{\#\#}$p<0.01 according to ordinary one-way ANOVA test. Data are presented as means ± SEM

4. Sexual dysmorphism in microglia in the mPFC

4.1 Microglia density

Stress has been demonstrated to have an impact on the immune system and, more specifically, in microglia cells. Even though the effects of stress in microglia cells reported so far are mostly focused on morphological alterations, some studies have pointed that chronic stress can also impact microglial density[226]. As such, we decided to test if ELS could have the same impact as chronic stress. To assess this hypothesis and verify if ELS could have an impact in microglia number in the mPFC, microglial density in different regions of the mPFC was assessed in adolescent male and female mice (P40) by unbiased stereology, a method that allows, using mathematical relationship equations, to consider the structural parameters that are lost during the generation of sections. For microglia quantification, we employed the optical fractionator method, where the total number of objects (N) in a defined region can be estimated by directly counting the objects (Q) in a known fraction (f) of the volume of that region. This is accomplished by counting the number of microglia in volume-fixed optical slices (dissectors) and multiplying the sum of the counted objects, $\sum Q^-$, by the reciprocal fraction to obtain the total number of microglia cells in the entire mPFC area of the slice. Density is then obtained by dividing the total number by the estimated volume of the region.

As it has been previously described in other studies, in several different brain regions[79], we could observe that microglia density, in adolescence, is affected by sex, as male mice present a higher density of microglia cells, in all regions of the mPFC, when compared to females (Figures 9b-e)

Even though statistical significance was not reached with the number of animals employed throughout this study (experiment is still ongoing), it is clear that MSUS male mice present a tendency to have higher microglial density than controls, especially in the PrL and IL regions of the mPFC (Figures 9d and 9e). Female MSUS mice, on the other hand, present a slight decrease in microglial density in the Cg1 (Figure 9c), but shown no alterations in other mPFC regions. These results suggest that microglia numbers in the mPFC present a sexual dysmorphism both under basal conditions and under ELS.

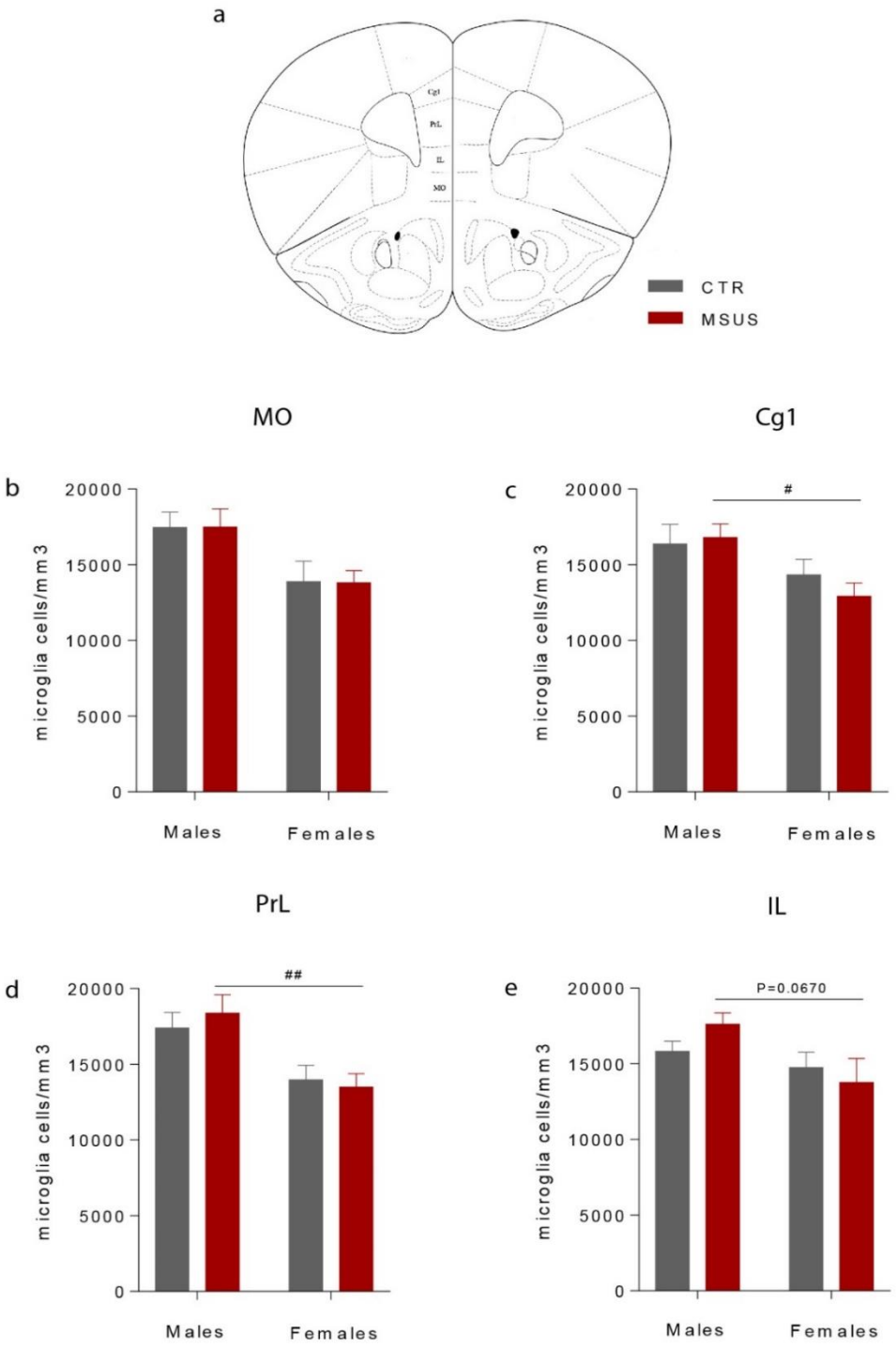


Figure 9 | Stereology-based evaluation of total number of microglia cells in the mPFC. (a) The mPFC was divided in four sub regions; Medial orbital region (MO) Cingulated cortex region (Cg1), prelimbic region (PL) and infralimbic region (IL). The total number of microglia cells in the total volume of each region was estimated by stereology, for each of the designated regions (b) AC, (c) Cg1, (d) PrL and (e) IL, using a Zeiss Z2 microscope and the StereoInvestigator software. Results are expressed as the number of cell/mm^3 and are representative of n=7 for each experimental group. $^{\#}p<0.05$, $^{\#\#}p<0.01$ according to two-way ANOVA test. Data is presented as mean ± SEM

4.2 IBA-1 intensity

Next, we decided to test if ELS could have an impact on microglia activation and morphology. Microglia cells from both male and female, control and MSUS adolescent mice were reconstructed using confocal microscopy. Cells were randomly chosen throughout the mPFC and a compilation of several images was taken across the Z plane (~30 planes) in order to obtain enough detail to perform total reconstruction using the Imaris software (Bitplane).

IBA-1 has been shown to cooperate with the G protein Rac, a key molecule in microglia activation, and to participate in the regulation of the actin cytoskeleton, which underlies crucial events of microglia activation, including migration, phagocytosis and proliferation[18]. Taking this into consideration, we decided to evaluate the intensity of IBA-1 signal in microglia cells from male and female, control and MSUS mice as a first indicator if ELS could induce changes in microglia phenotype.

IBA-1 intensity was observed to be significantly increased in MSUS males (Figure 10a) which indicates that maternal separation can induce an increase in IBA-1 levels as was expected to occur during activation. However, in females, no difference was observed (Figure 10a) in this parameter. Interestingly, our results also show that, under basal conditions, female microglia display a tendency to present a higher IBA-1 intensity sum values than males, which may be related with the increased cell size observed in female cells[77].

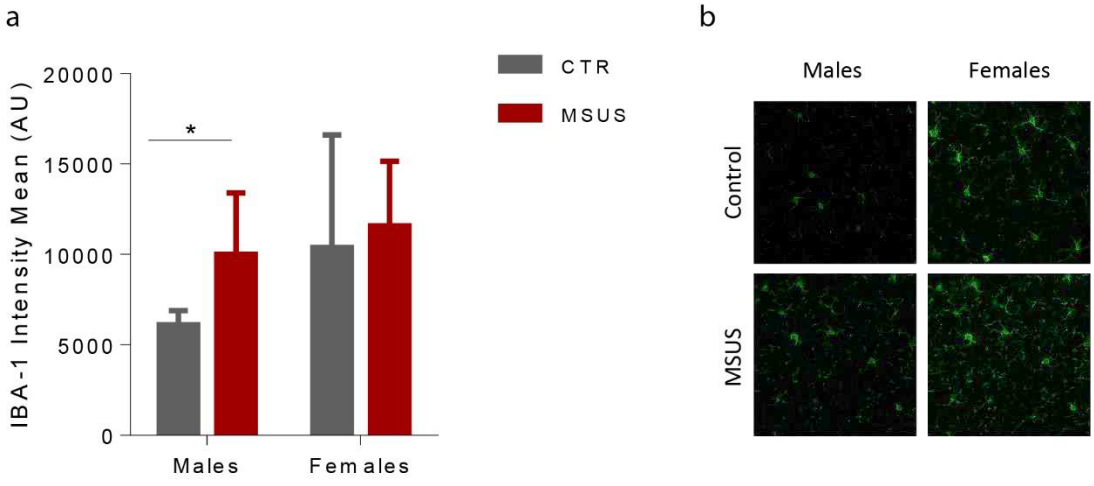


Figure 10 | IBA-1 Intensity is significantly increase in MSUS adolescent males compared to sex-matched controls. (a) IBA-1 mean intensity value of microglia cells in the mPFC of male and female, control and MSUS mice. (b) Representative image of microglia cells labelled with anti-Iba-1 antibody by immunohistochemistry and observed under a confocal microscope (400x amplification). n=10 for each experimental group. *p<0.05, according to two-way ANOVA test. Data is presented as mean ± SEM

4.3 Morphological analysis

These same cells were employed to perform surface and filament analysis using the Imaris software, which allowed us to obtain information regarding parameters such as total area, volume, sphericity and number and total length of microglia branches. Sholl analysis was also performed using this software, following automatic filling of microglia ramifications in the 3D model.

4.3.1 Filament and Sholl analysis

Sholl analysis revealed that independent of the experimental group, the major percentage of intersections between cellular processes and the radii imposed upon the cell occurred between 5 and 20 µm from the cell soma.

Sex comparisons revealed that microglia from male animals presented a higher number of intersections than female microglia between the radii 5 and 20, while microglia from females were more ramified between the radii 30 and 50 (Figure 11a), with respect to male microglia. These results indicated that males and females have a different distribution of ramifications, with males presenting a higher number closer to the cell body, that diminishes drastically as the processes get further away and females present fewer ramifications close to the cell body, but a higher number of ramifications at the periphery. It is also worth noticing that females present branch intersections with the radii until further away from the center, which may indicate that female microglia cells are larger and occupy a broader region than the ones from males.

In what concerns the effects of exposure to ELS, microglia from MSUS males tend to present a lower number of ramifications near and a higher number of ramifications further away from the cell body than age and sex-matched controls (Figure 11b), a morphology that resembles the one displayed by control females. In females, we can observe the opposite result, since MSUS female mice show a higher number of ramifications closer and a lower number further away from the cell body (Figure 11d). Therefore, our results indicate that not only male and female microglia in the mPFC are morphologically different under basal condition, ELS is able to alter microglia ramification in opposite ways in males and females, suggesting that the mechanisms underlying microglial cell response to ELS are gender-specific.

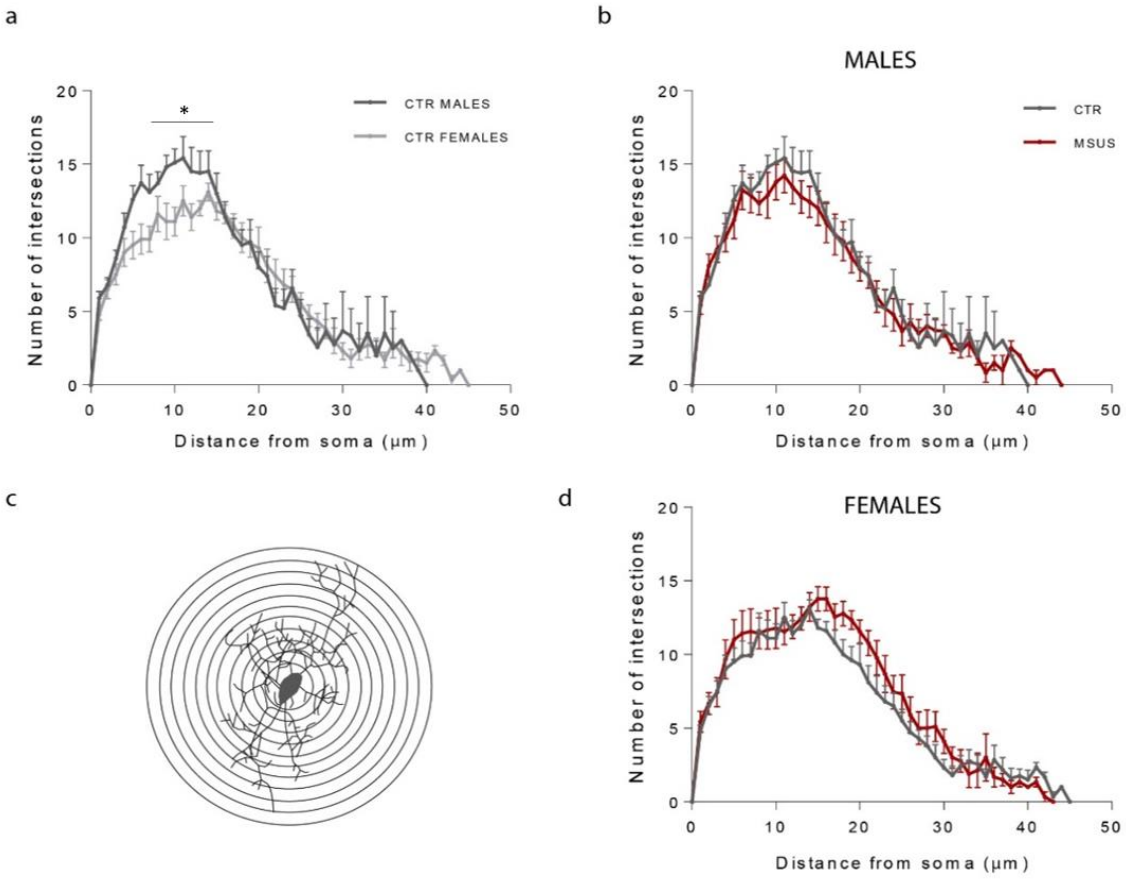


Figure 11 | MSUS induces alterations on microglia ramification profile in the mPFC of male and female adolescent mice. (a) effects of gender on microglia ramification profile. Effects of the MSUS protocol on mPFC microglia ramification in males (b) and females (d). Microglia complexity was measured by sholl analysis as the number of intersections from the ramifications in 1 µm radius increments (c). n=10 for each experimental group. *p<0.05, according to two-way ANOVA test. Data is presented as mean ± SEM

Other types of filament analysis were performed as a complement to sholl analysis, including determination of ramification length, total ramification area and volume, number of branching and ending points and total number of ramifications. We found no significant effects of sex on process length (Figure 11a), total ramification area and volume (Supplementary data 1), number of ending and branching points or in the total number of ramifications of the analyzed cells (Figure 11a-d).

While the MSUS protocol did not seem to induce alterations in process length in male mice, in females an increase near statistical significance was observed in this parameter (Figure 11a). Exposure to MSUS also induced a tendency for increased ramification volume and increased ramification area in males and females, respectively (Supplementary data 1b-c). As for the total number of ramifications and the number of branching and ending points in these

ramifications, the MSUS protocol induced opposite effects in female and male microglia. While female MSUS cells presented higher numbers than their control counterparts, male MSUS cells presented a slight decrease in all these parameters (Figure 11b-d).

Taken together, these results indicate that the MSUS protocol induces a more ramified phenotype in female microglia, with cells presenting more and longer ramifications.

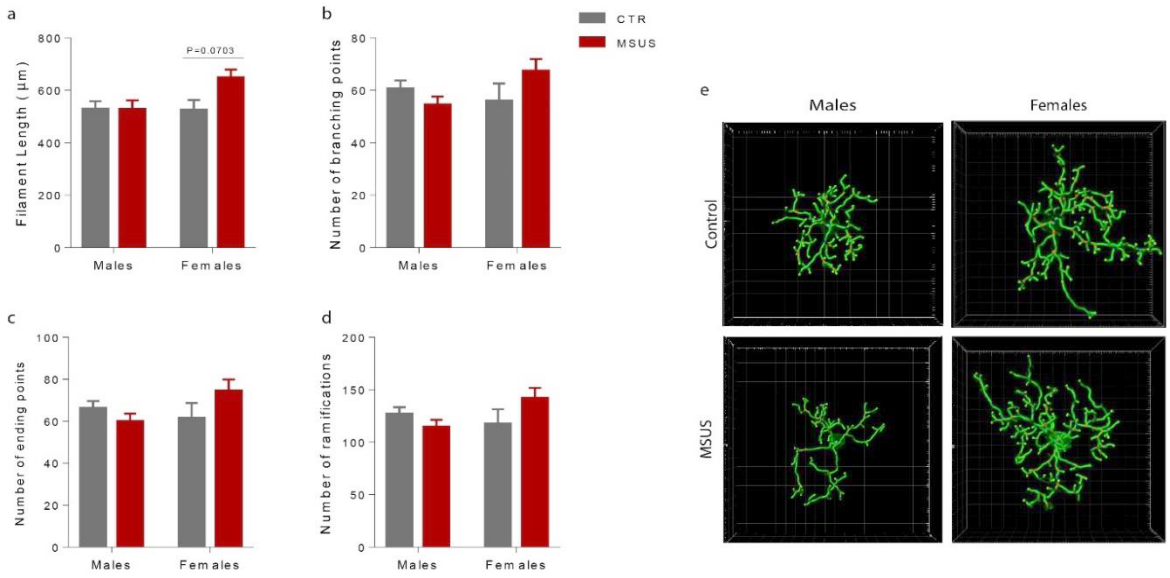


Figure 12 | MSUS increases filament length in the mPFC of adolescent female mice. Effects of ELS in filament length (a), number of branching points (b), number of ending points (c) and total number of ramifications (d). obtained by filament analysis using the IMARIS software (e). n=10 for each experimental group. P=0.0703, according to two-way ANOVA test. Data is presented as mean ± SEM

4.3.2 Surface analysis

Surface analysis was performed to assess cellular volume and surface area, as well as spatial distribution of processes.

We observed no significant impact of experimental group on microglia total surface area. However, MSUS females display a tendency to present higher total surface area when compared to controls (Supplementary data 1c).

Regarding volume (defined as the total volume of cell body and processes), no significant differences were observed between groups. Nevertheless, females appear to have a slightly higher cell volume than males and, among the last, MSUS mice present a tendency to exhibit increased cell volume when respect to controls (Supplementary data 1a).

Taking these parameters into account, a surface area/volume ratio was calculated. Although no significant differences were observed between genders (Figure 13a), control females appeared to display a lower ratio than control males. Interestingly, this ratio was significantly impacted by the MSUS protocol in both genders (Figure 13a) and changes were observed in opposite directions, with MSUS male mice showing a significant decrease and MSUS female mice a significant increase in surface area/volume ratio. These alterations can be correlated not only with the size of the cellular body but also with the length and thickness of microglia processes.

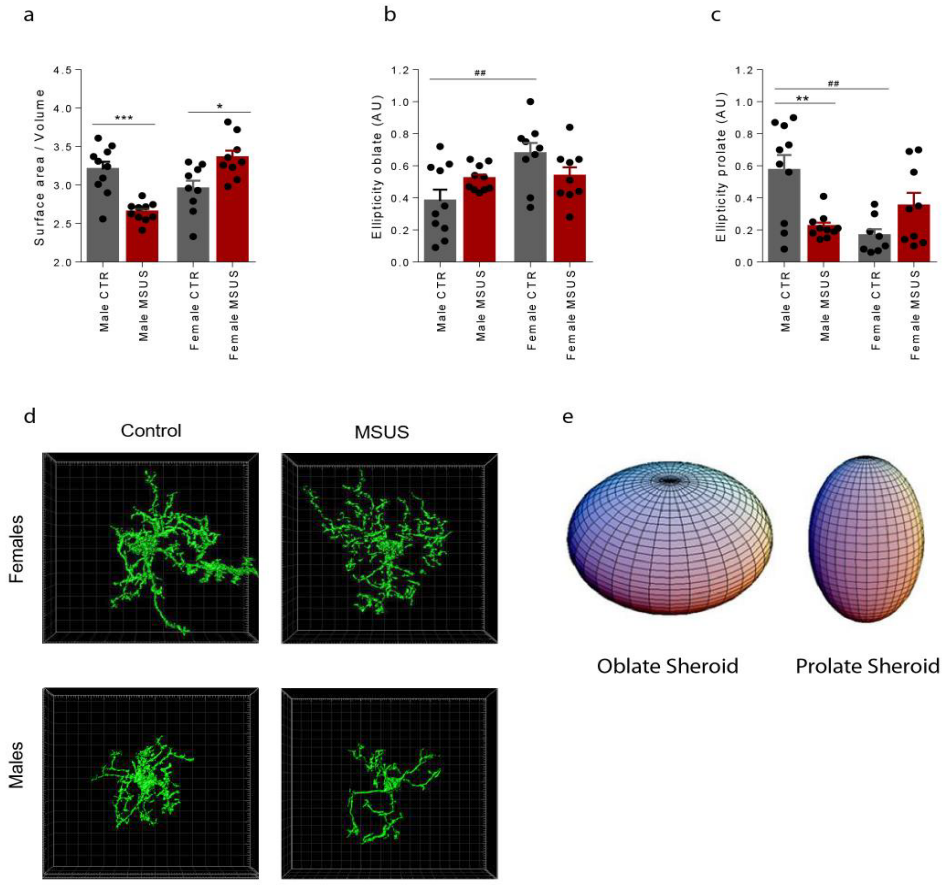


Figure 13 | MSUS significantly alters the spatial distribution of microglia processes in the mPFC.of adolescent male mice. Using Imaris, 3D reconstructions of microglia cells were performed (d) Considering total surface area and volume, we calculated the surface area/volume ratio for both males and female to assess MSUS impact on cell body size or processes thickness (a)**.** Spatial distribution of processes was also assessed and classified as oblate/flattened (Figures 10b and 10e) or prolate/elongated (Figures 10c and 10e). n=10 for each experimental group *P≤ 0.05, **p<0.01, ***p<0.001 and # P≤0.05 according to two-way ANOVA test. Data are presented as means ± SEM

The spatial distribution of ramifications was also assessed in the 3D reconstruction of each microglia cell and classified as either flattened (oblate spheroid like) or elongated (prolate spheroid like) (Figure13e).
Gender was shown to have a significant impact on the spatial dispersion of ramifications, with female mice displaying significantly more flattened (Figure 13b) and males significantly more elongated (Figure 13c) microglia morphologies. The MSUS protocol also resulted in an alteration in cell format. MSUS males displayed a significant decrease in cell elongation with respect to controls and a shift towards a more flattened profile, while female MSUS mice showed an opposite tendency, shifting towards a more elongated phenotype (Figures 13b-c).

Overall, gender was shown to have a strong impact in microglia cell morphology, in what concerns branching distribution, surface area and volume. Exposure to ELS interacts with these parameters in a gender dependent way, extremizing the observed phenotypes and increasing the differences observed between male and female microglia cells.

4.4 Final considerations

Microglia cells display significant differences between adolescent male and female mice in the mPFC, as presented in Table 4, with males displaying higher cellular densities, lower IBA-1 intensity, smaller and more elongated but equally ramified cells, presenting a smaller volume and a higher surface area/volume ratio, which is indicative of a smaller cell body and thinner processes.

Table 4| Microglia differences in the mPFC of adolescent male and female mice

	Males	Females
Microglia density	Tendency for higher density	Tendency for lower density
IBA-1 intensity	Tendency for lower intensity	Tendency for higher intensity
Ramification	More ramified closer to cell body and less ramified further away from cell body No differences in total number of ramifications	Less ramified closer to cell body and more ramified further away from cell body No differences in total number of ramifications
Area and volume	Tendency for lower volume Higher surface area/volume ratio	Tendency for higher volume Lower surface area/volume ratio
Spatial distribution of ramifications	Elongated	Flattened

Exposure to ELS in the form of the MSUS protocol also appears to induce alterations in the density and morphology of microglia as described in Table 5, in a sex depend way. MSUS male mice display higher microglial densities in some regions of the mPFC, increased IBA-1 intensity, an overall tendency for diminished ramification and increased volume, with significantly decrease elongation when compared to controls.

In what concerns females, the MSUS protocol tends to decrease microglia density in the Cg1 region of the mPFC, and to increase overall cell ramification, in particular closer to the cellular body, with an increase in process length close to statistical significance. MSUS females also present a significant increase in the surface area/volume ratio, as well as a tendency to increased elongation when compared to controls.

Table 5 | MSUS-induced alterations in the mPFC of male and female adolescent mice

	MSUS Males	MSUS Females
Microglia density	Tendency for ↑ density in the PrL and IL regions	Tendency for ↓ density in the Cg1 region
IBA-1 intensity	Significantly ↑ intensity	Not altered
Ramification	↓Ramification near and ↑ ramification further from the cell body Tendency to ↓ number of branching and ending points and total number of ramifications	↑ Ramification closer to cell body ↑ Ramification length, close to statistical significance Tendency to ↑ number of branching and ending points and total number of ramifications
Area and volume	↓surface area/volume ratio Tendency for ↑ total volume and filament Volume	↑ surface area/volume ratio Tendency for ↑ total surface area and filament area
Processes spatial distribution	Significantly ↓Elongation ↑ Flatness	↑ Elongation

5. MSUS shows a tendency to increase pro-inflammatory markers in the mPFC independent of gender.

Several studies have demonstrated that ELS can have a profound and long-lasting impact on the expression of several pro-inflammatory markers, such as *IL-1β, IL-6 and TNF-α,* among others. To test this hypothesis, we decided to perform quantitative real-time PCR at P90 animals in order to assess the expression of several genes already described as potentially susceptible to the effects of ELS and previously shown to be involved in the inflammatory cascade.

Although no statistically significant changes were observed in the expression of these genes when comparing the MSUS and control experimental groups (Figures 11a-d), the MSUS protocol displayed an overall tendency to increase the expression of some of the pro-inflammatory genes tested in this study, specially *IL-1β* (Figure 11a) and *IL-6* (Figure 11b), regardless of gender. Cox-2 expression was also shown to be slightly increased in MSUS animals, and this result almost reaches statistical significance in female mice (Figure 11c). These results point towards the existence of a more pro- inflammatory profile in the mPFC of MSUS animals, regardless of the gender.

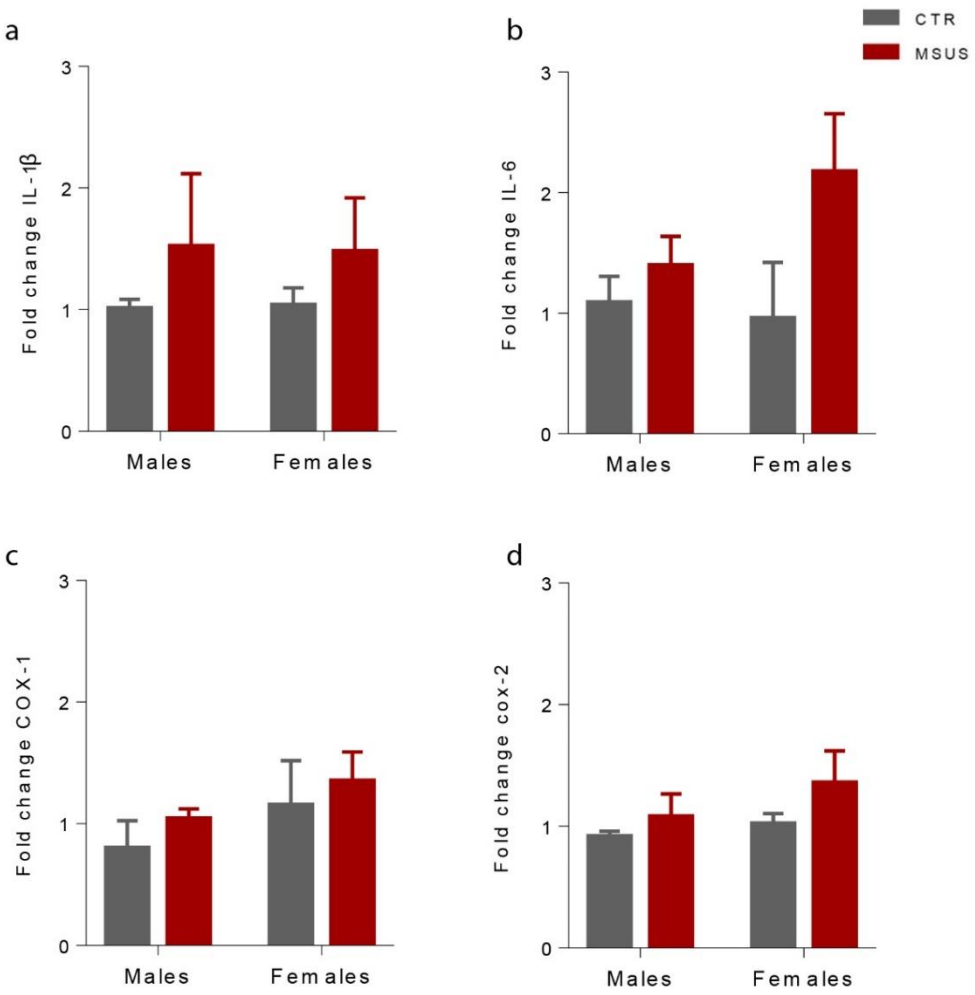
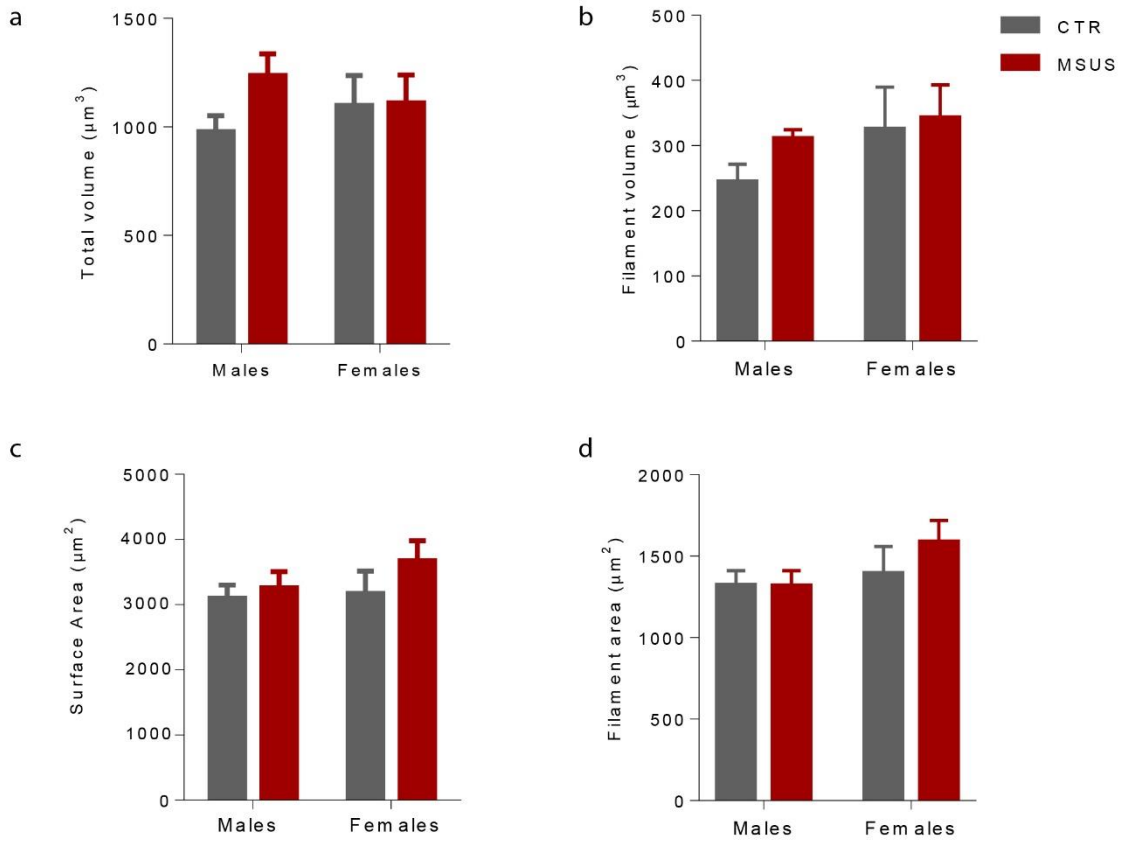


Figure 14 | MSUS adolescent male and female mice present an increase in the levels of pro-inflammatory markers in the mPFC. Expression levels of several pro-inflammatory markers, such as (a) *IL-1β, (b) IL-6, (c) COX-1 AND (d) COX-2* where quantified by quantitative real time PCR . The gene HPRT was used as reference gene throughout this study. Calculation of mRNA fold change for each gene was performed according to the Pfaffl Method. n=4 for each experimental group. Data is presented as mean ± SEM

6. Supplementary Data



Supplementary data 1 | MSUS increases filament volume in the mPFC of adolescent female mice. Effects of ELS in total cell volume (a), in filament volume (b), in total cell surface area (c) and in filament surface area (d). obtained by surface analysis using the IMARIS software (e). n=10 for each experimental group. Data is presented as mean ± SEM

CHAPTER 4 | DISCUSSION AND FUTURE DIRECTIONS

Childhood exposure to stress in forms of abuse or neglect is common worldwide. Reports have demonstrated that early life stress is associated with overall volume loss in several brain regions such as the hippocampus[227] and PFC[228], as well as with reduced neuronal density in the anterior cingulate[229]. Furthermore, an increasing number of clinical studies has demonstrated a clear relationship between harsh family environment, characterized by abuse, neglect and nonnurturing parents, and adverse health outcomes, specially neuropsychiatric disorders such as depression, autism spectrum disorders, schizophrenia, psychosis and PTSD[113,96,114,115,116,117]. Although the mechanisms underlying this relationship are not clear, a role has been suggested for microglia cells in this context, as mediators of the interactions between stress and neuronal circuits that result in the neurophysiologic alterations observed in these pathologies[230].

These cells, which until recently were believed to remain in a resting state until challenged, are now known to participate in several neurodevelopmental processes essential for the correct formation of brain networks, such as clearance of apoptotic debris, promotion of neuronal survival, synaptic pruning, remodeling and plasticity[231]. Being a part of the immune system, microglia cells are very sensitive to environmental cues and pro-inflammatory stimulus, such as stress in early-life stages. Stress-triggered alterations in these cells, during critical periods of development, can result in impairments in microglia-mediated processes and, consequently, result in neuronal circuit malfunction or aberrant neuronal wiring, ultimately leading to disease.

Most of the studies performed so far concerning the relation between early-life stress, inflammation and neuropsychiatric/neurodevelopmental disorders have been focused in male animals, with little information available concerning the impact of ELS in the female brain and without addressing the sexual dysmorphism that is known to exist in certain brain regions throughout brain development and under physiological conditions[232]. Therefore, in this work, we proposed to address this issue and investigate the impact of ELS in microglia morphology, density and function in the male and female mPFC, a brain region known to be particularly relevant in processes such as complex cognitive function, decision making and social cognition[183,180]. We also proposed to investigate the impact of ELS in social and anxiety-like behaviors during adolescence, focusing in the differences between genders.

Regarding overall behavior, we observed several differences between adolescent males and females and between MSUS and control animals. The FST revealed an increased depressive-like behavior in male mice, a phenotype well described in several maternal separation models at different ages [233,234,235], supporting the notion that ELS can increase the propensity for mood-disorders not only later in life but also during adolescence. In females, however, these

alterations were not present, which indicates that females are more resilient to the effects of stress during early life stages. Since anxiety and depressive-like behaviors often go hand-in-hand, we decided to evaluate the impact of ELS in anxiety using the EPM test. Contrary to the increased anxiety-like behaviors observed in several other ELS models, the MSUS model seams to induce a different phenotype, since both male and female MSUS animals exhibit a tendency to present a lower latency to first entry in the open arm of the maze, a trait that is indicative of reduced avoidance and fear of new situations ass it is indicative of increased risk-taking behavior. This type of behavior has also been associated with a lack of impulse control and, together with the depressive-like behavior, suggests that MSUS mice, in particular males, display characteristics reminiscent of several psychiatric disorders such borderline personality disorder, mood disorders and drug addiction, usually characterized by deficient behavioral control, impulsivity and predisposition to depressive states.

Since stress has also been described to have an impact in social skills[96] and promote alterations in sociability, such as those observed in neurodevelopmental and neuropsychiatric disorders, we decided to investigate social interaction and social memory following exposure to the MSUS protocol. We observed that MSUS male mice display a tendency for a more anti-social behavior during adolescence, spending less time interacting with a stranger animal during the first part of the three-chamber test, although this behavior is reversed later in life, during adulthood. This result may indicate a transient impairment in social skills during adolescence, caused by the impact of MSUS in maternal care, which is known to have significant influence in the formation of social bonds and in early social play and cognition[236]. At the physiological level, exposure to stress during early life may cause the hyperactivation of the HPA axis, which, ultimately, can lead to antisocial behavior.[96]

Interestingly, at P90, MSUS male mice display higher sociability that gender-matched controls. Although they are able to recognize the new social stimulus, in the second part of the three-chamber test MSUS male animals spend more time engaged in social interactions, regardless of the social partner. Therefore, we believe that, in adulthood, the risk-taking behavior may overcome the diminished social skills displayed in adolescence, leading MSUS male mice to spend more time exploring social partners.

On the other hand, female mice appear to be overall more resilient to ELS-induced changes in social behavior, since they do not display alterations in sociability during adolescence and in adulthood and they do not show signs of the depressive phenotype observed in males.

To investigate if some of these gender-specific behavior alterations could be linked to modifications in microglia function during development, caused by ELS-mediated immune activation, we evaluated microglia density and morphology in the mPFC.

We observed that under physiological conditions microglia from males are smaller but equally ramified when compared to microglia from females, with higher number of ramifications closer and fewer ramifications further from the cell body. In addition, adolescent males present a higher microglia density, allowing for a similar coverage and surveillance of the brain parenchyma in the mPFC. Another interesting difference is that male microglia present lower overall volume and lower filament volume and a higher surface area/volume ration, which indicates the presence of thinner processes than the ones displayed by microglia from female animals. These results corroborate other studies that show that although during development, males present microglia cells significantly rounder and less ramified than females, with thicker processes, during adolescence a shift occurs[79], and male cells also become ramified, despite retaining a smaller size and higher numbers.

As for the impact of MSUS on microglia morphology and density, we could observe that ELS triggers alterations in several parameters. MSUS males presented an increase in microglia densities in PrL and IL regions of the mPFC, regions that has been shown to be intimately linked to amygdala functions, such as fear expression and fear extinction.[237] Of note, brief uncontrollable stress has been shown to alter fear response and cause dendritic retraction of terminal branches in the mouse IL cortex[238], presenting evidence that stress can have an impact in these circuits.

As for females, MSUS was shown to trigger a decrease in microglia density in the Cg1 region. This region has long been implicated in emotional and cognitive processing, as well as in cognitive control of behavior. In addition, alterations in Cg1 activity have been linked to novelty seeking and highly impulsive behaviors, which were, in fact, the only behavior changes observed in adolescent females subjected to MSUS.

These region-specific ELS-mediated alterations in microglia density might be due to alterations in microglia activation and gene expression and can signalize relevant changes in microglia physiological functions during development. For example, these changes may lead to impairments in synaptic pruning and circuit refinement during development, which may help explain the risk taking-behavior observed in MSUS mice in the EPM. These results also indicate that ELS impacts are gender-specific. However, significantly more research is needed to understand the mechanisms underlying these alterations. We believe that an important

experiment going forward is to investigate the impact of the higher microglia density observed in males in the number and morphology of synapses from inhibitory mPFC neurons, since alterations in inhibitory networks in this region have already been linked to a diminished fear response[239] and increased risk-taking behavior[240]. Another important question that needs further investigation is whether MSUS microglia present alterations in the expression of genes that regulate cell-cycle progression, survival and apoptosis, since these alterations could help to elucidate the alterations in microglia density and activation upon ELS.

IBA-1 intensity was also shown to be significantly increased in MSUS males but not in MSUS females. Since IBA-1 is known to be involved in alterations in the actin cytoskeleton upon microglia activation, this result points towards the presence of an activated phenotype in microglia cells for these animals. In MSUS females, and even though statistical significance was not reached, a trend for higher IBA-1 intensities was also observed. This result is in accordance with our previous observations (behavior tests and microglia morphology analysis), further suggesting that even though ELS can impact IBA-1 expression in females, they seem to be somewhat more resistant than males.

Moreover, overall morphology profiles from microglia cells were also impacted by ELS in opposite directions. MSUS males displayed an overall tendency for diminished ramification, increase in total volume and filament volume and a significant decrease in filament elongation when compared to controls. This alteration in morphology is reminiscent of a transition from a ramified state (or surveillant state) to a reactive state, characterized by a shortening and thickening of cellular processes. This morphological alterations usually occur as a response to injury or to the presence of inflammatory signals, and represents a transitional phase between the surveillant, highly ramified state and the ameboid, highly motile state [241]. On the other hand, in females, the MSUS protocol induced changes in the opposite direction of the spectrum, leading to an increase in ramification, ramification length, total surface and filament area and to increased elongation, all characteristics of an hipper-ramified state, which has been described to occur after exposure to chronic stress in adult mice[220]. This gender-related dysmorphism observed in microglial response to ELS can be explained, at least in part, by the process of brain masculinization that happens during the early stages of post-natal development and which coincides with exposure to the MSUS protocol.

The developing brain organizes itself in a sexually-dysmorphic way resulting from the indirect effects of sexual hormones. Elevated levels of testosterone are released by the developing testis and are converted to estradiol in the brain, through a process of aromatization. Estradiol acts on microglia cells, leading to the production of PGE2, which helps reorganizes dendritic

spines and establishes male sexual behavior. During the first post-natal week the number of activated microglia in males is highly superior to that of females and inhibiting the activation process during this critical period prevents overall brain masculinization[74]. This means that during this time window male brains are naturally more susceptible to pro-inflammatory factors and present an overall more pro-inflammatory profile. Taking this into account, it is not strange that early-life insults capable of inducing neuroinflammation, as is the case of the MSUS protocol, can have a stronger impact in males than in females.

Since microglial remodeling is known to occur in response to activating stimuli present in the microenvironment, surveillant microglia that exhibit a highly ramified appearance, upon detection of an activating signal, may react in a stimulus-dependent way. Depending on the nature, strength and chronicity of a certain stimulus and on the receptor portfolio expressed by microglia, these cells can change into a hyper-ramified, reactive or ameboid/phagocytic state[242], sometimes going through all these phenotypes. It is possible that because the female brain is less dependent on sexual hormones during the first weeks of life, it is also less susceptible to the damage induced by early life stress. This may help explain why microglia from females does not shift towards a reactive state upon exposure to MSUS, and, instead, present a hyper-ramified profile, which is believed to correspond to a lesser degree of activation.

We are currently assessing if the observed alterations in microglia number and morphology remain until adulthood, by performing the same morphological analysis in P90 animals. So far, the gene expression analysis of several pro-inflammatory mediators at this age revealed that adult MSUS mice present a stronger pro-inflammatory profile when compared to controls, regardless of gender. Male and female MSUS animals showed higher expression of *IL-1β* and *IL-6,* two major pro-inflammatory cytokines expressed by microglia upon activation, as well as a higher expression of *Cox-2*, a key enzyme in the conversion of arachidonic acid to prostaglandins and other lipid mediators, whose expression is induced by inflammatory stimuli, and *Cox-1,* an enzyme that is known to play a major role in the neuroinflammatory process[243]. These results suggest that ELS is able to induce changes in microglia that are long-lasting and remain until adulthood.

Taken together, our results corroborate the hypothesis that microglia activation can be one of the missing links between exposure to ELS and the higher risk of developing neuropsychiatric disorders, such as depression, PTSD and ASD. This idea is further supported by studies showing that high levels of pro-inflammatory cytokines, including IL-1β, can reduce the availability of dopamine and serotonin by increasing the expression and function of reuptake

transporters and reducing the synthesis of monoamine precursors[244]. Moreover, chronic microglia activation has also been shown to lead to neuronal apoptosis, neurogenesis inhibition, reduced neurotransmitter synthesis and cytotoxicity[245] which can, in turn, impact neuronal circuits and trigger disease states.

As a conclusion, this work has shown that MSUS can have an important impact in microglia activation, morphology and density, aspects that can ultimately contribute to the behavioral changes observed in MSUS animals. Moreover, throughout this study, we observed several gender-specific differences in microglia morphology and behavior in physiological conditions and upon exposure to ELS, such as an MSUS male-specific increase in depressive-like and risk-taking behaviors. These sexually dysmorphic observations, that were exacerbated by exposure to the MSUS protocol, highlight the importance of further studying the impact of ELS and other forms of stress separately in males and females, in order to shed light into the amply described gender-related differences in the incidence of several mental disorders.

CHAPTER 5 | REFERENCES

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