SEX DIFFERENCES IN THE ENDURING NEUROINFLAMMATORY AND BEHAVIOURAL SEQUELAE OF SYSTEMIC IMMUNE CHALLENGE DURING PUBERTY

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Abstract

Puberty is a critical period for sexual maturation during which the sex-specific reorganization and remodelling of the pubertal brain facilitate sex biases in stress sensitivity. Pubertal (i.e., six-week-old) CD-1 mice treated with the bacterial endotoxin lipopolysaccharide (LPS; 1.5 mg/kg body weight, *ip*) show several sex-specific changes to the neuroendocrine and behavioural systems of several reproductive and non-reproductive functions. One promising explanation for the elusive mechanisms driving the sex-specific outcomes of pubertal immune challenge may lie in the cascade of neuroimmune events induced by this systemic immune stressor. This doctoral thesis tested the hypothesis that sex-specific responses of the pubertal neuroimmune network contribute to sex differences in the enduring outcomes of pubertal immune challenge on hippocampus-dependent cognitive processes. Male and female CD-1 mice are equally vulnerable to enduring impairments in spatial memory following pubertal LPS exposure. Across brain regions for cognition and stress regulation, pubertal LPS treatment alters baseline sex differences in microglial expression and morphology in a sex-dependent manner. The temporary female-specific increase in whole-brain blood-brain barrier permeability during LPS-induced sickness may have facilitated the apparent female bias in LPS-induced changes to pubertal microglia. In the context of sex- and region-specific residual effects of pubertal LPS-induced sickness on microglial expression and morphology, pubertal LPS treatment may accelerate certain neurodevelopmental processes in males but not females. The innate sex differences in the pubertal neuroimmune network highlighted by these studies underscore how a systemic immune challenge precipitates sex biases in immune-mediated disorders of brain and behaviour during adulthood.

General Introduction

Statement of the Problem

Puberty is a pivotal developmental period distinguished by its sexually differentiated maturation of systems required for reproductive competence (Romeo, 2003; Sisk & Foster, 2004; Sisk & Zehr, 2005). Sexual dimorphisms in pubertal development arise from variations in the secretion of gonadal steroid hormones (i.e., androgens, estrogens, and progestogens) by the hypothalamic-pituitary-gonadal (HPG) axis at the onset of puberty (Herbison, 2016; Sisk & Foster, 2004). The extensive steroid-dependent organization and activation of neurocircuitry during puberty creates a vulnerability towards external stressors, particularly immune challenges (Holder & Blaustein, 2014; Kane & Ismail, 2017). In mice, a systemic immune challenge at six weeks of age (i.e., stress-sensitive pubertal period) permanently impairs neuroendocrine and behavioural systems that underlie both reproductive and non-reproductive processes (e.g., Girard-Joyal & Ismail, 2017; Ismail & Blaustein, 2013; Ismail et al., 2013). Given that the sex-specific organizing effects of the pubertal hormonal milieu also extend to the central stress response system, systemic immune stressors during puberty can elicit different outcomes between males and females (Oyola & Handa, 2017). The processes governing these sex differences in vulnerability towards pubertal immune challenge have not been fully established. This research, therefore, aimed to delineate possible routes by which a systemic immune challenge during puberty exerts its sex-specific outcomes on the brain and on behaviour.

The critical role of gonadal steroid hormones in pubertal development points towards a hormonal component underlying some of the sex-specific repercussions of a pubertal immunostressor. LPS appears to exert some of its effects by blocking or reversing the protective effects of estradiol and progesterone (Ismail et al., 2011; Olesen et al., 2011). For example,

pubertal LPS appears to block the cognition-enhancing effects of estradiol among adult female mice (Ismail & Blaustein, 2013). These LPS-induced hormonal changes during puberty have also been linked to several maladaptive ovarian hormone-dependent behaviours during adulthood, including depression-like and anxiety-like behaviours (Ismail et al., 2013; Murray et al., 2019, 2020; Olesen et al., 2011) and reduced sexual receptivity (Ismail et al., 2011; Laroche et al., 2009 a, b). Sex differences in endocrinological systems during puberty and their effects on immune and stress reactivities make it unclear whether the behavioral changes seen among females following a pubertal immune challenge can extend to males.

Sex differences in the neuroinflammatory responses to immune stressors may explain how a peripheral immune challenge during puberty permanently alters the brain and behaviour in males and females. Systemic LPS treatment can activate microglia, the brain's innate immune cells, to fight the infection (Banks et al., 2015; Holder & Blaustein, 2017). However, microglia can become uncontrollably toxic when primed by intense or frequent immune stressors (e.g., Cunningham et al., 2005; Puntener et al., 2012). Microglia are particularly vulnerable to this exaggerated activation during critical periods of neurodevelopment (Bilbo et al., 2005; Hoeijmakers et al., 2016; Lively et al., 2018). Furthermore, sex-, age-, and region-dependent differences in microglial expression and phenotype (e.g., Bollinger et al., 2019; Matcovitch-Natan et al., 2016; Weinhard et al., 2018; Yang et al., 2013) may account for the divergences in behavioural and neurophysiological outcomes seen in males and females following a systemic immune challenge.

Disruption of the blood-brain barrier (BBB) during sickness may be a key regulator of neuroinflammatory processes (Erickson et al., 2012). The BBB maintains the brain's homeostasis by controlling the passage of substances at the blood-brain interface (for review see

Erickson & Banks, 2018). Regulatory features of the BBB continue to be influenced by immune processes (Banks et al., 2015; Li et al., 2015) and circulating gonadal steroid hormones throughout the lifespan (e.g., Bake & Sohrabji, 2004; Wilson, et al., 2008). Under certain conditions, immune responses disrupt the integrity of the BBB and lead to disturbances in brain functioning (e.g., Geng et al., 2018; Stolp et al., 2005; Strbian et al., 2008). Therefore, differences in endocrine-immune interactions on BBB integrity during infection may explain some sex differences in the acute responses to pubertal immune challenge and its long-term consequences.

The doctoral thesis tested the hypothesis that sex differences in the pubertal neuroimmune responses to systemic LPS facilitate sex-specific effects of pubertal LPS treatment on hippocampus-dependent cognitive systems. This work first examined whether sex and circulating gonadal steroid hormones mediate the enduring effects of pubertal LPS treatment on hippocampus-dependent spatial memory processes and hippocampal cell development (Study 1; Kolmogorova et al., 2019). Follow-up studies focused on the functionally and structurally interconnected brain regions for cognition (i.e., hippocampus, prefrontal cortex, and the cerebellum) and stress regulation (i.e., hypothalamus) and examined sex differences in pubertal neuroimmune responses to systemic LPS (Study 2; Kolmogorova et al., 2021) and the residual effects of pubertal LPS-induced sickness on baseline expression of key proteins in inflammation, oxidative stress, apoptotic cell death, and synaptic transmission (Study 3).

Conceptual and Theoretical Background

Puberty as a Critical Period of Steroid-Dependent Organization

Pubertal Development

Puberty is a critical period that encompasses the biological transitions for sexual maturity (Romeo, 2003, 2010; Sisk & Foster, 2004). This phase of development initiates the changes in psychological and social functions characteristic of adolescence (Sisk & Foster, 2004). Puberty begins with the reawakening of gonadotropin releasing hormone (GnRH) neurons in the hypothalamic-pituitary-gonadal (HPG) axis (Avendaño et al., 2017; Blakemore et al., 2010; Herbison, 2016). The pulsatile secretion of GnRH during puberty indirectly acts on the testes in males and the ovaries in females to promote gametogenesis and the production of gonadal steroid hormones (i.e., androgens, estrogens, and progestogens) (Abreu & Kaiser, 2016; Sisk & Foster, 2004). Gross structural changes in the pubertal brain are the result of region-specific proliferation of cells, apoptosis, growth of axonal projections, axonal sprouting, myelination, reshaping of the dendritic tree, and synaptic remodelling (for reviews see Giedd et al., 2006; Schulz et al., 2009; Sisk & Foster, 2004). Sex differences in circulating gonadal steroid hormones during puberty facilitate the appearance of secondary sexual characteristics and sculpt neural circuits relevant to sexual functioning for adulthood in a sex-specific manner (Arnold, 2009; Schulz et al., 2009; Sisk & Foster, 2004). The transition into adulthood is considered complete upon maturation of the gonadal system (i.e., puberty) and social and cognitive behaviours (i.e., adolescence) (Sisk & Foster, 2004).

The involvement of gonadal steroid hormones in peripubertal development lends to puberty's conceptualization as a postnatal period for the steroid-dependent organization of brain and behaviour (Arnold, 2009; Schulz & Sisk, 2016; Sisk & Foster, 2004). These organizational

properties of gonadal steroid hormones are permanent and limited to critical periods during early development (Sisk & Foster, 2004; Schulz & Sisk, 2016). In general, testicular-derived hormones masculinize (i.e., enhance male-typical attributes) and defeminize (i.e., suppress female-typical attributes) social and reproductive behaviours, whereas ovarian-derived hormones exert both feminizing (enhance female-typical attributes) and defeminizing effects on female behaviour depending on the social behaviour in question (Bakker & Baum, 2008; McCarthy et al., 2012; Schwarz & McCarthy, 2008). Once the underlying neural circuits have been organized, gonadal steroid hormones can also temporarily activate certain reproductive behaviours (McCarthy et al., 2012; Schulz & Sisk, 2016; Sisk & Foster, 2004). Therefore, experiences that alter the hormone-driven organization of neural systems during puberty can have profound consequences that persist into adulthood.

Peripubertal Reactivity to Stressors

The hypothalamic-pituitary-adrenal (HPA) axis manages the central stress response together with the brainstem noradrenergic neurons, sympathetic andrenomedullary circuits, and parasympathetic systems (for reviews see Heck & Handa, 2019; Herman et al., 2016; Smith & Vale, 2006). This neuroendocrine-immune network serves to stabilize physiological processes in the face of stressors (i.e., allostasis) (McEwen, 1998, 2000). The HPA axis' response to stress results in a cascade of hormonal changes that lead to the gradual release of glucocorticoids (i.e., cortisol in primates and corticosterone in many rodent species) from the adrenal cortex (Smith & Vale, 2006). Circulating glucocorticoid levels peak 10 to 20 minutes following a stressor, with genomic effects appearing approximately one hour after stress exposure (Sapolsky et al., 2000; Schommer et al., 2003). These stress-evoked hormonal changes facilitate survival of the host by promoting acute adaptations aimed at coping with the internal and external demands of the

stressor (e.g., reduction of inflammation, mobilization of energy stores, and perception of and coping with temperature variations in their environment) (for reviews see Goncharova, 2013; Ramsay & Woods, 2014; Straub et al., 2011).

The magnitude and duration of the hormonal stress response is impacted by various factors, including the age of the organism (Goncharova, 2013; Herman et al., 2016). Significant discrepancies in the time course of ACTH and corticosterone release between prepubertal and adult rats point towards robust influences of pubertal development on the HPA axis (Gunnar et al., 2009; Romeo et al., 2006). Pubertal maturation of the HPA axis precedes that of the HPG axis and is initiated by the increased production and secretion of adrenal hormones (i.e., adrenarche) (Byrne et al., 2017).

Sex differences in pubertal stress exposure. Sex differences in age of pubertal onset and circulating gonad-derived steroid hormones are thought to contribute to the differing trajectories in HPA axis development between the sexes (Green & McCormick, 2016; Heck & Handa, 2019; Oyola & Handa, 2017). Although there is a considerable gap in our understanding of how gonadal steroid hormones influence the development of the HPA axis during puberty, the existing literature suggests that pubertal gonadal steroid hormones initiate maturational processes that facilitate the development and expression of sex differences in HPA axis stress reactivity (Goel et al., 2014; Panagiotakopoulos & Neigh, 2014). Despite similar increases in HPA reactivity in males and females following puberty, sex differences in the pubertal development of neural and peripheral circuitries appear to bias females to greater glucocorticoid release relative to their male counterparts (Goel et al., 2014; Green & McCormick, 2016). However, sex differences in basal and stress-induced HPA axis activity during puberty are somewhat dependent on subject age, the experimental model (e.g., human versus animal models, animal

strain), the stressor (e.g., acute versus chronic), and biological sample of interest (e.g., brain region, serum).

The implications of sexually dimorphic responses to stressors during a critical postnatal period of neurodevelopment are widespread. First, pubertal stress exposure can help explain the predisposition or exacerbation of stress-related disorders that appear during puberty or early adulthood but do not originate in the pre-/post-natal periods or during adulthood. Preclinical and clinical models generally show a strong positive relationship between stress burden during puberty and risk for internalizing and externalizing disorders, reactivity to future stressors, and cognitive functioning in young adulthood (e.g., Angold et al., 1998; Byrne et al., 2017; Ellis et al., 2019; Graber, 2013; Hamilton et al., 2014). Secondly, sex differences in stress reactivity (Oyola & Handa, 2017) may also provide a framework for the pathogenesis of sex biases in stress-related disorders such as depression (Angold & Costello, 2006; Marcotte et al., 2002). However, it is presently unclear why puberty is more susceptible to the effects of stress compared to adults and why males and females have substantially different propensities in coping with stress during puberty.

Puberty as a Stress-Sensitive Period in Development: Focus on Immunostressors

Seminal work by Laroche and colleagues (2009 a, b) demonstrated that the enduring effects of external stressors during puberty appear to be limited to a stress-vulnerable period that spans approximately four to six weeks of age in the inbred C57Bl/6 mouse strain. They found that commercial shipping of C57Bl/6 females between five and six weeks of age impaired sexual receptivity during adulthood, with the most robust effects seen among females shipped at six weeks of age (Laroche et al., 2009 b). Follow-up studies confirmed that shipping stress at six weeks of age reduces adult sexual functioning in both sexes and impairs responsiveness to

estradiol and progesterone in females and to testosterone in males (Laroche et al., 2009 b).

Similar outcomes on sexual behaviour were observed following treatment with LPS, a bacterial endotoxin that causes robust stress and innate immune responses (Dantzer, 2001; Laroche et al., 2009 b; Rivest, 2009). These persistent effects of LPS on male and female sexual behaviour appear to be limited to the six-week period in both the inbred C57Bl/6 and the outbred CD-1 mice, as LPS treatment at younger and older ages did not significantly change reproductive behaviours (Ismail et al., 2011; Laroche et al., 2009 a). Furthermore, the effects of systemic LPS at six weeks of age are not limited to reproductive behaviours, but extend to several non-reproductive processes, such as cognitive functions (Ismail & Blaustein, 2013) and the expression of depression-like and anxiety-like behaviours (Murray et al., 2019, 2020). This line of work points towards a distinct stress-sensitive period during puberty that is susceptible to robust sex differences in the acute and enduring effects of an immune challenge.

Acute Responses to Pubertal Immune Challenge

Systemically administered LPS triggers a cascade of neuroimmune events that induces the expression of physiologically adaptive "sickness behaviours" (Dantzer, 2009; Kawasaki & Kawai, 2014; Konsman et al., 2002; Pålsson-McDermott & O'Neill, 2004). Peripheral LPS gains access to the brain via the circumventricular organs and choroid plexus, where it activates the innate immune system via its interactions with cells in the brain that express toll-like receptor 4 (TLR-4) and with peripheral cytokines acting on afferent neural pathways (Banks & Robinson, 2010; Dantzer, 2001; Pålsson-McDermott & O'Neill, 2004). These activated immune cells in turn induce the production and release of cytokines (Dantzer 2001; Konsman et al., 2002; McCusker & Kelley, 2013; Zhang & An, 2007). These intracellular messenger proteins coordinate the immune response in an autocrine and paracrine manner by exerting pro-

inflammatory or anti-inflammatory effects (Dantzer, 2001; Lu et al., 2008; Zhang & An, 2007). Sickness behaviours and accompanying fever subside when anti-inflammatory cytokines predominate over pro-inflammatory cytokines in the body and the sickness response circuitry has been downregulated by glucocorticoids, neuropeptides (e.g., vasopressin), and growth factors (e.g., insulin-like growth factor I) (Aronoff & Neilson, 2001; Dantzer, 2009; Konsman et al., 2002; Lu et al., 2008; Zhang & An, 2007).

The intensity and duration of pubertal immune responses differ significantly between the sexes. In mice, pubertal females exhibit fewer sickness behaviours, less fluctuation in body temperature, and quicker recovery from sickness relative to their male counterparts (Cai et al., 2016; Sharma et al., 2018). Sex differences in immunocompetence are regulated in part by the actions of circulating gonadal steroid hormones on humoral and cell-mediated immune reactions (Bouman et al., 2005; Gaillard & Spinedi, 1998; Giefing-Kröll et al., 2015). Gonadal steroid hormones can directly modulate the immune response through hormone receptors on immune cells (Bhatia et al., 2014; Giefing-Kröll et al., 2015). Estrogenic steroids generally demonstrate immune-enhancing characteristics, whereas testosterone and other androgens tend to be immunosuppressive (Bouman et al., 2005; Gaillard & Spinedi, 1998; Giefing-Kröll et al., 2015; Schuurs & Verheul, 1990). These hormone-mediated functional differences contribute to the more adaptive and balanced cellular machinery for fighting infection in females relative to males (Bouman et al., 2005; Giefing-Kröll et al., 2015).

Enduring Outcomes of Pubertal Immune Activation

Given the extensive neuroplasticity during puberty, stress exposure during such critical periods can modify the trajectories of neurodevelopment (Andersen, 2003). As mentioned earlier, a pubertal immune challenge permanently disrupts sexual functioning and behaviours

regulated by gonadal steroid hormones and influences the development of several non-reproductive adult functions (Holder & Blaustein, 2014; Kane & Ismail, 2017). In CD-1 mice, systemic LPS treatment at six weeks of age affects the programming of adult stress and immune responses (Sharma et al., 2019), increases the expression of anxiety-like and depression-like behaviours (Murray et al., 2019, 2020), impairs hippocampus-dependent learning (Kolmogorova et al., 2019) and recognition memory (Ismail & Blaustein, 2013), and worsens dopamine-sensitive processes (Girard-Joyal & Ismail, 2017). In females, pubertal LPS exposure reduces the anti-depressive, anxiolytic, and cognition-enhancing properties of estrogenic steroids such as estradiol (Ismail & Blaustein, 2013; Ismail et al., 2011, 2013; Olesen et al., 2011).

As with the acute responses to LPS, there are sex differences in the permanent effects of this pubertal immune challenge on brain and behaviour. For instance, Girard-Joyal and Ismail (2017) observed that the permanently impaired performance on dopamine-dependent tasks (e.g., buried pellet test) following a systemic treatment to LPS during puberty was limited to males. Similarly, unlike their male counterparts, CD-1 females exposed to LPS during puberty do not show anxiety-like behaviours and increased reactivity to novel stressors in adulthood (Murray et al., 2019). Instead, the pubertal LPS-treated females showed more depression-like behaviours during adulthood. However, the extent of sex differences in the long-term effects of a pubertal immune challenge and their underlying mechanisms have not been thoroughly examined.

The Pubertal Link in the Development of Immune-Based Brain Pathology

The emerging literature on pubertal immune challenge presents the intriguing hypothesis that a peripheral infection during this stress-sensitive critical period can precipitate sex-biased disorders of brain and behaviour in adulthood. Nevertheless, many questions remain about the breadth of behavioural and brain changes induced by a pubertal immune challenge and the

mechanisms underlying these enduring effects, especially in the context of sex differences. One promising explanation for the sex-specific outcomes of pubertal immune challenge lies in the neuroinflammatory responses to a systemic immune challenge during puberty.

Structure of the Immune System

The immune system is a highly specialized network of cells, tissues, and organs which serves to recognize and eliminate pathogens (for reviews see Nicholson, 2016; Smith, 2012 a, b). The immune system of vertebrates is layered and can be divided into its innate and adaptive subsystems. The innate immune system produces an immediate but non-specific response that activates inflammatory, phagocytic, and lytic processes which destroy evolutionarily conserved pathogens such as bacterial and viral proteins (Medzhitov & Janeway, 2000). Pathogens that successfully evade the innate response are then faced with the adaptive immune system (Iwasaki & Medzhitov, 2010). This branch of the immune system uses B- and T-lymphocytes to mount immune responses tailored to each pathogen. This targeted response is achieved by the production of memory cells which then allow for more effective responses upon each subsequent encounter to that pathogen (Janeway, 2001).

Despite several physical barriers between the central nervous system (CNS) and the periphery, the brain is not privileged to immunological events originating in the body (Carson et al., 2006; Forrester et al., 2018; Klein & Hunter, 2017). It is now generally well-established that the immune system and the CNS use a coordinated bidirectional means of communication to collaborate in the maintenance and reestablishment of homeostasis (Dantzer, 2017; Perry & Teeling, 2013; Trakhtenberg & Goldberg, 2011). Immune cells and factors (cytokines) can travel through the semi-permeable fluid-brain barriers which separate the brain from the periphery (e.g., blood-brain barrier [BBB] and the blood-cerebrospinal fluid barrier) to assist in and

promote the development of acquired defenses against encephalopathic agents (e.g., Banks et al., 1995 a, b; Williams & Hickey, 1995).

The CNS also maintains its own structurally distinct line of defense against pathogens referred to as the neuroimmune axis (Erickson & Banks, 2018; Kofler & Wiley, 2011). This complex network maintains neuronal health by controlling the passage of substances between the periphery and the CNS, mobilizing host defenses against pathogens, and mediating immune processes in the brain (e.g., neuroinflammation, wound healing) (Glezer et al., 2007; Hawkins & Davis, 2005; Streit et al., 2005). Neuroimmune responses are facilitated by peripheral immune cells that can cross into the CNS, as seen, for example, with the post-injury migration of peripheral macrophages and T cells into the spinal cord (Costigan et al., 2009).

Microglia: Resident Immune Cells of the Brain

Microglia represent the key cellular components of the brain's immune system, maintaining a relatively permanent network of cells in healthy adults (approximately 15 percent of all adult CNS cells) (Prinz et al., 2017; Tremblay et al., 2011). When microglia detect immune threats (e.g., plaque, apoptotic cells), they migrate to the site and adapt their phenotype along a graded continuum which culminates in a fully activated, phagocytic state (i.e., amoeboid cell) (Kabba et al., 2018; Kierdorf & Prinz, 2013; Ransohoff & Perry, 2009; Town et al., 2005). Activated microglial states can be broadly categorized into its classical proinflammatory (i.e., M1) and repair-related (i.e., M2) states (for reviews see Fernández-Suárez, 2015; Orihuela et al., 2016; Tang & Le, 2016). Although all microglial states upregulate immune molecules such as cytokines and major histocompatibility complex classes I and II, the expression profiles of these immune substances differ significantly between microglial phenotypes (Chhor et al., 2013; Cunha et al., 2016; Jablonski et al., 2015).

Age and sex differences. Microglia play diverse roles in healthy brains throughout the lifespan (for reviews see Cowan & 2018; Lenz & Nelson, 2018; Tay et al., 2017). During critical periods of neurodevelopment like puberty, microglia contribute to the formation and refinement of mature neural networks (Cunningham et al., 2013; Ji et al., 2013; Paolicelli, et al., 2011; Schafer et al., 2012). Most developing microglia are fully activated amoeboid-like cells (Cengiz et al., 2019; Hammond et al., 2019; Schwarz et al., 2012), as they assist in synaptic pruning, neuronal proliferation and differentiation, shaping of axonal projections, and phagocytic scavenging (for review see Schafer et al., 2012; VanRyzin et al., 2018; Wu et al., 2015). On the other hand, adult microglia primarily maintain homeostatic properties as they scavenge the CNS for pathogens, debris, and unnecessary or damaged neurons and synapses (Sierra et al., 2010; Nimmerjahn et al., 2005; Parkhurst et al., 2013).

Sex-specific patterns of microglial colonization of the brain (Mouton et al., 2002; VanRyzin et al., 2016; Weinhard et al., 2018) facilitate the development of sex differences in neural networks and sex-dependent and sex-independent behaviours (for review see Lenz et al., 2018). Male rats express more microglia, particularly of the activated phenotype, in the hippocampus, parietal cortex, amygdala during the postnatal period relative to their female counterparts (Schwarz et al., 2012). This pattern reverses later on in development such that juvenile and adult females show more activated microglial cells in these regions relative to their male counterparts (Schwarz et al., 2012). Sex-specific features in maturation and immune reactivity are also observed among fully-formed adult microglia (e.g., Crain et al., 2013; Guneykaya et al., 2018; Hanamsagar et al., 2017; Lively et al., 2018; Villa et al., 2018), although it is unclear how much gonadal hormones contribute to these sex differences (for review see Osborne et al., 2018; Villa et al., 2019).

Microglial Priming and Inflammation-Mediated Neurodegeneration

Given that microglial populations in the brain are relatively stable throughout the lifespan, events that disturb microglial functioning can permanently impact the CNS' immune defenses (Bilbo et al., 2012; Cunningham, 2012; Norden et al., 2015; Perry & Teeling, 2013). Such immunological perturbations are particularly impactful during critical periods because they can "prime" microglia to produce exaggerated and prolonged pro-inflammatory responses to subsequent challenges (Bilbo et al., 2012; Brenhouse & Schwarz, 2016). Under certain circumstances, the unregulated activation of microglia can result in a self-propelling cycle of inflammation which propagates neuronal injury (for reviews see Block & Hong, 2005; Cunningham, 2012; Patterson, 2015; Glass et al., 2010). Microglia-mediated neurotoxicity is increasingly implicated in the onset and progression of neurodegenerative conditions such as Alzheimer's disease (AD) (Cunningham et al., 2005, 2009; Streit et al., 2009) and Parkinson's disease (PD) (Qin et al., 2007). Yet, many questions remain regarding when and how microglial functioning turns awry.

Puberty may be a candidate period for establishing toxic neuroinflammatory patterns seen in neurodegenerative conditions given this period's sensitivity to stressors and sex-specific stress and immune functions. Girard-Joyal and Ismail (2017) recently explored this hypothesis by examining whether a systemic immune challenge could induce behavioural and cellular features consistent with PD in an age- (pubertal versus adulthood) and sex-dependent manner. They found that systemic LPS treatment during puberty led to more PD-like behaviours in adulthood, although the behavioural impairments were greater among males compared to females. Pubertal LPS treatment, however, did not impact the expression of tyrosine hydroxylase (TH), a rate-limiting enzyme involved downstream in the biosynthesis pathway of dopamine. On the other

hand, systemic LPS challenge during adulthood increased TH expression without globally affecting dopamine-dependent behaviours in both males and females.

One challenge to this model of microglial priming is delineating how peripheral immune challenges exert these effects on the CNS in males and females during puberty. Sex differences in BBB integrity during sickness may offer a promising explanation. It is now generally accepted that the BBB imposes a relative immune privilege to the CNS by regulating the influence of peripheral immune events on the CNS (Forrester et al., 2018; Galea et al., 2017). Although the BBB constitutes the largest interface for blood-brain exchange (12-18 m^2 for the average human adult) (Abbott et al., 2010; Zhao et al., 2015), the role of the BBB during pubertal immune challenges is not fully understood. The BBB is of particular interest here because BBB-mediated disruptions in the CNS' microenvironment induced by peripheral factors are increasingly implicated in the exacerbation and development of neuroimmune-related dysfunctions, including AD and PD (for reviews see Banks & Erickson, 2010; Erickson & Banks, 2013; Hawkins & Davis, 2005; Maiuolo, 2018).

Blood-Brain Barrier Integrity During Puberty

The BBB is part of a dynamic multicellular network called the neurovascular unit which regulates regional cerebral blood flow and delivery of nutrients into the CNS (Obermeier et al., 2013; Muoio et al., 2014; Wolburg et al., 2009). The BBB refers to the cerebral microvasculature of the CNS which controls the exchange of substances between the periphery and the brain parenchyma (Nico & Ribatti, 2012). In a healthy individual, the BBB limits the passage of immune substances like cytokines into the CNS (<0.1% of peripherally-derived cytokines) (Banks et al., 1995 a, b). This selective permeability is achieved by transmembrane tight junction protein complexes between the cerebral endothelial cells, the choroid plexus, and cells of the

arachnoid epithelium (Abbott et al., 2010; Daneman & Brat, 2015; Engelhardt & Liebner, 2014). Tight junctions are composed of transmembrane protein dimers of claudin, occludin, or junctional adhesion molecules anchored into the endothelial cell by cytoplasmic accessory proteins (e.g., cingulin, zona occludens [ZO]-1, ZO-2, ZO-3). Cadherin-catenin complexes called adherens junctions, the second of the junctional complexes, in turn provide structural support to tight junctions.

Regulators of the BBB: Age, sex, and sickness. Although the structure of the BBB is fully developed at birth, BBB permeability remains dynamic and changes throughout the lifespan (Erdö et al., 2016; Erickson & Banks, 2019; Mizee & de Vries, 2013; Obermeier et al., 2013). As with most other physiological functions, the BBB is subjected to normal age-related changes in structure and functioning which diminishes its integrity with age (Bors et al., 2018; Goodall et al., 2018; Wilson et al., 2008). Regional differences in these age-related changes in BBB permeability point towards a possible cerebrovascular role in the onset of early cognitive dysfunctions (e.g., Montagne et al., 2015).

Given that the microvasculature of the BBB is an important target for gonadal steroid hormones (Banks, 2012; Krause et al., 2006 a, b; Stirone et al., 2003), some of the changes in the BBB during development and senescence are likely secondary to similar age-related changes in circulating sex hormones (e.g., Atalla et al., 2016; Bake et al., 2009; Sunday et al., 2006, 2007). Estrogens' effects on the BBB generally differ to those of androgens and progesterone (e.g., estrogens dilate cerebrovascular tone and increase cerebral flow, whereas androgens such as testosterone generally constrict vascular tone) (Krause et al., 2002; Ospina et al., 2003). Similarly, the cerebrovascular inflammatory response is affected differently by estrogens (suppression/anti-inflammatory) and androgens and progesterone (enhancement/pro-

inflammatory) (Gonzales et al., 2005, 2009; Ospina et al., 2004; Sunday et al., 2006, 2007). However, our understanding of androgenic effects on the BBB are confounded by its natural conversion to estrogenic steroids in the body (Krause et al., 2011) and the presence of both androgenic and estrogenic receptors on the cerebral vessels of males (Gonzales et al., 2007). The clinical implications of hormonal differences in BBB regulation generally point towards neuroprotective effects of estradiol during certain cerebrovascular-related diseases (e.g., ischemic stroke, cerebrovascular inflammation), whereas testosterone can exacerbate these pathologies (for reviews see Krause et al., 2006; Robison et al., 2019).

Neuroimmune responses. The BBB is a critical interface in the communication between the CNS and periphery (Dantzer, 2017; Erickson & Banks, 2018; Trakhtenberg & Goldberg, 2011). The BBB's role in neuroimmune functions and interactions can be categorized into five pathways: BBB disruption (i.e., non-specific leakage of normally BBB impenetrable substances); uptake and transport of immunoactive substances; immune cell trafficking; immune-mediated regulation of barrier and interface functions; and secretion of immunoactive substances by BBB cells (Erickson & Banks, 2018). Systemic LPS disrupts BBB permeability by increasing the expression of inflammatory substances (e.g., interleukin [IL]-1ß, IL-6, IL-10) which subsequently alter tight junction function (Banks et al., 2015; Erickson et al., 2018; Nishioku et al., 2009). These changes then promote the influx of immune factors such as tumor necrosis factor (TNF)-α into the CNS, which then directly activate neuroinflammatory responses (Gutierrez et al., 1993). The LPS-induced effects on BBB integrity appear to be dose- and time-dependent. In male CD-1 mice (6-8 weeks of age), BBB disruption to small (^{14}C-sucrose and sodium fluorescin) and large (^{13}I-albumin) radioisotopes was maximal at 24 hours post-treatment but limited to the higher treatment doses (i.e., 3.0 mg/kg versus 0.3 mg/kg and 0.03 mg/kg of

LPS) (Banks et al., 2015; Nishioku et al., 2009). The most significant changes in LPS-induced BBB disruption occurred in the parietal cortex (36%), striatum (42%), hippocampus (50%), cerebellum (52%), pons-medulla (54%), frontal cortex (55%), and thalamus (56%) (Banks et al., 2015). Interestingly, all doses of LPS were able to activate neuroimmune pathways and processes (e.g., sickness behaviour, body weight loss). Age-matched females show similar patterns of LPS-induced BBB disruptions, although they are significantly more vulnerable than males to repeated LPS administrations (three doses of 3.0 mg/kg, LPS, *ip*) (Erickson et al., 2018).

Objectives and Hypotheses

A systemic immune challenge during puberty leads to various enduring and sexually dimorphic physiological and behavioural sequelae, although the mechanisms driving these sex differences are not fully understood. Moreover, the compartmentalization of disciplines complicates this matter further by limiting the examination of such multi-system events using an integrated, whole systems approach. Converging evidence suggests that sex differences in BBB integrity and neuroinflammatory processes during a pubertal immune challenge may explain some of the behavioural and neurological sequelae of this event in males and females.

The overarching goal of this doctoral research was to examine whether pubertal neuroimmune responses to systemic LPS treatment alter hippocampus-dependent memory systems during adulthood in a sex- and hormone-dependent manner. This work first examined whether circulating gonadal steroid hormones regulate sex differences in the effects of a systemic LPS exposure during puberty on adult spatial learning and memory and hippocampal cellular proliferation and neurogenesis (i.e., Study 1; Kolmogorova et al., 2019). Focusing on the interconnected brain regions for cognition and stress regulation, this thesis then examined sex

differences in LPS-induced changes to the pubertal neuroimmune system (i.e., Study 2; Kolmogorova et al., 2021) and the baseline expression of key proteins in inflammation, oxidative stress, apoptosis, and synaptic plasticity one week post-treatment (i.e., Study 3). Sex differences in the pubertal neuroimmune network's responses to systemic LPS were expected to moderate the enduring effects of a pubertal immune challenge on hippocampus-dependent functions in a sex-specific manner.

Study 1: Pubertal Immune Stress Transiently Alters Spatial Memory Processes in Adulthood.

Author Note

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Abstract

Pubertal immune challenge can permanently alter hippocampus-dependent memory processes in a sex-specific manner. Although gonadal hormones can influence various cognitive processes, their role in regulating the cognitive sequelae to pubertal immune challenge has not been thoroughly assessed. We examined whether a pubertal immune challenge could affect hippocampus-dependent memory functions in adulthood and whether these effects are regulated by gonadal steroid hormones. We hypothesized that exposure to an immune challenge during puberty would induce sex-specific deficits in the behavioral and cellular correlates of hippocampus-dependent memory during adulthood. At six weeks of age, during the stress-vulnerable pubertal period, male and female CD-1 mice were injected with either saline or the bacterial endotoxin lipopolysaccharide (LPS). Three weeks later, mice underwent either gonadectomy or sham-surgery. At ten weeks of age (i.e., in adulthood), mice began behavioral testing in an open field, Barnes maze, and Morris water maze. Brain tissue was collected at 17 weeks of age and stained for doublecortin and Ki67 to examine migrating neuronal progenitor cells and cellular proliferation in the neurogenic subgranular zone (SGZ) and the cornus ammonis (CA)1 and CA3 regions of the hippocampus. Pubertal LPS treatment impaired learning during adulthood in both sexes and increased cellular proliferation in the CA1 region in castrated males only. Although adult sex hormones did not reliably modulate these changes, gonadectomy impaired learning during the Morris water maze in both sexes. Learning deficits were more prominent during the Barnes maze, which suggests a stress-dependent expression of LPS-induced cognitive deficits. Neurogenesis in the SGZ and cellular proliferation in the CA3 were not affected by pubertal LPS treatment or gonadectomy. These novel findings emphasize the sensitivity of developing cognitive processes during puberty to immune challenges and suggest a possible mechanism for learning-based difficulties in adulthood.

Keywords: puberty, lipopolysaccharide, sex differences, gonadal steroid hormones, Barnes maze, Morris water maze.

1. Introduction

Puberty is a critical period for sexual maturation characterized by sex-specific changes to behavioral and neuroendocrine systems and their underlying neural circuitry (Brenhouse and Andersen, 2011; McCarthy et al., 2015; Sisk and Foster, 2004). Sexual dimorphisms in the reorganization and restructuring of the pubertal brain are regulated by the influx of gonad-derived hormones (i.e., androgens, estrogens, and progestogens) into the brain that follows pre-pubertal reactivation of the hypothalamic-pituitary-gonadal axis. These sex-specific organizing effects also extend to the body's central stress response system, the hypothalamic-pituitary-adrenal axis (Green and McCormick, 2016; Oyola and Handa, 2017). The extensive neuroplasticity and sex-based distinctions in physiological stress responses during puberty contribute to sex differences in stress reactivity and vulnerability, particularly towards immune challenges (e.g., Girard-Joyal et al., 2015; Sharma et al., 2018). In pubertal mice, immune challenges such as the bacterial endotoxin lipopolysaccharide (LPS) induce the most robust effects at six weeks old, a stress-sensitive peripubertal period (Laroche et al., 2009a,b). Systemic LPS exposure during this stress-sensitive pubertal period permanently impairs various reproductive and non-reproductive functions (e.g., sexual receptivity, anxiety-like and depression-like behaviors, recognition memory, and dopamine-sensitive functions) (Girard-Joyal and Ismail, 2017; Ismail and Blaustein, 2013; Ismail et al., 2011; Olesen et al., 2011).

Adult hippocampus-dependent cognition is sensitive to these dynamic immune-endocrine interactions during the stress-sensitive pubertal period. In female CD-1 mice, pubertal LPS exposure significantly impairs the recognition of familiar social and object stimuli during adulthood and permanently blocks estradiol's cognition-enhancing properties (Ismail and Blaustein, 2013). Translation of these findings to males, however, is complicated by the apparent male performance bias in rodent models of spatial learning and memory and the potential variability in performance associated with the estrous cycle phase and circulating estrogen levels among pubescent and adult female rodents during long-term hormone-dependent tasks (Duarte-Guterman et al., 2015; Frick et al., 2015; Jonasson, 2005).

One possible mechanism driving these long-term effects of pubertal LPS exposure on

hippocampus-dependent cognition may be via LPS-induced changes in hippocampal neurogenesis. Performance on hippocampus-dependent tasks is thought to correlate with the production, survival, integration, and activation of new neurons in the subgranular zone (SGZ) of the dorsal hippocampus (for review see Duarte-Guterman et al., 2015). Neurogenic processes are also sensitive to estrous phase, circulating estrogen levels, and to age and sex (e.g., greater cellular proliferation but lower expression of immature neurons in the hippocampus among adult females compared to adult males) (for reviews see Duarte-Guterman et al., 2015; Frick et al., 2015). Chronic LPS-induced neuroinflammation suppresses basal neurogenesis in the SGZ by decreasing the short-term survival of new neurons (Bastos et al., 2008; Ekdahl et al., 2003; Monje et al., 2003). However, age, sex, and hormonal differences in neurogenesis complicate the generalizability of findings from chronic LPS paradigms to hypothesize how neuroinflammatory agents during puberty impact basal neurogenesis in males and females.

Our understanding of hippocampus-dependent cognitive sequelae to pubertal immune challenge is currently limited to social and object recognition memory in female CD-1 mice. Therefore, we assessed sex differences in how pubertal LPS exposure impacts spatial learning and memory during adulthood in CD-1 mice. Given the association between learning and hippocampal neurogenesis, we also examined how basal neurogenesis is affected by this paradigm. Compared to saline-treated controls, mice treated with LPS during puberty were expected to show poorer spatial learning and memory and reduced hippocampal expression of Ki67 (i.e., cellular proliferation) and doublecortin (DCX) (i.e., neuronal precursor cells). Males were expected to show better spatial learning and memory and greater hippocampal neurogenesis than females, regardless of treatment and surgery. Lastly, mice were gonadectomized or sham-operated during early adulthood to examine whether circulating gonadal hormones regulate the enduring cognitive and neurogenic effects of pubertal LPS treatment in both males and females. Gonadectomy was expected to impair cognitive performance and hippocampal neurogenesis in both sexes.

2. Material and methods

2.1. Animals

Ninety CD-1 male and female mice were shipped from Charles River Laboratories (Saint-Constant, Québec, Canada) at three weeks old, an age that is resistant to the enduring effects of shipping stress (Laroche et al., 2009a,b). Mice were segregated by sex and housed in groups of three in rooms maintained on a reversed light cycle (lights off at 1000 h) under standard conditions (14 h:10 h light/dark cycle; 24 ± 2 °C; relative humidity of 40 ± 5). The polycarbonate Lexan housing cages (17 × 28 × 12 cm [width x length x height]) were bedded with Teklad Corn Cob bedding (Harlan Laboratories, Inc., Madison, WI, USA, .25 in. diameter) and enriched with one square piece of Nestlet (Ancare Corp., Bellmore, NY, USA) and a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, Canada). Food (Harlan Laboratories, Inc., Madison, WI, US, T2018 – Global 18% rodent) and water were available ad libitum. All behavioral tests were completed during the dark phase under red light unless otherwise specified. All experiments were approved by the Animal Care Committee of the University of Ottawa.

2.2. Experimental procedures

Mice were exposed to LPS or saline at the stress-sensitive age of six weeks. Nine-week-old mice underwent gonadectomy (i.e., orchiectomy [ORX] and ovariectomy [OVX]) or sham-surgery (SHAM) to examine the role of gonad-derived hormones. Following one week of recovery, adult (i.e., ten-week-old) mice completed the open field test, the Barnes maze (BM), and the Morris water maze (MWM) over five weeks to limit carryover effects (Fig. 1). Vaginal smears were collected during the learning (every other day) and probe phases of the BM and MWM. Brain tissue was collected two weeks after behavioral testing to examine group differences in basal hippocampal neurogenesis and cellular proliferation.

2.3. Pubertal immune stress

2.3.1. Systemic exposure to lipopolysaccharide and saline

Mice were treated intraperitoneally (*ip*) with either 0.9% sterile saline control ($n = 42$) or LPS (from *Escherichia coli* serotype O26:B6; #L3755; Sigma Chemical Co., St. Louis, MO, USA; 1.5 mg/kg

body weight; *n* = 48) diluted in 0.2 mg/mL sterile saline. This LPS dosage during puberty elicits mild sickness symptoms for approximately 48 h in both sexes and impairs hippocampus-dependent cognition in adult females (Cai et al., 2016; Ismail and Blaustein, 2013). Injections were performed towards the end of the light phase.

2.3.2. Sickness monitoring

Behavioral signs of sickness were assessed at 30 min and 2, 4, 8, 12, 24 and 48 h following saline or LPS exposure by two experienced observers blind to the treatment conditions. As described by Kolmogorova et al. (2017), all mice received a score between 0 and 4 at each time point to reflect the number of sickness symptoms (lethargy, huddling, piloerection, and ptosis) observed. Final sickness scores were calculated as the average of the two raters' individual scores.

2.3.3. Body weight analyses

The body weights were recorded at the time of injection (i.e., baseline) and at 12, 24, and 48 h after injection. Percentage changes in body weight from baseline were calculated as described by Kolmogorova et al. (2017).

2.4. Sham-surgery and gonadectomies

Gonadectomy and sham-surgeries for both sexes were completed as described by Cai et al. (2016). All mice were provided ad libitum access to water bottles filled with 3% children's Tylenol® (acetaminophen) during the 48 h before surgery and for three days after surgery. Post-operative care included a subcutaneous injection of Carprofen (5 mg/kg body weight) and topical application of bupivacaine to the sutured skin. The cages were placed on Gaymar® T/Pump® classic heating pads set to 38 °C (± 1 °C) for two days post-surgery.

2.5. Behavioral testing

2.5.1. Open field test

General locomotor activity was assessed by examining free exploration of an open Plexiglass arena (90 cm x 90 cm x 45 cm walls) over five minutes. Olfactory cues were reduced by wiping the arena with 70% alcohol before introducing each mouse. Video recordings captured by a closed-circuit

Panasonic video camera were analyzed for distance travelled (cm) and speed (cm/s) with Noldus EthoVision® XT software. Noise in the tracking analyses (e.g., body wobble and precision-level noise) was smoothed by a robust Locally Weighted Scatterplot Smoothing (LOWESS) with a 10-second half-window (Benjamini et al., 2010).

2.5.2. Barnes maze

Hippocampus-dependent spatial learning and memory were examined with the BM (Sunyer et al., 2007). In order to reduce potential anxiety, mice were first familiarized with the escape box and the elevated circular arena (120 cm diameter, 90 cm high). Mice were then provided four days (four daily trials with a 15-minute inter-trial interval [ITI]) to learn to distinguish the entrance into the escape box amongst 15 other identical holes (9 cm diameter) evenly spread out along the perimeter. Reference cues were available on the periphery. Briefly, mice were placed in an opaque chamber in the arena center. The chamber was removed after 10 s and the mouse was exposed to two aversive stimuli – an overhead 27 W bright white bulb and an 85-dB piezo buzzer (3500 ± 500 Hz; Nexxtech, Barrie, Ontario, Canada). The trial ended either upon entry into the target box (i.e., minimum three paws touching the ramp into the target box) or after 3 min had elapsed. Mice were allowed 1 min in the target box. Short-term and long-term spatial memory (days 5 and 12, respectively) were evaluated from 90-second probe trials during which the target hole was closed.

All trials were recorded by an overhead closed-circuit Panasonic video system. One observer blind to group conditions analyzed the videos for primary and total latency (s), primary and total errors, search strategies (direct, mixed, and serial), and head pokes into the target and opposite holes according to Sunyer et al. (2007). Total latency and search strategies were assessed only for the acquisition phase, whereas frequency of head pokes in the target and opposite holes were examined during the memory probes. Acquisition parameters are reported as daily averages ($M \pm S.E.M.$).

2.5.3. Morris water maze

Spatial memory (acquisition and retention) and cognitive flexibility were assessed using the MWM (Vorhees and Williams, 2006). The arena, a circular pool (120 cm diameter, 50 cm tall) filled with

water (35 cm deep; 21 ± 1 °C), was placed in a room dimly illuminated by an 8 W white bulb. Spatial cues were available on the pool edge. The pool was arbitrarily divided into north, east, south, and west quadrants. To habituate to the arena and the escape platform (5 cm diameter), mice first completed three 60-second sessions in clear water. The remainder of the MWM was performed in water made opaque by non-toxic tempera paint. The acquisition phase (days 1–4) involved four 60-second trials (30-second ITI) daily with the objective of locating the unmarked platform submerged 0.5 cm below water level in the target quadrant (north). Trial starting positions were semi-randomized to different non-target quadrants opposite the platform. Spatial retention was assessed 24 h after the last acquisition trial using a 60-second trial (day 5) during which the platform was removed. To assess cognitive flexibility (days 7–11), starting locations were reversed and the platform was moved to the south quadrant.

All trials were recorded by an overhead closed-circuit Panasonic video system. Total distance (cm) travelled in the arena and in the target quadrants, duration (s) in the target and opposite quadrants, and average distance (cm) to the platform in each phase were obtained from videos analyzed with Noldus EthoVision® XT software. Mice were tracked using the whole body “differencing” detection method with a 10-second half-window LOWESS to reduce noise (Benjamini et al., 2010). Latency (s) to reach the platform and distance (cm) travelled in the target quadrant were also examined during the learning phases and probes, respectively. Non-probe data are reported as daily averages (*M* ± *S.E.M.*).

2.6. Estrous cycling

Estrous stage (i.e., proestrus, estrus, metestrus, and diestrus) was determined from vaginal smears collected as described by Byers et al. (2012). A trained rater examined all samples at 10 x and 40 x magnification under a light microscope (Olympus BX61).

2.6.1. Perfusion and tissue collection

All mice were deeply anesthetized with Euthanyl (40 mg/kg body weight, *ip*) at 17 weeks of age. Upon confirmation of deep anesthesia, mice were intracardially perfused with 20 mL saline followed by 20 mL of 4% paraformaldehyde (PFA). The excised brains were post-fixed in 4% PFA for 2 h and then placed in fresh 30% sucrose solution at 24 and 48 h after extraction. The brain tissue was sliced by

vibratome into 40 μm free-floating sections (1 in 3 series) and then stored in Eppendorf tubes filled with cryoprotectant solution at −20 °C.

2.6.2. Immunocytochemistry

All washes and incubations were performed at room temperature with gentle agitation, unless otherwise specified. Briefly, free-floating sections were washed with 1X phosphate-buffered saline (PBS; three x 5 min.) and then blocked with 1% bovine serum albumin / .3% Triton X-100 / 1X PBS for one hour. The tissue was incubated for 24 h with goat DCX (1:1000; Santa Cruz Biotechnology; cat: SC-806) and rabbit Ki67 (1:5000; Abcam; cat: ab15580) in a solution of 1X PBS / .3% Triton X-100. The sections underwent another 1X PBS wash (three x 5 min.) before a 30-minute incubation in a solution of donkey anti-rabbit Alexa Fluor 488 (1:2000; ThermoFisher Scientific; cat: A21206), donkey anti-goat Alexa Fluor 594 (1:1000; ThermoFisher Scientific; cat: A11058), and 1X PBS / .3% Triton X-100 at 30 °C in the dark. After a final set of three five-minute washes in 1X PBS, hippocampal sections were mounted onto slides coated with anti-fade (.1% P-phenylenediamine in 90% glycerol) and sealed with nail polish.

2.6.3. Cell quantification

All analyses were performed manually on one representative section of the dorsal hippocampus for each mouse (i.e., bregma: -2.18 mm, Franklin and Paxinos, 2007) by two raters blind to experimental conditions. DCX^+, $Ki67^+$, and $DCX^+/Ki67^+$ cell expression in the SGZ were examined from the middle 11 images of z-stacks (.95 μm step intervals) collected over the entire 40 μm section (Oishi et al., 2016). Images were captured by sequential excitation at 488 nm and 559 nm at 40 x magnification using an Olympus FV1000 confocal laser microscope coupled with Olympus FluoView FV10-ASW (version 4.2a) software (Center Valley, PA, USA). Emission was collected by spectral detectors in separate channels with user-defined minimum and maximum wavelengths. Fluorescing DCX^+ and $Ki67^+$ cells in the SGZ were counted if a clear circular cell body was visible and the cell bordered the SGZ. Cells that met both criteria and showed clear overlap in morphology were considered double-labelled ($DCX^+/Ki67^+$). $Ki67^+$ cell expression was similarly assessed in the CA1 and CA3 regions of the dorsal hippocampus using

representative images captured at 20 x by an Olympus BX61 microscope.

2.7. Statistical analyses

LPS-treated mice that did not show the characteristic sickness behavior response and body weight loss ($n_{\text{males}} = 3$; $n_{\text{females}} = 2$) were excluded from all analyses to eliminate the possibility of outliers due to improper treatment dosage and/or uncharacteristic LPS tolerance. The remaining dataset was then screened for statistical outliers. In order to maintain sample sizes and to reduce the effects of extreme outliers, cases that fell outside the 1.5 interquartile range in boxplots were adjusted using winsorization (Lotan et al., 2017; Pollet and van der Meij, 2017). A saline-treated [SAL]-OVX female and a SAL-ORX male had died during the study; therefore, sickness monitoring data was available for both mice, whereas open field activity was only available for the male.

Group differences in sickness-related parameters were analyzed with a three-way (sex x treatment x time) mixed-design repeated-measures analysis of variance (ANOVA). Acquisition parameters in the BM and MWM were analyzed with a four-way mixed-design repeated-measures ANOVA to examine the effects of Sex, Treatment, and Surgery over the four learning days. *F*-values were adjusted with the Greenhouse-Geisser correction when sphericity was violated according to Mauchly's test (i.e., $\varepsilon_{\text{Greenhouse-Geisser}} < .75$). The remaining behavioral and cellular data were examined using three-way (Sex x Treatment x Surgery) between-subjects ANOVAs. Measures of effect size are reported using partial eta-squared (η_p^2). ANOVAs were followed by Bonferroni-corrected pairwise comparisons when appropriate. Statistical significance was set to $p < .05$. All statistical analyses were conducted with IBM® SPSS® (version 20.0.0) software.

3. Results

3.1. Sickness behavior and body weight changes

As expected, LPS treatment induced more sickness behaviors than saline treatment ($F_{(1, 86)} = 942.39$, $p < .001$, $\eta_p^2 = .916$) and elicited greater sickness behavior responses among males compared to females ($F_{(1, 86)} = 4.50$, $p = .037$, $\eta_p^2 = .050$) (Fig. 2A and B). The expression of sickness behaviours changed significantly over time ($F_{(4.17, 358.89)} = 211.83$, $\eta_p^2 = .711$) and was influenced by time-point, sex,

and treatment ($F_{(4.17, 358.89)} = 4.45$, $\eta_p^2 = .049$) (all $p \leq .001$) such that LPS-treated males displayed more sickness behaviors than females at 30 min (*MD* = .54, *SE* = .14) and at 24 and 48 h (*MD* = .88, *SE* = .13 and *MD* = .63, *SE* = .07, respectively) (all $p < .001$).

Main effects of Time ($F_{(2.24, 192.77)} = 71.38$, $\eta_p^2 = .454$) and Treatment ($F_{(1, 86)} = 294.81$, $\eta_p^2 = .774$) and a significant Time x Treatment interaction ($F_{(2.24, 192.77)} = 94.57$, $\eta_p^2 = .524$) (all $p < .001$) were also seen for percentage change in body weight (Fig. 2C and D).

3.2. Locomotor activity in open field

Gonadectomized mice were faster than intact mice ($F_{(1, 76)} = 5.32$, $p = .024$, $\eta_p^2 = .065$). Velocity and distance travelled varied significantly by sex and treatment ($F_{(1, 76)} = 6.54$, $\eta_p^2 = .079$ and $F_{(1, 76)} = 8.22$, $\eta_p^2 = .098$, all $p < .05$), where LPS-SHAM females were slower and travelled less than SAL-SHAM and LPS-OVX females and their male counterparts (*MD* = -2.60, *SE* = .97 and *MD* = -720.87, *SE* = 279.38, respectively, all $p < .05$), and LPS-ORX males travelled further than SAL-ORX males (Fig. 2E – H).

3.3. Hippocampus-dependent learning and memory

3.3.1. BM acquisition

All groups appeared to learn the location of the target hole. Consistent with these observations, there was a significant main effect of Time for primary and total latencies ($F_{(3, 225)} = 30.92$, $\eta_p^2 = .292$ and $F_{(2.61, 195.97)} = 43.76$, $\eta_p^2 = .368$, respectively) and primary and total errors ($F_{(2.50, 187.83)} = 52.74$, $\eta_p^2 = .077$ and $F_{(2.21, 165.82)} = 47.39$, $\eta_p^2 = .387$, respectively) (all $p < .001$). Intact mice made more errors than gonadectomized mice before finding the target hole (primary errors: $F_{(1, 75)} = 6.78$, $\eta_p^2 = .083$), whereas LPS-treated mice took longer than saline-treated mice to find the target hole (primary latency: $F_{(1, 75)} = 7.68$, $\eta_p^2 = .093$) (all $p < .05$). LPS-treated mice were also slower to escape relative to saline-treated mice (total latency: $F_{(1, 75)} = 7.61$, $\eta_p^2 = .092$) (Fig. 3C and D). The analyses also revealed significant interactions of Time x Surgery for primary latency ($F_{(3, 225)} = 62.90$, $\eta_p^2 = .037$) and primary errors ($F_{(2.50, 187.83)} = 2.92$, $\eta_p^2 = .037$), Time x Treatment for primary and total latencies ($F_{(3, 225)} = 6.30$, $\eta_p^2 = .077$ and $F_{(2.61, 195.97)} = 6.23$, $\eta_p^2 = .077$, respectively), and Time x Sex ($F_{(2.61, 195.97)} = 2.94$, $\eta_p^2 = .038$) and Time x Treatment x Surgery ($F_{(2.61, 195.97)} = 3.03$, $\eta_p^2 = .039$) for total latency (all $p < .05$).

On the first day, saline-treated sham-operated (i.e., control) males took longer to escape relative to SAL-ORX males (Fig. 3A). LPS-ORX males and LPS-SHAM females had longer primary latencies on the second day than LPS-SHAM males (*MD* = 11.23, *SE* = 4.89, and *MD* = 11.99, *SE* = 5.01, respectively) (all $p < .05$). On the third day, LPS-SHAM females had longer primary latencies and made more primary errors compared to their saline-treated counterparts (*MD* = 1.51, *SE* = .62 and *MD* = 20.60, *SE* = 7.89, respectively, all $p < .05$). Relative to their saline-treated counterparts, LPS-intact females and males also committed more errors overall (Fig. 3C and D). On the final day, female controls made fewer total errors than LPS-SHAM females (Fig. 3D) and control males (*MD* = -7.53, *SE* = 2.88, $p = .011$).

Although mice adjusted their search strategies to locate the target hole over time, LPS-treated groups implemented more disorganized, random searches than saline-treated mice (Fig. 3E and F). Accordingly, we observed significant main effects of Time for direct, serial, and mixed searches ($F_{(3, 225)} = 7.90$, $\eta_p^2 = .095$; $F_{(3, 225)} = 6.17$, $\eta_p^2 = .075$; and, $F_{(3, 225)} = 10.36$, $\eta_p^2 = .121$, respectively, all $p \leq .001$) and a significant main effect of Treatment for mixed searches ($F_{(1, 75)} = 6.75$, $p = .011$, $\eta_p^2 = .083$). The ANOVAs also revealed significant interactions of Treatment x Surgery ($F_{(1, 75)} = 4.16$, $p = .045$, $\eta_p^2 = .052$) for direct approaches, Time x Treatment and Time x Sex x Treatment for serial searches ($F_{(3, 225)} = 6.23$, $\eta_p^2 = .077$ and $F_{(3, 225)} = 4.03$, $\eta_p^2 = .051$, respectively, all $p \leq .001$), and Time x Sex x Treatment x Surgery for mixed searches ($F_{(3, 225)} = 2.72$, $p = .045$, $\eta_p^2 = .035$). On the first day, control females used direct searches more often than LPS-SHAM females (*MD* = .19, *SE* = .09, $p = .030$). Compared to LPS-OVX females, mixed approaches were initially more common among LPS-ORX males (*MD* = .24, *SE* = .11, $p = .041$). By the third day, LPS-OVX males used direct searches more often than their female counterparts (*MD* = .24, *SE* = .11, $p = .034$) but implemented serial searches less often than SAL-OVX males (*MD* = -.23, *SE* = .10, $p = .028$). SAL-OVX females implemented direct searches less often than control females on the final day (*MD* = .28, *SE* = .10, $p = .007$).

3.3.2. BM memory probes

During the short-term memory probe, females made more primary errors than males ($F_{(1, 75)} = 6.11$, $\eta_p^2 = .075$) and LPS-treated mice made more primary errors and target-opposite pokes than

saline-treated mice ($F_{(1, 75)} = 5.69$, $\eta_p^2 = .070$ and $F_{(1, 75)} = 4.04$, $\eta_p^2 = .051$, all $p < .05$). Furthermore, LPS-SHAM females committed more primary errors than LPS-SHAM males ($MD = 3.82$, $SE = 1.56$, $p = .016$). Primary latencies, target-hole pokes, and total errors did not differ significantly between groups.

During the long-term memory probe, females made more total errors than males ($F_{(1, 74)} = 6.41$, $p = .013$, $\eta_p^2 = .080$). All other parameters were similar across groups.

3.3.3. MWM acquisition

The analyses suggest that all mice learned the platform location (north). Accordingly, the analyses revealed significant main effects of Time for escape latency ($F_{(3, 225)} = 19.33$, $\eta_p^2 = .205$), path length ($F_{(2.73, 204.58)} = 21.28$, $\eta_p^2 = .221$), target-quadrant duration ($F_{(3, 225)} = 2.95$, $\eta_p^2 = .038$), average distance to the platform ($F_{(3, 225)} = 9.19$, $\eta_p^2 = .109$), and opposite-quadrant duration ($F_{(3, 225)} = 13.94$, $\eta_p^2 = .157$) (all $p < .05$). Overall, females swam closer to the platform than males ($F_{(1, 75)} = 4.93$, $p = .029$, $\eta_p^2 = .062$) (Fig. 4C and D).

LPS-SHAM females initially swam closer to the platform than their male and OVX counterparts ($MD = -7.75$, $SE = 3.26$ and $MD = -6.55$, $SE = 3.26$, respectively) and took shorter paths to locate the platform than LPS-SHAM males ($MD = -288.68$, $SE = 121.82$) (all $p < .05$). By the second day, control females explored the south longer and swam further to find the platform than SAL-OVX females ($MD = 4.64$, $SE = 2.23$ and $MD = 273.99$, $SE = 106.91$, respectively), whereas LPS-ORX males spent more time in the south than SAL-ORX males ($MD = 5.11$, $SE = 2.16$) (all $p < .05$).

3.3.4. MWM acquisition probe

All groups spent similar amounts of time in the target quadrant (north). However, gonadectomized mice travelled more in the north and spent less time in the south than intact mice ($F_{(1, 75)} = 4.18$, $\eta_p^2 = .053$ and $F_{(1, 75)} = 4.17$, $\eta_p^2 = .053$, all $p < .05$). The ANOVA also identified significant interactions of Sex x Treatment for path length ($F_{(1, 75)} = 4.15$, $p = .045$, $\eta_p^2 = .042$) and Sex x Treatment x Surgery for target proximity ($F_{(1, 75)} = 5.58$, $p = .021$, $\eta_p^2 = .069$). SAL-OVX females swam closer to the platform than intact counterparts ($MD = 10.10$, $SE = 4.56$), as did SAL-OVX and LPS-SHAM females relative to their male counterparts ($MD = -9.86$, $SE = 4.32$ and $MD = -8.80$, $SE = 4.32$, respectively) (all

$p < .05$). LPS-ORX males swam shorter distances than LPS-OVX females ($MD = -263.56$, $SE = 95.71$, $p = .007$) and LPS-SHAM males ($MD = -215.74$, $SE = 95.71$, $p = .027$).

3.3.5. MWM spatial reversal

All groups appeared to learn the new platform location (south) given the significant main effects of Time for escape latency ($F_{(2.60, 195.15)} = 26.44$, $\eta_p^2 = .261$), path length ($F_{(2.56, 191.92)} = 24.57$, $\eta_p^2 = .247$), duration in the target and opposite quadrants ($F_{(2.57, 193.06)} = 7.67$, $\eta_p^2 = .093$ and $F_{(2.28, 171.20)} = 24.59$, $\eta_p^2 = .247$, respectively), and average distance to the platform ($F_{(2.51, 188.39)} = 17.97$, $\eta_p^2 = .193$) (all $p < .001$). Overall, gonadectomized mice took longer to escape and spent more time exploring the south than intact mice ($F_{(1, 75)} = 5.78$, $\eta_p^2 = .072$ and $F_{(1, 75)} = 6.09$, $\eta_p^2 = .075$, respectively, all $p < .05$) (Fig. 5A – B and E – F).

We also observed significant Time x Sex x Treatment x Surgery interactions for target-quadrant duration, escape latency, and path length ($F_{(2.57, 193.06)} = 4.15$, $\eta_p^2 = .052$; $F_{(2.60, 195.15)} = 3.94$, $\eta_p^2 = .050$; and, $F_{(2.56, 191.92)} = 3.47$, $\eta_p^2 = .044$, respectively) (all $p < .05$). LPS-OVX females initially spent less time in the south than SAL-OVX females (Fig. 5D) and LPS-ORX males ($MD = 4.08$, $SE = 1.60$) and swam further away from the platform than LPS-ORX males ($MD = 9.64$, $SE = 3.96$) and their intact counterparts ($MD = 11.10$, $SE = 5.17$) (all $p < .05$). On the second day, SAL-ORX males took longer to escape than their female counterparts ($MD = 12.34$, $SE = 5.72$). Furthermore, compared to their saline-treated and sham-operated counterparts, LPS-OVX females took longer to escape and explore the north (Fig. 5B and D), swam further away from the platform ($MD = 14.58$, $SE = 5.04$ and $MD = 12.19$, $SE = 5.04$, respectively), and took longer paths to reach the platform ($MD = 376.10$, $SE = 127.58$ and $MD = 465.15$, $SE = 127.58$, respectively) (all $p < .05$). Longer durations in the north, path lengths, and target proximities were also seen among LPS-OVX females relative to LPS-ORX males on the second day ($MD = 4.43$, $SE = 1.52$; $MD = 340.18$, $SE = 130.73$; and, $MD = 4.43$, $SE = 1.52$, respectively, all $p < .05$). On the third day, control males took longer swim paths than LPS-SHAM males ($MD = 265.30$, $SE = 129.08$, $p = .043$), were quicker to escape than LPS-SHAM males (Fig. 5A) and their female counterparts ($MD = 10.25$, $SE = 5.04$, $p = .046$), and spent more time in the south than their LPS-treated counterparts (Fig. 5C). On

the last day, LPS-OVX females were again slower to escape and spent more time in the north than SAL-OVX females (Fig. 5E and F) and LPS-ORX males (*MD* = 9.27, *SE* = 3.72 and, *MD* = 3.56, *SE* = 1.18, respectively) (all $p < .05$). LPS-ORX males also took shorter paths to reach the platform than LPS-OVX females (*MD* = -252.03, *SE* = 103.47) and intact counterparts (*MD* = -231.62, *SE* = 103.47) (all $p < .05$).

3.3.6. MWM spatial reversal probe

Gonadectomized mice travelled less in the target south quadrant and had shorter path lengths than intact mice ($F_{(1, 75)} = 4.76$, $\eta_p^2 = .060$ and $F_{(1, 75)} = 5.65$, $\eta_p^2 = .070$, respectively, all $p < .05$). Compared to males, females spent more time in the north, had longer swim paths, and swam further away from the platform ($F_{(1, 75)} = 8.50$, $\eta_p^2 = .102$; $F_{(1, 75)} = 6.35$, $\eta_p^2 = .078$; and, $F_{(1, 75)} = 4.75$, $\eta_p^2 = .060$, respectively, all $p < .05$). Control males and females travelled more than their gonadectomized counterparts (*MD* = 203.85, *SE* = 97.55 and *MD* = 197.43, *SE* = 97.55, respectively, all $p < .05$).

3.4. Cellular expression

The significant Treatment x Surgery interaction ($F_{(1, 56)} = 5.11$, $p = .028$, $\eta_p^2 = .084$) showed significantly higher $Ki67^+$ cells in the CA1 of LPS-SHAM males compared to their saline-treated and castrated counterparts (Fig. 6C).

All groups showed similar expression of DCX^+, $Ki67^+$, and $DCX^+/Ki67^+$ cells in the SGZ and $Ki67^+$ cells in the CA3.

4. Discussion

As expected, LPS treatment during the stress-sensitive pubertal period impaired learning in adulthood. In the BM, LPS-treated mice used more inaccurate and inefficient searches to locate and enter the escape hole than saline-treated mice. Pubertal LPS treatment also negatively impacted short-term but not long-term retention of the target location, although females tended to show poorer spatial retention than males overall. In contrast, all groups showed similar learning and retention of the escape platform in the MWM, but females generally swam closer to the platform than males while learning its location. Intact mice travelled less in the target quadrant and spent more time in the opposite quadrant during the probe trial than gonadectomized mice. When tasked with learning a new platform location, only

gonadectomized groups were significantly slower to adapt to this change relative to their intact counterparts, although LPS-treated groups showed slower learning, particularly among LPS-OVX females.

The discrepant findings between the BM and MWM can be attributed to differences in stress experienced by the mice during behavioral testing. This stressor-dependent performance is particularly relevant for sex differences because testosterone and estradiol cause opposing stress responses under certain conditions (Boivin et al., 2017; Romeo et al., 2016; ter Horst et al., 2012). The dry-land BM is a less stressful and more ethologically-relevant task than the MWM and is therefore more advantageous at elucidating learning/navigation behaviors (D'Hooge and De Deyn, 2001; Sharma et al., 2010; Whishaw and Tomie, 1996). Furthermore, the predominantly rat-based findings from water mazes can be difficult to translate to mice (Sharma et al., 2010; Whishaw and Tomie, 1996). Although both rats and mice perform similarly on dry-land mazes, mice are more sensitive to the aversive nature of the MWM and show an inverse relationship between spatial learning and corticosterone levels only in the water maze (D'Hooge and De Deyn, 2001; Harrison et al., 2009; Whishaw and Tomie, 1996).

Given these considerations in the dry-land and water mazes, pubertal LPS treatment appears to impair hippocampus-dependent task performance in adulthood during low-stress but not high-stress conditions. Additional support for this stress-dependent expression of LPS-induced cognitive deficits comes from the increase in treatment interactions seen during the reversal phase of the MWM once the mice have presumably become more familiarized and thus less stressed with the testing environment. These findings are generally consistent with the impairments seen among adult females treated with LPS during puberty in other dry-land hippocampus-dependent measures (Ismail and Blaustein, 2013). Future studies should follow up on our findings and examine whether physical markers of stress correlate with performance on hippocampus-dependent measures.

Interestingly, sex or surgery did not modulate LPS-induced learning deficits, which suggests that these LPS-induced cognitive changes occur independently of gonadal hormonal influences. This absence of sex differences in learning the BM and MWM coincide with other studies of mice at this age (e.g.,

Frick et al., 1999; Harrison et al., 2006; Rogers et al., 2017). Nevertheless, several noteworthy significant sex and surgery differences were observed. First, females swam significantly closer to the platform than males during the acquisition and retention trials of the MWM which suggests a more accurate search strategy among females. This finding reflects Jonasson's (2005) report of better performance of females compared to males in water maze tasks in mice but not rats. Furthermore, females made more spatial retention errors in both probe trials of the BM and showed poorer memory for the new platform location in the reversal probe of the MWM. Similar male advantages in reference learning are attributed to age and sex differences in motivational tendencies, task-dependent displays of anxiety, and spatial processing (e.g., search strategies and utilization of different extra-maze and intra-maze cues on different tasks) (Grissom et al., 2013; LaBuda et al., 2002; Lamberty and Gower, 1988; Mishima et al., 1986). Some of these sex-specific results on hippocampus-dependent tasks may be due to age-related sex differences in the organizational and activational effects of gonadal steroid hormones on hippocampal physiology and function (for review see Koss and Frick, 2016).

Contrary to our hypotheses, gonadal steroid hormones did not impact performance on both spatial tasks or regulate the cognitive effects of pubertal LPS exposure. Instead, gonadectomy during early adulthood impacted cognitive flexibility in the MWM later in adulthood. This task-specific finding may be due to the timing of the behavioral test. Several studies have demonstrated that the extent of OVX-induced impairments in adult rodents differ between hippocampus-dependent tasks (for review see Tuscher et al., 2015). The cognitive effects of castration among adult male rodents are more ambiguous but generally point towards task-dependent impairments in spatial working but not reference memory paradigms (for review see Celec et al., 2015). Gonadectomy-induced impairments in spatial learning during the reversed MWM paradigm were more prominent among female mice compared to male counterparts, which is in line with the aforementioned task-dependent cognitive deficits in castrated males. Therefore, spatial reference memory in females may be more reliant on circulating gonadal steroid hormone levels than in males.

Given the association between adult hippocampus-dependent memory processes and hippocampal

neurogenesis, we also examined whether group differences in learning behaviors translated to expression of DCX^+ and $Ki67^+$ cells in the dorsal hippocampus. Neither pubertal immune challenge nor gonadectomy during early adulthood permanently affected basal expression and co-labelling of these markers in the SGZ in males and females. Although the literature has generally shown decreases in DCX^+ and $Ki67^+$ cells in the SGZ following gonadectomy, our findings may be explained by species differences and the timing of our surgery. For example, Lagace et al. (2007) found similar proliferation and neurogenesis in adult C57BL/6 mice, regardless of OVX and estrous stage, whereas others have found that significant sex differences in meadow voles are impacted by breeding season (Galea and McEwen, 1999). The lack of treatment effect in SGZ cellular proliferation also suggests that these treatment-induced effects may have recovered by the time of euthanasia. In fact, the current literature on stress-related changes in hippocampal neurogenesis points towards greater vulnerabilities and longer recoveries following chronic versus acute stressors in older ages compared to younger groups (for reviews see Hueston et al., 2017; Loi et al., 2014; Lucassen et al., 2015).

In contrast, our experimental manipulations induced sex-specific changes in cellular proliferation in the CA1, a region involved in detecting spatial novelty and episodic-like memory (Barbosa et al., 2012; Drieskens et al., 2017). Intact males exposed to LPS during puberty expressed more $Ki67^+$ cells in the CA1 than their saline-treated and castrated counterparts, whereas females were not significantly impacted. Although we did not examine the functional significance of these cellular changes, similar stress-induced neurogenic changes have been implicated in the expression of depressive-like and anxiety-like behaviours (for reviews see Loi et al., 2014; Lucassen et al., 2015). Future studies should also explore whether pubertal LPS treatment similarly impacts other aspects of hippocampal neurogenesis (e.g., integration, activation).

4.1. Conclusions

We conclude that pubertal immune challenge elicits similar learning deficits in adult males and females on low-stress hippocampus-dependent spatial memory tasks but impairs cognitive flexibility more in females. High-stress tasks, however, mask the cognitive effects of pubertal LPS exposure. These

enduring cognitive deficits do not appear to be modulated by gonad-derived hormones or changes in basal neurogenesis. Pubertal males, nevertheless, are more sensitive than age-matched females to LPS-induced priming of basal CA1 cellular proliferation, whereas both sexes show resiliency towards LPS-induced changes in basal hippocampal neurogenesis. These novel findings provide invaluable insights into our growing understanding of the stress-sensitive pubertal period and is the first to examine sex differences in the enduring cognitive effects of pubertal immune challenge.

Conflicts of interest

None.

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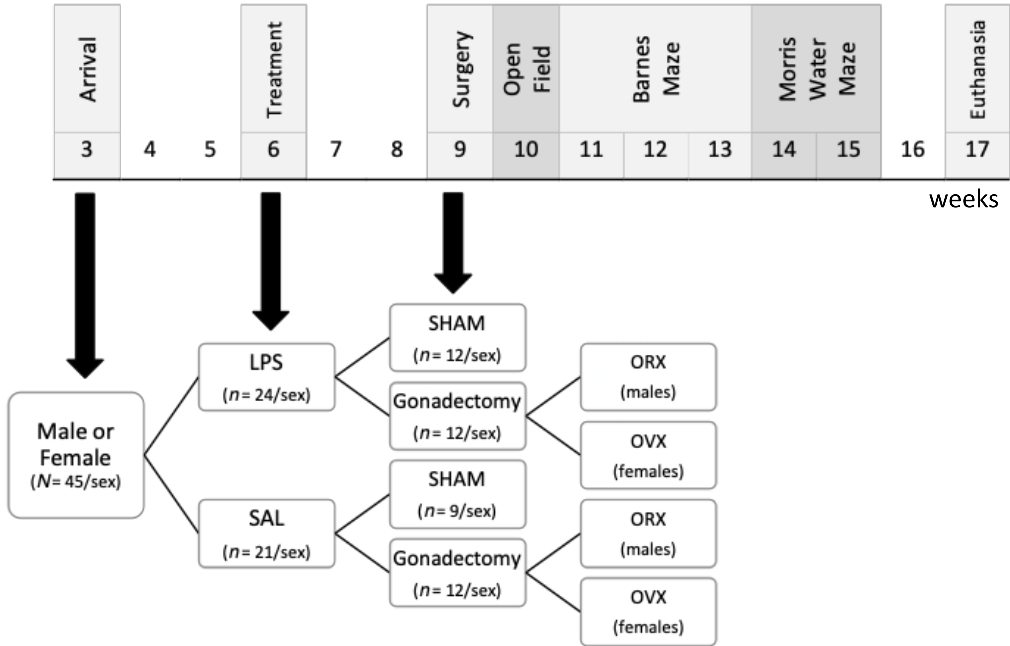
Figure 1.

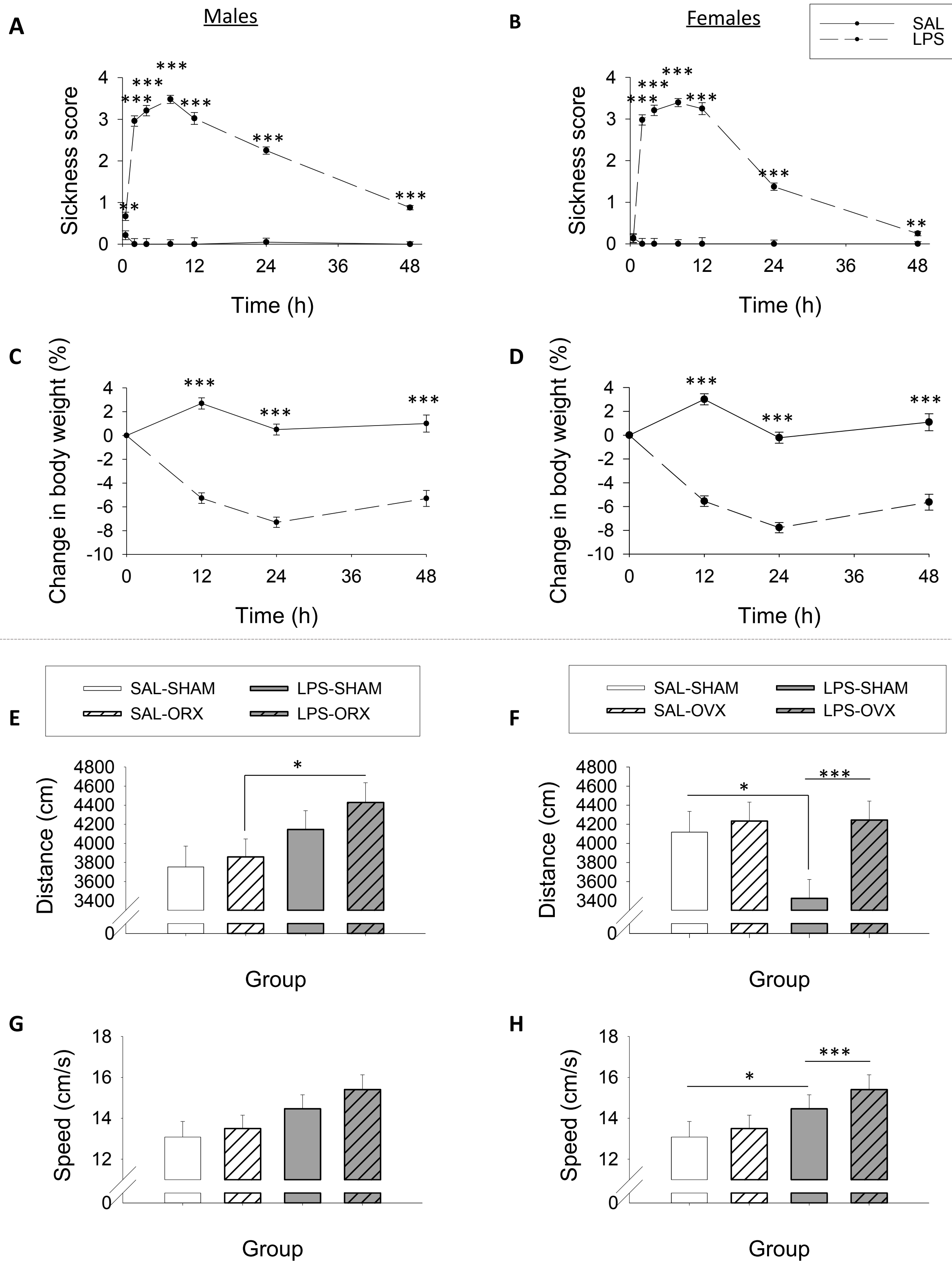
Experimental timeline and experimental groups.



Note: SAL = saline; LPS = lipopolysaccharide; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy.

Figure 2.

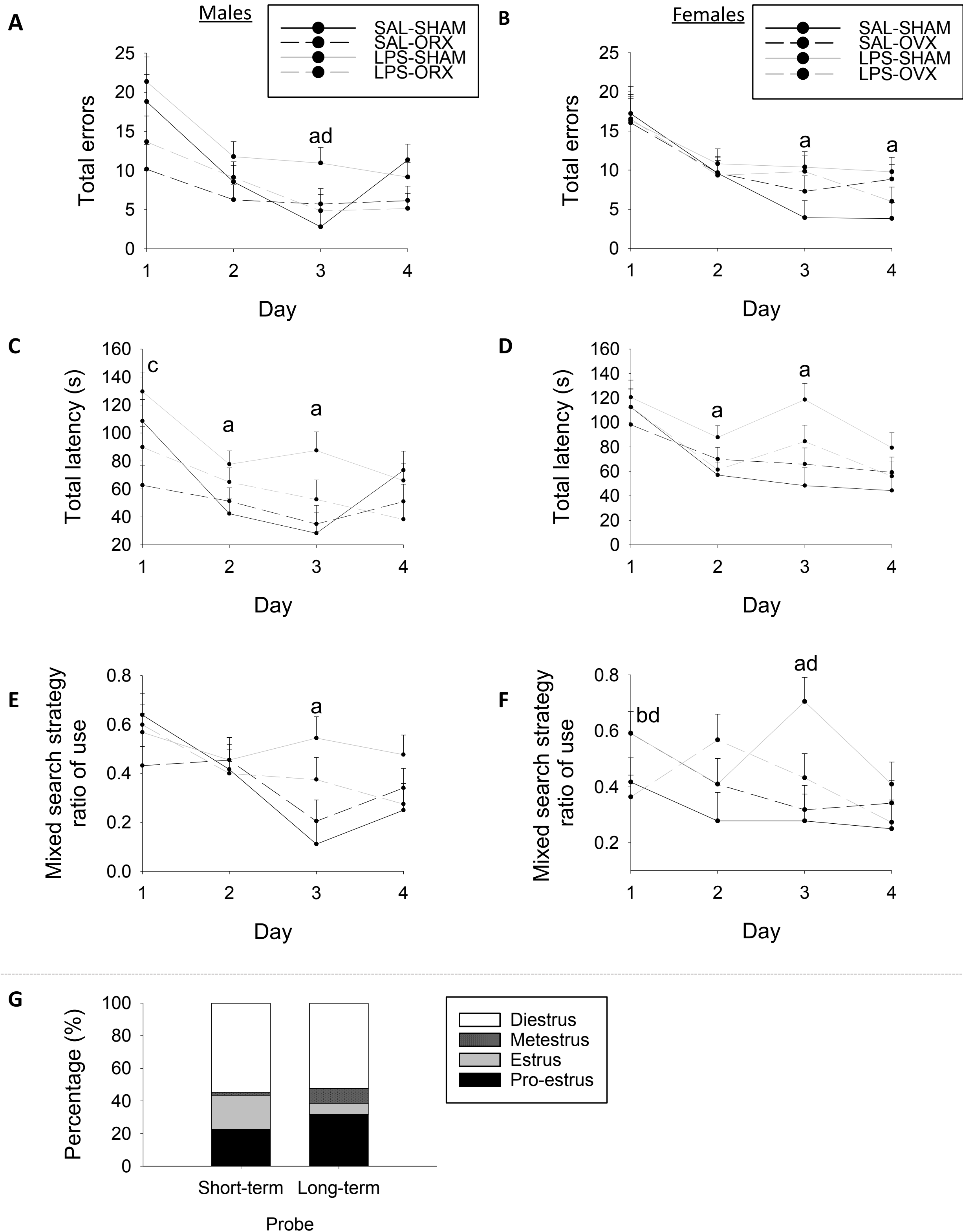
Acute sickness responses to saline or LPS treatment and open field locomotor activity.



Note. Figures A and B refer to acute changes in sickness behaviour following saline or LPS treatment in males and females, respectively. Figures C and D refer to acute changes in percent body weight (g) change from baseline following saline or LPS treatment in males and females, respectively. Figures E and F refer to treatment (saline versus LPS) and surgery (intact versus gonadectomized) differences in distance (cm) travelled in the open field in males and females, respectively. Figures G and H refer to treatment and surgery differences in velocity (cm/s) in the open field in males and females, respectively. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

Figure 3.

Treatment and surgery differences in Barnes maze acquisition among males and females.



Note. Figures A and B refer to treatment (saline versus LPS) and surgery (intact versus gonadectomized) differences in total errors made by males and females, respectively. Figures C and D refer to treatment and surgery differences in total latencies (s) among males and females, respectively. Figures E and F refer to treatment and surgery differences in mixed search strategy use among males and females, respectively. Figure G depicts percentage of female mice in pro-estrus, estrus, metestrus, and diestrus during the short-term and long-term memory probes. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; *a* = significant treatment difference ($p < .05$) among intact subjects, *b* = significant treatment difference ($p < .05$) among gonadectomized subjects, *c* = significant surgery difference ($p < .05$) among saline-treated subjects, *d* = significant surgery difference ($p < .05$) among LPS-treated subjects.

Figure 4.

Treatment and surgery differences in learning in the acquisition phase of the Morris water maze among males and females.

A Males
SAL-SHAM
SAL-ORX
LPS-SHAM
LPS-ORX
Escape latency (s)
Day

B Females
SAL-SHAM
SAL-OVX
LPS-SHAM
LPS-OVX
Escape latency (s)
c
Day

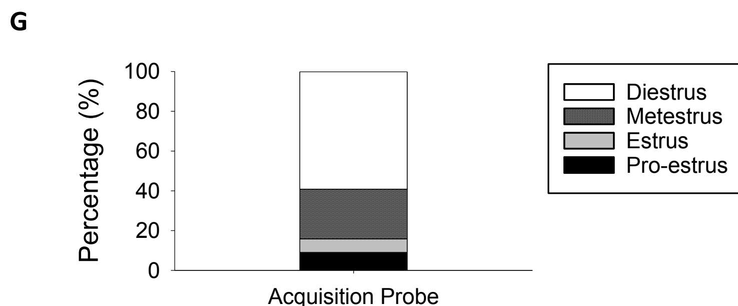
C
Average distance (cm) to platform
Day

D
Average distance (cm) to platform
d
Day

E
Duration (s) in target quadrant
Day

F
Duration (s) in target quadrant
Day

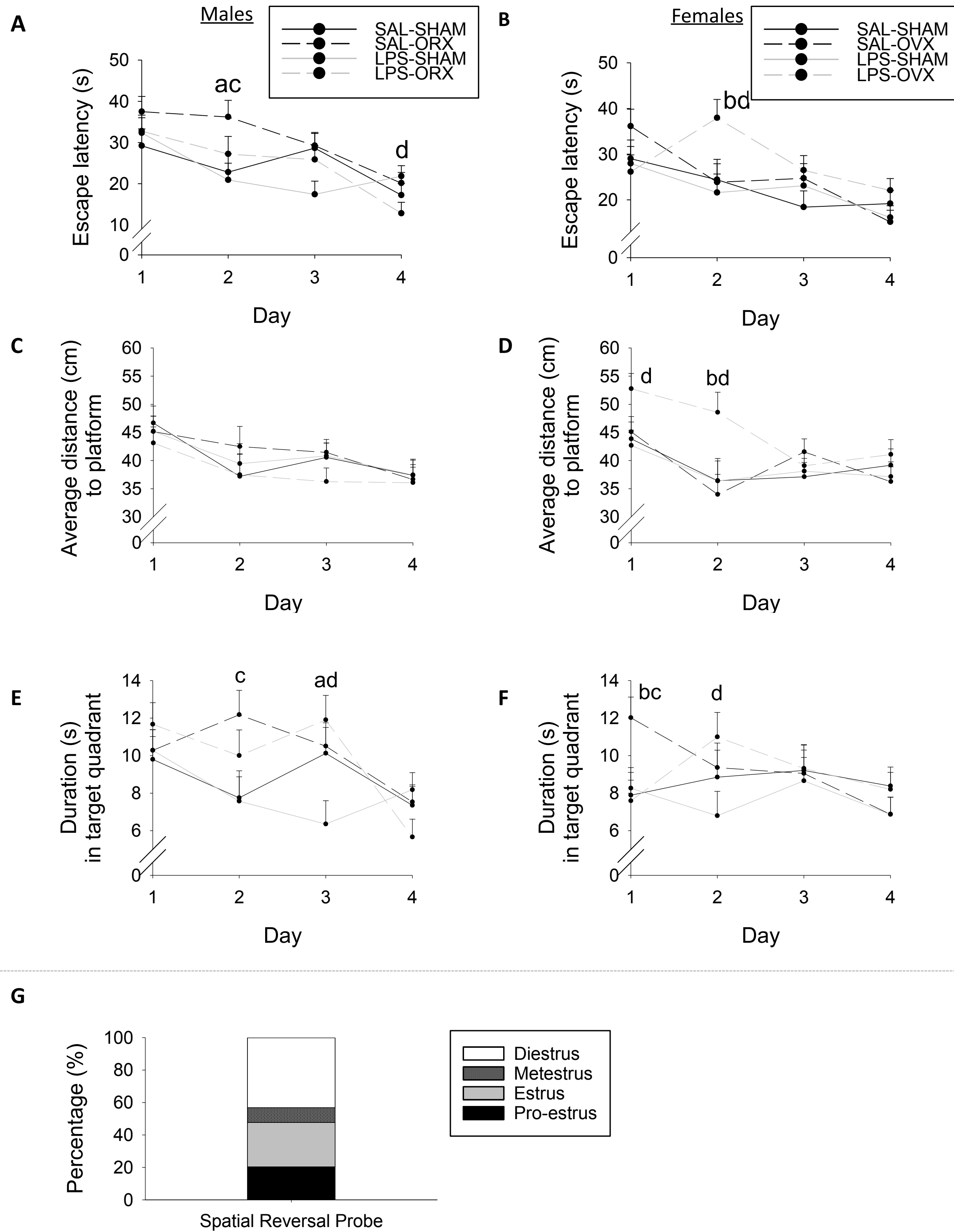
G



Note. Figures A and B refer to treatment (saline versus LPS) and surgery (intact versus gonadectomized) differences in escape latency (s) among males and females, respectively. Figures C and D refer to treatment and surgery differences in average distances (cm) to the platform among males and females, respectively. Figures E and F refer to treatment and surgery differences in duration (s) in the target quadrant (north) among males and females, respectively. Figure G depicts percentage of female mice in pro-estrus, estrus, metestrus, and diestrus during the acquisition memory probe. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; *a* = significant treatment difference ($p < .05$) among intact subjects, *b* = significant treatment difference ($p < .05$) among gonadectomized subjects, *c* = significant surgery difference ($p < .05$) among saline-treated subjects, *d* = significant surgery difference ($p < .05$) among LPS-treated subjects.

Figure 5.

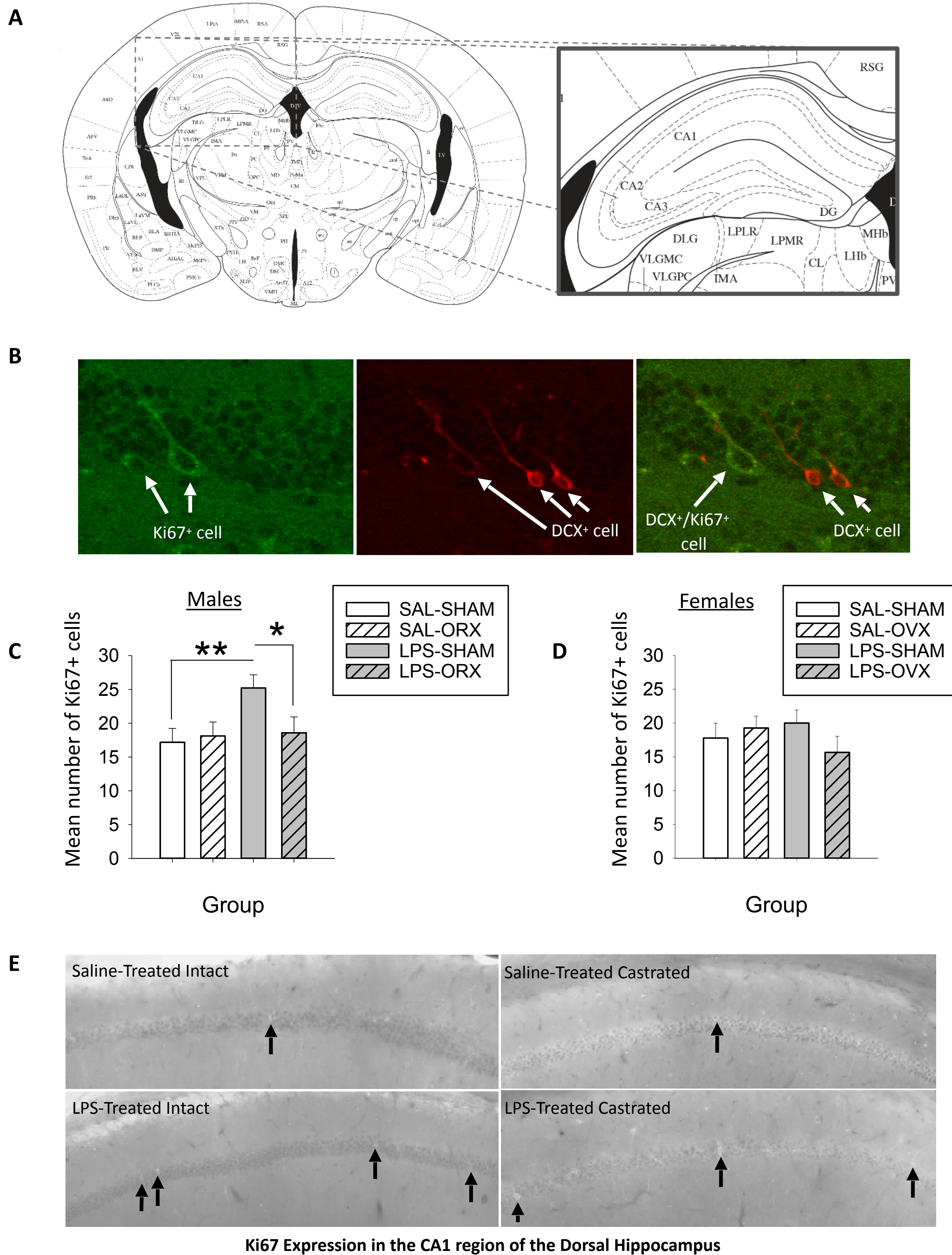
Treatment and surgery differences in learning in the spatial reversal phase of the Morris water among males and females.



Note. Figures A and B refer to treatment (saline versus LPS) and surgery (intact versus gonadectomized) differences in escape latency (s) among males and females, respectively. Figures C and D refer to treatment and surgery differences in average distances (cm) to the platform among males and females, respectively. Figures E and F refer to treatment and surgery differences in duration (s) in the target quadrant (south) among males and females, respectively. Figure G depicts percentage of female mice in pro-estrus, estrus, metestrus, and diestrus during the acquisition memory probe. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; *a* = significant treatment difference ($p < .05$) among intact subjects, *b* = significant treatment difference ($p < .05$) among gonadectomized subjects, *c* = significant surgery difference ($p < .05$) among saline-treated subjects, *d* = significant surgery difference ($p < .05$) among LPS-treated subjects.

Figure 6.

Treatment and gonadal effects on DCX and Ki67 expression in the dorsal hippocampus.



Ki67 Expression in the CA1 region of the Dorsal Hippocampus

Note. Figure A displays the dentate gyrus (DG), cornus ammonis (CA1) and CA3 regions of the dorsal hippocampus of the mouse brain at -2.18 mm from bregma (reproduced from Franklin and Paxinos, 1997). Figure B displays photomicrographs of DCX^+, $Ki67^+$, and double-labelled $DCX^+/Ki67^+$ cells in the subgranular zone of the dorsal hippocampus. Figures C and D show treatment (saline versus LPS) and surgery (intact versus gonadectomized) effects in mean $Ki67^+$ cell expression in the CA1 region of the dorsal hippocampus of adult (10-week-old) males and females, respectively. Figure D displays photomicrographs of treatment and surgery differences in Ki67 expression in the CA1 region of the dorsal hippocampus of adult male mice. *Note*: SAL = saline; SHAM = sham-surgery; ORX = orchiectomy; OVX = ovariectomy; * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

Study 2: Sex-Specific Responses of the Pubertal Neuroimmune Axis in CD-1 Mice.

Author Note

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Abstract

The mechanistic relationship between the sexually dimorphic neuroimmune system and the sex-specific outcomes of a pubertal immune challenge is unclear. Therefore, we examined sex differences in the progression of cytotoxic microglial responses and blood-brain barrier (BBB) disruption to a peripubertal lipopolysaccharide (LPS) treatment in brain regions relevant to stress responses and cognitive function. Six-week-old (i.e., stress-sensitive pubertal period) male and female CD-1 mice were treated with LPS (1.5 mg/kg body weight, *ip*) or 0.9% saline (LPS-matched volume, *ip*). Sex and treatment differences in microglial ($Iba1^+$) and apoptotic neuronal ($caspase\text{-}3^+$/ $NeuN^+$) and non-neuronal ($caspase\text{-}3^+$/$NeuN^-$) expression were examined in the hippocampus, medial prefrontal cortex (mPFC), and paraventricular nucleus 24 h (sickness), one week (symptomatic recovery) and four weeks (early adulthood) post-treatment (n = 8/group). Microglial morphology was quantified with fractal analyses. Group differences in BBB permeability to ^{14}C-sucrose were examined 24 h (whole-brain, hippocampus, prefrontal cortex, hypothalamus, and cerebellum) and one week (whole-brain) post-treatment. The acute effects of pubertal LPS were specific to females (i.e., global BBB disruption, altered microglial expression and morphology in the mPFC and hippocampus, increased hippocampal apoptosis). The residual effects of pubertal LPS-induced sickness observed in microglia persisted into adulthood in a sex- and region-specific manner. In addition to highlighting these sex-specific responses of the pubertal neuroimmune system, we report baseline region-specific sex differences in microglia spanning puberty through adulthood. We propose that these sex differences in neuroimmune-neurovascular interactions during the stress-sensitive pubertal period create sex biases in stress-related disorders of brain and behaviour.

Keywords: lipopolysaccharide, blood-brain barrier, fractal analysis, Iba1, caspase-3,

NeuN.

1. Introduction

Sex-specific patterning of the brain and the stress response system during puberty contribute to sex differences in stress vulnerability and reactivity during this critical period, particularly towards a systemic immune challenge (Holder and Blaustein, 2014; Kane and Ismail, 2017; Schulz and Sisk, 2016; Sisk and Foster, 2004; Vigil et al., 2011). Sex differences in the reorganization and remodelling of pubertal neuro- circuitry facilitate the sex-specific outcomes of a pubertal immune chal- lenge on brain and behaviour. For example, CD-1 mice treated with the bacterial endotoxin lipopolysaccharide (LPS) (1.5 mg/kg body weight, intraperitoneal [*ip*]) during the stress-sensitive pubertal period (i.e., six weeks of age) show female-skewed increases in depression-like behav- iours and male-biased increases in anxiety-like behaviours and reactivity to novel stressors (Murray et al., 2019). Similar sex-dependent effects of pubertal LPS treatment are observed in stress and immune responses (Cai et al., 2016; Girard-Joyal et al., 2015; Sharma et al., 2019), behavioural responsiveness to gonadal hormones (Ismail et al., 2013; Laroche et al., 2009 a, b; Olesen et al., 2011), spatial learning and hippocampal cell development (Kolmogorova et al., 2019), and dopamine-sensitive behaviours (Girard-Joyal and Ismail, 2017). The neural mechanisms driving the sex-specific acute and enduring effects of LPS treatment during the stress-sensitive pubertal period remain elusive.

The brain's primary line of immune defense is a system of microglia cells whose phagocytic properties lend themselves well to addressing immune threats (e.g., cellular debris, bacteria), shaping of neural circuits during critical periods, and maintaining neural networks (Lenz and Nelson, 2018; Li and Barres, 2018; Tay et al., 2017). Age-related sex differences in cell density and phenotype (e.g., Crain et al., 2013; Hanamsagar et al., 2017; Villa et al., 2018) establish region-dependent sexual dimorphisms in microglial functions. When faced with an

immune threat, the highly ramified surveillant "resting" microglia alter their morphology along a graded continuum towards the amoeboid-like form of their fully "activated" phagocytic state (Hoogland et al., 2015). Intense systemic immune stressors can dysregulate adaptive microglia-mediated inflammatory responses (Ling et al., 2006; Puntener et al., 2012; Qin et al., 2007; Williamson et al., 2011). Maladaptive microglial responses generate excess cytotoxic factors (e.g., nitric oxide, tumour necrosis factor alpha) which activate programmed cell death pathways such as apoptosis (e.g., Cunningham et al., 2005; Khan et al., 2016; 2017; Noh et al., 2014). Due to genomic and hormonal influences, neonatal and young females adopt predominantly caspase-dependent apoptotic path- ways during inflammatory events compared to the caspase-independent pathways more commonly seen among age-matched males (Alfonso-Loeches et al., 2013; Du et al., 2004; Liu et al., 2009; Rau et al., 2003; Waters and Simerly, 2009; Zhu et al., 2005, 2006).

As a key interface between the central nervous system (CNS) and the periphery, the blood-brain barrier (BBB) moderates neuroinflammatory responses to systemic immune events by restricting passage of peripheral immune-activating factors into the CNS (Abbott et al., 2006; Engelhardt and Liebner, 2014; Langen et al., 2019; Sweeney et al., 2019). High doses of systemic LPS (e.g., 3.0 mg/kg body weight) disrupt BBB integrity to small (^{14}C-sucrose [342 Da], sodium fluorescin [376 Da], ^{99m}Tc-DTPA [487 Da]) and large (^{13}I-albumin [66 kDa]) radioisotopes, with the most significant LPS-induced BBB disruption occurring 24 h post-treatment (Banks et al., 2015; Nishioku et al., 2009). The magnitude of regional and global BBB vulnerability to systemic immune events is influenced by age-related sex differences in circulating gonadal steroid hormones (Abi-Ghanem et al., 2020; Duckles and Krause, 2007; Erdö et al., 2016; Erickson and Banks, 2019; Krause et al., 2006). Although our understanding of the

androgenic influences on the BBB is complicated by the natural conversion of androgens to estrogenic steroids in the body, androgens and progesterone appear to oppose the cerebrovascular effects of estrogens (Abi-Ghanem et al., 2020; Krause et al., 2002; Ospina et al., 2003; Robison et al., 2019). Androgens and progesterone generally constrict cerebrovascular tone and exert pro-inflammatory effects, whereas estrogens tend to dilate vascular tone, increase cerebral flow, and show immune-suppressing or anti-inflammatory properties (Gonzales et al., 2005, 2008; Krause et al., 2002; Ospina et al., 2003, 2004; Sunday et al., 2006).

This study tested the mechanistic involvement of the pubertal neuroimmune network in the sex-specific outcomes of systemic immune challenge during this critical period. We examined sex differences in the progression of LPS-induced effects on BBB permeability, microglial expression and morphology, and caspase-3-dependent apoptosis of neuronal and non-neuronal cells, focusing on the sexually dimorphic and interconnected brain regions for stress regulation and cognitive functioning. Acutely, systemic LPS treatment was expected to disrupt BBB integrity (i.e., whole brain and in the hippocampus, prefrontal cortex [PFC], hypothalamus, and cerebellum) and to induce apoptotic microglial responses in the hippocampus, medial PFC (mPFC), and paraventricular nucleus (PVN). LPS-induced BBB disruption and apoptosis were expected to subside upon symptomatic recovery, whereas the microglial changes were expected to persist into early adulthood. The magnitude of all LPS-induced effects was expected to differ betwen regions. Baseline sex differences in the pubertal neuroimmune network predict a generally female-biased vulnerability to LPS-induced changes in the examined systems.

2. Methods

2.1 Animals

Three-week-old male and female CD-1 mice were shipped to our animal facility from

Charles River Laboratories (Saint-Constant, Québec). All mice were housed in groups of three in polycarbonate Lexan cages (17 x 28 x 12 cm [width x length x height]) in sex-segregated rooms maintained on a reversed light cycle (lights off at 1000 h) under standard conditions (14 h:10 h light/dark cycle; 24 ± 2 ºC; relative humidity of 40 ± 5%). Dusk and dawn were induced gradually over 1 h. The cages included Teklad Corn Cob bedding (Harlan Laboratories, Inc., Madison, WI, USA; 0.25 in. diameter), a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, Canada), and one square piece of Nestlet (Ancare Corp., Bellmore, NY, USA). Ad libitum access to food (Harlan Laboratories, Inc., Madison, WI, US, T2018 – Global 18% rodent) and water was provided. The Animal Care Committee of the University of Ottawa approved all experimental procedures.

2.2 Treatment Administration

Six-week-old mice mice received either LPS (from Escherichia coli serotype O26:B6; L#3755; Sigma Chemical Co., St. Louis, MO, USA; 1.5 mg/kg body weight, *ip*) or 0.9% sterile saline (LPS-matched volume, *ip*) towards the end of the light phase (n = 96/treatment). During puberty, this LPS dose induces sickness for approximately 48 h in both sexes and impairs stress- and cognition-related neurocircuitry and behaviour in a sex-dependent manner (Cai et al., 2016; Girard-Joyal et al., 2015; Kolmogorova et al., 2019; Sharma et al., 2019).

2.3 Sickness Monitoring

Two experienced raters blinded to treatment examined each mouse 0.5, 2, 4, 8, 12, and 24 h post-treatment for behavioural signs of sickness (i.e., lethargy, piloerection, ptosis, and huddling). Scores between 0 and 4 were assigned to reflect the number of sickness behaviours observed (Kolmogorova et al., 2017). Mean scores at each time-point were used for analysis.

2.4 Body Weight

Body weights were measured at time of injection (i.e., baseline) and 12 and 24 h post-treatment. Changes in body weight were examined as percent change in body weight from baseline (Kolmogorova et al., 2017).

2.5 Brain Tissue Collection

All mice were deeply anesthetized with sodium pentobarbital (500 mg/kg body weight, *ip*) at either 24 h, one week, or four weeks after LPS or saline exposure (n = 8/group). Upon confirmation of deep anesthesia, mice were intracardially perfused with sterile saline (20.0 mL) followed by 4% paraformaldehyde (PFA) (20.0 mL). The excised brains were post- fixed in 4% PFA for 2 h and then placed in fresh 30% sucrose solution 2 and 24 h after post-fixing. The brain tissue was sliced by vibratome into 30 μm free-floating sections into four equal series and stored in cryoprotectant solution at –20 °C.

2.6 Immunohistofluorescence for Iba1

Staining for microglia was completed on one series of brain tissue. All steps were performed at room temperature with gentle agitation, unless otherwise specified. Free-floating sections were first washed in 0.3% TritonX-100 / 1X phosphate-buffered saline (PBS; three x 5 min). After 2 h blocking with a 1% bovine serum albumin (BSA) / 0.3% TritonX-100 / 1X PBS solution, the tissue was incubated for 24 h at 4 °C in a solution of rabbit anti-Iba1 (1:1000; Wako Laboratory Chemicals; cat: 019–19741) / 1% BSA / 0.3% TritonX-100 / 1X PBS. The sections were again rinsed in 0.3% TritonX-100 / 1X PBS (three x 5 min) and then incubated with Alexa Fluor® 488 (1/2000; ThermoFisher Scientific; cat: A21206) in a 1% BSA / 0.3% TritonX-100 / 1X PBS solution for 2 h. A final set of washes in 0.3% TritonX-100 / 1X PBS (three x 5 min) was completed before mounting onto glass slides using DPX mountant (Sigma-Aldrich; cat: 06522) and coverslip.

2.7 Double-labelling for Caspase-3 and NeuN

A second series of brain tissue was used to double-label for neurons (i.e., NeuN) and apoptotic cells (cleaved caspase-3). Caspase-3 is a key executioner of programmed cell death in both the mitochondrial (intrinsic) and death receptor (extrinsic) pathways of apoptosis (Elmore, 2007; Shalini et al., 2015). The procedures were performed at room temperature with gentle agitation, unless otherwise specified. Briefly, the tissue was rinsed in 0.3% Triton X-100 / 1X PBS (three x 5 min) and then blocked with 1% donkey serum / 0.3% TritonX-100 / 1X PBS for 2 h. NeuN and caspase-3 labelling was achieved with guinea pig anti-NeuN (1/2000; Millipore; cat: ABNP) and rabbit anti-caspase-3 (1/1000; Cell Signaling Technologies; cat: 9661) diluted in 1% donkey serum / .3% TritonX-100 / 1X PBS (24 h incubation at 4 °C). Following a second wash (0.3% TritonX-100 / 1X PBS; three x 5 min), the staining was visualized using donkey anti-guinea pig Alexa Fluor® 488 (1/1000; Jackson Immuno Research; cat: 706-545-148) and donkey anti-rabbit Alexa Fluor® 594 (1/2000; Life Technology; cat: A21207) diluted in 1% donkey serum / 0.3% TritonX-100 / 1X PBS (2 h incubation). The tissue was again washed in 0.3% TritonX-100 / 1X PBS (three x 5 min). Nuclear counterstaining was achieved by immersing the tissue in Hoechst 33342 (1/20,000; Invitrogen; cat: H3570) / 1X PBS for 10 min. After a final set of washes (0.3% TritonX-100 / 1X PBS; three x 5 min), the tissue was mounted onto glass slides using with DPX mountant (Sigma-Aldrich; cat: 06522) and coverslip.

2.8 Cell Quantification

Quantification of Iba1^{+}, caspase-3^{+}, caspase-3^{+}/NeuN^{-}, and caspase-3^{+}/NeuN^{+} cells in the hippocampus, hippocampal sub-regions (i.e., dentate gyrus [DG], cornus ammonis [CA]1, CA2, and CA3), mPFC, and PVN was assessed by averaging the total cell counts of two trained raters blinded to experimental group. Cell quantification was completed on one representative bilateral

image of the dorsal hippocampus (i.e., bregma: –2.18 mm), mPFC (i.e., bregma: +1.78 mm), and the PVN (i.e., bregma: –0.82 mm) (Franklin and Paxinos, 2007). All images were captured using a 20x objective on an Olympus BX61 microscope. $Iba1^+$ cells were counted only when a cell body was clearly identifiable. Colocalization with Hoechst was required for counting both caspase-3^+ and $NeuN^+$ cells. Caspase-3^+/$NeuN^+$ cells showed identical Hoechst co-labelling (see Fig. 1A).

2.9 Quantitative Analyses of Microglial Morphology

Fractal analyses were performed using Fiji software (Schindelin et al., 2012). $Iba1^+$ cells for fractal analysis were randomly selected using a grid and random number generator (n = 24 cells/bilateral region) (Morrison et al., 2017). Photomicrographs of selected $Iba1^+$ cells were first made binary and then converted to outlines in Fiji (Karperien et al., 1999–2013; Morrison et al., 2017) (see Fig. 1B). The FracLac plugin (Karperien, 1999–2013) was then applied to the outline of each microglia cell to quantify density, fractal dimension (using a box plot protocol), lacunarity (using a box plot protocol), span ratio, and cell circularity (see Table 1).

2.10 Radioactive Measures of *In Vivo* BBB Disruption

In vivo BBB disruption was assessed at 24 h (whole-brain and region) and at one week (whole-brain) after saline or LPS treatment (n = 8/ group). In brief, mice were deeply anesthetized with 0.1–0.2 mL of 40% urethane (*ip*) and then injected with ^{14}C-sucrose (10^6 *dpm* in 0.2 mL of lactated Ringer's solution / 1% BSA) into the jugular vein (Banks et al., 2015). After 10 min, arterial blood was collected from the descending abdominal aorta, the descending thoracic aorta was clamped, the left and right jugular veins were severed, and 20.0 mL of lactated Ringer's solution was perfused through the left heart ventricle in under 1 min. Whole brains and hippocampal, PFC, cerebellar, or hypothalamic tissue were then excised, weighed,

and solubilized. Serum was obtained by centrifuging the collected blood for 5 min at 10,000xg. Total levels of radioactivity in the brain and serum were measured using a PerkinElmer Tri-Carb 2910 TR scintillation counter. Data are expressed as mean counts per minute (CPM) per brain sample (g) divided by the CPM per μL in the corresponding serum (μL/g).

2.11 Experimental Procedures

Male and female CD-1 mice were treated with LPS or saline at six weeks of age, a stress-sensitive period during puberty (Laroche et al., 2009 a, b). A subset of mice was used for immunohistofluorescence to assess sex and treatment differences in microglial expression and morphology and caspase-3-dependent apoptosis of neuronal and non-neuronal cells in the hippocampus, mPFC, and PVN over time (i.e., 24 h [sickness], one week [symptomatic recovery], and four weeks post-treatment [early adulthood]). Sex- and treatment-dependent changes in BBB disruption during sickness and symptomatic recovery were examined in the remaining mice. BBB disruption during sickness was examined in the whole-brain and regionally in the hippocampus, PFC, cerebellum, and hypothalamus; whole-brain BBB disruption was examined during symptomatic recovery. Sample sizes were consistent across all parameters (n = 8/experimental group).

2.12 Statistical Analyses

Extreme statistical outliers (i.e., cases that exceed the 3 x interquartile range in boxplots) were adjusted by winsorization. Sex (male versus female) and treatment (saline versus LPS) effects on sickness parameters over time were examined with a three-way mixed-design analysis of variance (ANOVA) (Kolmogorova et al., 2017). The Greenhouse-Geisser correction was applied to F-values that violated Mauchly's test of sphericity (i.e., $\varepsilon_{\text{Greenhouse-Geisser}} < 0.75$). The expression of Iba1$^+$, caspase-3$^+$, caspase-3$^+$/NeuN$^-$, and caspase-3$^+$/NeuN$^+$ cells, and the

morphometric profiles of Iba1^{+}cells, and ^{14}C-sucrose whole-brain/serum ratios were analyzed using a three-way (time x sex x treatment) between-subjects ANOVA. ^{14}C-sucrose whole-brain/serum ratios for hippocampal, PFC, hypothalamic, and cerebellar tissue were analyzed with a two-way (sex x treatment) between-subjects ANOVA. Statistical significance was set to $p <$ .05. To protect from Type I error, Bonferroni correction was applied to post hoc pairwise comparisons of statistically significant interactions (Abdi, 2007). Trending interactions (i.e., $.05 < p < .10$) are only reported when corresponding Bonferroni-corrected pairwise comparisons were statistically significant. Effect sizes were estimated using partial eta-squared (η^2_p). All analyses were completed with IBM® SPSS® (version 20.0.0) statistical software.

3. Results

3.1 Acute Behavioural and Physical Responses to Treatment

The ANOVA of sickness behaviours showed a significant time x sex x treatment interaction ($F_{(3.85, 724.68)} = 2.45$, $p = .047$, $\eta_p^2 = .013$) (see Fig. 2A). As expected, sickness behaviour responses changed significantly over the 24 h observation period ($F_{(3.85, 724.68)} = 202.47$, $p < .001$, $\eta_p^2 = .519$) and were consistently higher among LPS-treated mice compared to their saline-treated counterparts ($F_{(1, 188)} = 4427.20$, $p < .001$, $\eta_p^2 = .959$). Sickness behaviour responses were also significantly affected by sex ($F_{(1, 188)} = 16.56$, $p < .001$, $\eta_p^2 = .081$). Among LPS-treated mice, males and females showed similar increases in sickness behaviours 30 mins post-treatment; however, mean sickness behaviour responses were significantly higher among LPS-treated males relative to their female counterparts at 2, 8, 12, and 24 h post-treatment (mean difference [*MD*] = .53, standard error [*SE*] = .11; *MD* = .32, *SE* = .09; *MD* = .47, *SE* = .08; and *MD* = .56, *SE* = .12, respectively; all $p \leq .001$).

Analyses of percent change in body weight from baseline revealed a significant time x

treatment interaction ($F_{(2, 376)} = 1273.13$, $p < .001$, $\eta_p^2 = .684$) and significant main effects of time ($F_{(2, 376)} = 155.37$, $p < .001$, $\eta_p^2 = .452$) and treatment ($F_{(1, 188)} = 728.74$, $p < .001$, $\eta_p^2 = .795$) (see Fig. 2B). Baseline body weight did not differ between saline- and LPS-treated mice ($p > .05$). LPS treatment induced significant body weight loss in both male and female mice than their saline-treated counterparts 12 h and 24 h post-treatment (all $p < .001$). Males and females showed similar treatment-related percent changes in body weight (all $p > .05$).

3.2 Treatment and Sex Differences in Hippocampal Microglial Expression

3.2.1 Total. Hippocampal microglia expression revealed a significant time x sex interaction ($F_{(2, 84)} = 3.15$, $p = .048$, $\eta_p^2 = .070$), as well as significant main effects of treatment ($F_{(1, 84)} = 5.43$, $p = .022$, $\eta_p^2 = .061$) and sex ($F_{(1, 84)} = 6.07$, $p = .016$, $\eta_p^2 = .067$) (see Fig. 3B). Saline-treated males expressed more Iba1$^+$ cells in the hippocampus than their female counterparts at both 24 h and one week post-treatment (*MD* = 357.75, *SE* = 123.94, $p = .005$ and *MD* = 302.63, *SE* = 123.94, $p = .017$, respectively). LPS-treated females showed significantly more total Iba1$^+$ cells than saline-treated females during symptomatic recovery (*MD* = 342.06, *SE* = 123.94, $p = .007$). Microglial expression in the hippocampus was similar across groups during adulthood (all $p > .05$).

3.2.2 DG. DG expression of microglia showed significant time x sex ($F_{(2, 84)} = 3.19$, $p = .046$, $\eta_p^2 = .071$) and sex x treatment ($F_{(1, 84)} = 5.22$, $p = .025$, $\eta_p^2 = .058$) interactions, as well as significant main effects of treatment ($F_{(1, 84)} = 4.01$, $p = .049$, $\eta_p^2 = .046$) and sex ($F_{(1, 84)} = 8.42$, $p = .005$, $\eta_p^2 = .091$) (see Fig. 4A). Iba1$^+$ cell counts in the DG were significantly higher 24 h and one week post-treatment among saline-treated males relative to saline-treated females (*MD* = 161.69, *SE* = 49.15, $p = .001$ and *MD* = 137.63, *SE* = 49.15, $p = .006$, respectively). At one week post-treatment, Iba1$^+$ cell counts were significantly higher among LPS-treated females relative to

saline-treated females (*MD* = 128.69, *SE* = 49.15, $p = .010$). Microglial expression in the DG during adulthood was similar across groups (all $p > .05$).

3.2.3 CA1. Microglial expression in the CA1 revealed a significant time x sex interaction ($F_{(2, 84)} = 3.87$, $p = .025$, $\eta_p^2 = .084$) and significant main effects of treatment ($F_{(1, 84)} = 5.95$, $p = .017$, $\eta_p^2 = .066$) and sex ($F_{(1, 84)} = 8.81$, $p = .004$, $\eta_p^2 = .095$) (see Fig. 4B). Saline-treated males expressed more $Iba1^+$ cells in the CA1 than saline-treated females 24 h and one week post-treatment (*MD* = 126.75, *SE* = 45.39, $p = .006$ and *MD* = 124.82, *SE* = 45.39, $p = .007$, respectively). LPS-treated females expressed more $Iba1^+$ cells in the CA1 than their saline-treated counterparts one week post-treatment (*MD* = 105.63, *SE* = 45.39, $p = .022$). Microglial expression in the CA1 was similar across groups during adulthood (all $p > .05$).

3.2.4 CA2. Microglial expression in the CA2 was similar across groups at each time-point (all $p > .05$) (see Fig. 4C).

3.2.5 CA3. Microglial expression in the CA3 differed significantly across time ($F_{(2, 84)} = 5.26$, $p = .007$, $\eta_p^2 = .111$) (see Fig. D). Although the interactions were not statistically significant (all $p > .05$), the time-point x sex x treatment interaction was trending towards significance ($F_{(2, 84)} = 2.79$, $p = .067$, $\eta_p^2 = .067$). Microglial expression in the CA3 was similar across groups 24 h post-treatment. $Iba1^+$ cell expression was significantly higher one week post-treatment among LPS-treated females relative to saline-treated females (*MD* = 72.25, *SE* = 32.88, $p = .031$). During adulthood, LPS-treated males had significantly higher $Iba1^+$ cell counts relative to their saline-treated counterparts (*MD* = 66.50, *SE* = 32.88, $p = .046$).

3.3 Morphometric Features of Hippocampal Microglia

Analyses of microglial morphology in the hippocampus (see Fig. 5A-E) revealed a significant time x sex interaction for density ($F_{(2, 84)} = 4.10$, $p = .020$, $\eta_p^2 = .089$) and a significant

sex x treatment interaction for D_B ($F_{(1, 84)} = 5.90$, $p = .017$, $\eta_p^2 = .066$). Trends towards statistical significance were observed for the time x sex interaction for span ratio ($F_{(2, 84)} = 3.04$, $p = .053$, $\eta_p^2 = .068$) and the sex x treatment interaction for lacunarity ($F_{(1, 84)} = 3.41$, $p = .068$, $\eta_p^2 = .039$). D_B values differed significantly across time ($F_{(2, 84)} = 3.41$, $p = .038$, $\eta_p^2 = .075$). A significant main effect of treatment was observed for density ($F_{(1, 84)} = 8.38$, $p = .005$, $\eta_p^2 = .091$) and D_B ($F_{(1, 84)} = 10.91$, $p = .001$, $\eta_p^2 = .115$), as well as a significant main effect of sex for density ($F_{(1, 84)} = 11.44$, $p = .001$, $\eta_p^2 = .120$). Hippocampal microglia of LPS-treated mice consistently showed significantly higher circularity and lacunarity compared to those of saline-treated controls ($F_{(1, 84)} = 8.78$, $p = .004$, $\eta_p^2 = .095$ and $F_{(1, 84)} = 5.00$, $p = .028$, $\eta_p^2 = .056$, respectively). Span ratios were significantly lower among LPS-treated mice relative to controls ($F_{(1, 84)} = 16.13$, $p < .001$, $\eta_p^2 = .161$).

During LPS-induced sickness, hippocampal microglia showed significantly higher D_B values among LPS-treated females relative to saline-treated females (*MD* = .05, *SE* = .02, *p* = .005). One week post-treatment, density was significantly higher among saline-treated females relative to their LPS-treated counterparts (*MD* = .02, *SE* = .01, *p* = .014) and saline-treated males (*MD* = .02, *SE* = .01, *p* = .015). Hippocampal microglia four weeks post-treatment showed significantly greater lacunarity and density among LPS-treated females compared to saline-treated females (*MD* = .07, *SE* = .03, *p* = .008 and *MD* = .03, *SE* = .01, *p* = .001, respectively) and LPS-treated males (*MD* = .05, *SE* = .03, *p* = .042 and *MD* = .02, *SE* = .01, *p* = .028, respectively). Microglial span ratios were significantly higher in saline-treated females relative to male counterparts during adulthood (*MD* = .17, *SE* = .08, *p* = .041).

3.4 Hippocampal Apoptosis

Hippocampal caspase-3-dependent apoptosis showed a significant time x sex x treatment

interaction ($F_{(2, 84)} = 15.50$, $p < .001$, $\eta_p^2 = .270$) and significant main effects of time ($F_{(2, 84)} = 9.93$, $p < .001$, $\eta_p^2 = .191$), sex ($F_{(1, 84)} = 7.14$, $p = .009$, $\eta_p^2 = .078$), and treatment ($F_{(1, 84)} = 18.29$, $p < .001$, $\eta_p^2 = .179$) (see Table 2). Mean hippocampal caspase-3^+ cell expression 24 h post-treatment was significantly higher among LPS-treated females relative to saline-treated females (*MD* = 3.25, *SE* = .38, $p < .001$) and LPS-treated males (*MD* = 3.25, *SE* = .38, $p < .001$). Significant sex and treatment differences were not observed at later time-points (all $p > .05$).

3.4.1 Neuronal and non-neuronal apoptosis. Analyses of hippocampal apoptosis also revealed significant time x sex x treatment interactions for apoptosis of neuronal (i.e., caspase-3^+/NeuN$^+$) ($F_{(2, 84)} = 9.30$, $p < .001$, $\eta_p^2 = .181$) and non-neuronal (i.e., caspase-3^+/NeuN$^-$) cells ($F_{(2, 84)} = 5.73$, $p = .005$, $\eta_p^2 = .120$) (see Table 2). Significant main effects of time, sex, and treatment were observed for neuronal ($F_{(2, 84)} = 7.14$, $p = .001$, $\eta_p^2 = .145$; $F_{(1, 84)} = 6.07$, $p = .016$, $\eta_p^2 = .067$; and $F_{(1, 84)} = 10.37$, $p = .002$, $\eta_p^2 = .110$, respectively) and non-neuronal cells ($F_{(2, 84)} = 5.73$, $p = .005$, $\eta_p^2 = .120$; $F_{(1, 84)} = 5.73$, $p = .019$, $\eta_p^2 = .064$; and $F_{(1, 84)} = 5.73$, $p = .019$, $\eta_p^2 = .064$, respectively). At 24 h post-treatment, LPS-treated females had significantly more caspase-3^+/NeuN$^+$ and caspase-3^+/NeuN$^-$ cells than saline-treated females (*MD* = 1.88, *SE* = .27, $p < .001$ and *MD* = .75, *SE* = .13, $p < .001$, respectively) and LPS-treated males (*MD* = 1.88, *SE* = .27, $p < .001$ and *MD* = .75, *SE* = .13, $p < .001$, respectively). Neuronal and non-neuronal apoptosis was similar across groups one week and four weeks post-treatment (all $p > .05$).

3.5 Temporal Sex and Treatment Effects on Total Microglial Expression in the mPFC

Microglial expression in the mPFC revealed a significant time x sex interaction ($F_{(2, 84)} = 5.22$, $p = .007$, $\eta_p^2 = .110$) and a significant main effect of sex ($F_{(1, 84)} = 10.43$, $p = .002$, $\eta_p^2 = .110$) (see Fig. 6B). Iba1$^+$ cell numbers in the mPFC did not differ between groups 24 h and one week post-treatment (all $p > .05$). Four weeks post-treatment, however, mean Iba$^+$ cell counts

were significantly higher among males relative to females in saline-treated and LPS-treated mice (*MD* = 171.19, *SE* = 56.66, $p = .003$ and *MD* = 188.56, *SE* = 56.66, $p = .001$).

3.6 Morphometric Features of Microglia in the mPFC

Fractal analyses in the mPFC (see Fig. 7A-E) identified significant time x sex ($F_{(2, 84)} = 3.16$, $p = .048$, $\eta_p^2 = .070$) and time x sex x treatment interactions ($F_{(2, 84)} = 3.51$, $p = .034$, $\eta_p^2 = .077$) for circularity. There were trends towards statistical significance for the time x treatment interaction for span ratio ($F_{(2, 84)} = 2.94$, $p = .059$, $\eta_p^2 = .065$) and the time x sex x treatment interaction for D_B values ($F_{(2, 84)} = 2.55$, $p = .084$, $\eta_p^2 = .057$). Circularity, D_B, and span ratios varied significantly across time ($F_{(2, 84)} = 4.30$, $p = .017$, $\eta_p^2 = .093$; $F_{(2, 84)} = 3.32$, $p = .041$, $\eta_p^2 = .073$; and $F_{(2, 84)} = 6.94$, $p = .002$, $\eta_p^2 = .142$, respectively). A significant main effect of sex was observed for circularity ($F_{(1, 84)} = 22.85$, $p < .001$, $\eta_p^2 = .214$). Span ratios and density values were overall significantly higher among females compared to males ($F_{(1, 84)} = 16.13$, $p < .001$, $\eta_p^2 = .161$ and $F_{(1, 84)} = 23.40$, $p < .001$, $\eta_p^2 = .218$, respectively). In contrast, D_B and lacunarity values were significantly skewed towards males ($F_{(1, 84)} = 22.81$, $p < .001$, $\eta_p^2 = .214$ and $F_{(1, 84)} = 42.90$, $p < .001$, $\eta_p^2 = .338$, respectively).

Microglia in the mPFC 24 h post-treatment showed significantly higher span ratios among saline-treated females relative to LPS-treated females (*MD* = .15, *SE* = .05, $p = .006$) and saline-treated males (*MD* = .14, *SE* = .05, $p = .012$). Female controls also showed significantly lower D_B values than saline-treated males (*MD* = -.04, *SE* = .02, $p = .025$) and less circularity than LPS-treated females (*MD* = -.03, *SE* = .01, $p = .027$) and saline-treated males (*MD* = -.02, *SE* = .01, $p = .028$) at this time-point. One week post-treatment, D_B values, lacunarity, circularity were significantly skewed towards males in saline-treated (*MD* = .04, *SE* = .02, $p = .045$; *MD* = .04, *SE* = .02, $p = .015$ and *MD* = .04, *SE* = .01, $p = .001$, respectively) and LPS-treated mice

(*MD* = .08, *SE* = 02, $p < .001$; *MD* = .08, *SE* = .02, $p < .001$; and *MD* = .04, *SE* = .01, $p = .002$, respectively). Conversely, span ratios one week post-treatment were significantly greater among females relative to males in saline-treated and LPS-treated mice (*MD* = .19, *SE* = .05, $p = .001$) and LPS-treated mice (*MD* = .16, *SE* = .05, $p = .003$). This female bias in span ratios among saline-treated and LPS-treated mice persisted into adulthood (*MD* = .11, *SE* = .05, $p = .039$ and *MD* = .13, *SE* = .05, $p = .014$, respectively). LPS-treated females displayed significantly less circularity in microglia than females (*MD* = -.02, *SE* = .01, $p = .045$) and LPS-treated males (*MD* = -.03, *SE* = .01, $p = .003$) four weeks post-treatment. D_B and lacunarity values during adulthood were significantly higher among LPS-treated males relative to LPS-treated females (*MD* = .04, *SE* = .02, $p = .049$ and *MD* = .05, *SE* = .02, $p = .003$, respectively). The microglia of adult male controls showed significantly greater lacunarity than their female counterparts (*MD* = .04, *SE* = .02, $p = .029$).

3.7 Apoptosis in the mPFC

Caspase-3 activity in the mPFC showed significant interactions of time x treatment ($F_{(2, 84)} = 3.18$, $p = .047$, $\eta_p^2 = .070$) and time x sex x treatment ($F_{(2, 84)} = 3.18$, $p = .047$, $\eta_p^2 = .070$) and a significant main effect of sex ($F_{(1, 84)} = 6.24$, $p = .014$, $\eta_p^2 = .069$) (see Table 3). Mean caspase-3$^+$ cell numbers were similar across groups 24 h and one week post-treatment (all $p > .05$). During adulthood, however, LPS-treated males had significantly higher mean capase-3$^+$ cell expression compared to saline-treated males (*MD* = .38, *SE* = .14, $p = .010$) and LPS-treated females (*MD* = .38, *SE* = .14, $p = .010$).

3.7.1 Neuronal and non-neuronal apoptosis. Among apoptotic cells in the mPFC, neuronal cells showed significant interactions of time x treatment ($F_{(2, 84)} = 3.18$, $p = .047$, $\eta_p^2 = .070$) and time x sex x treatment ($F_{(2, 84)} = 3.18$, $p = .047$, $\eta_p^2 = .070$) and a significant main effect

of sex ($F_{(1, 84)} = 6.24$, $p = .014$, $\eta_p^2 = .069$) (see Table 3). Neuronal apoptosis in the mPFC was similar across groups 24 h and one week post-treatment (all $p > .05$). Caspase-3$^+$/NeuN$^+$ cell expression during adulthood was significantly higher among LPS-treated males compared to their saline-treated and female counterparts ($MD = .38$, $SE = .14$, $p = .010$ and $MD = .38$, $SE = .14$, $p = .010$, respectively). Non-neuronal apoptosis in the mPFC was similar across groups at each time-point (all $p > .05$).

3.8 LPS-induced Changes in Microglial Expression in the PVN

Microglial expression in the PVN revealed a significant time x sex x treatment interaction ($F_{(2, 84)} = 5.64$, $p = .005$, $\eta_p^2 = .118$) and significant main effects of treatment ($F_{(1, 84)} = 5.63$, $p = .020$, $\eta_p^2 = .063$) and sex ($F_{(1, 84)} = 10.42$, $p = .002$, $\eta_p^2 = .110$) (see Fig. 8B). During LPS-induced sickness, LPS-treated females had significantly more Iba1$^+$ cells in the PVN than their male counterparts ($MD = 32.69$, $SE = 12.42$, $p = .010$). Iba1$^+$ cell expression one week post-treatment was significantly lower among saline-treated males compared to LPS-treated males ($MD = -32.38$, $SE = 12.42$, $p = .011$) and saline-treated females ($MD = -36.75$, $SE = 12.42$, $p = .004$). Iba1$^+$ cell numbers in the adult PVN were significantly higher among LPS-treated females relative to saline-treated females and LPS-treated males ($MD = 35.13$, $SE = 12.42$, $p = .006$ and $MD = 28.25$, $SE = 12.42$, $p = .025$, respectively).

3.9 Time-Related Sex and Treatment Effects on Microglial Morphology in the PVN

Fractal analyses in the PVN (see Fig. 9A-E) revealed a significant sex x treatment interaction for D_B ($F_{(1, 84)} = 8.63$, $p = .004$, $\eta_p^2 = .093$) and density ($F_{(1, 84)} = 4.89$, $p = .030$, $\eta_p^2 = .055$) and a significant time x treatment interaction for lacunarity ($F_{(1, 84)} = 3.42$, $p = .037$, $\eta_p^2 = .075$). A main effect of treatment was also observed for lacunarity ($F_{(1, 84)} = 4.26$, $p = .042$, $\eta_p^2 = .048$). The ANOVAs failed to reveal statistically significant main effects and interactions for

circularity and span ratio of microglia in the PVN (all $p > .05$).

The morphometric features of microglia in the PVN were similar across groups 24 h post-treatment (all $p > .05$). Following symptomatic recovery from treatment, LPS-treated females had significantly higher D_B and lacunarity values relative to saline-treated females (*MD* = .04, *SE* = .02, *p* = .023 and *MD* = .04, *SE* = .01, *p* = .004, respectively). Saline-treated males also showed significantly higher D_B and density values than LPS-treated males (*MD* = .04, *SE* = .02, *p* = .026 and *MD* = .03, *SE* = .01, *p* = .032, respectively) and saline-treated females (*MD* = .06, *SE* = .02, *p* = .001 and *MD* = .04, *SE* = .01, *p* = .006, respectively) at this time-point. During adulthood, lacunarity values were significantly higher among saline-treated males relative to saline-treated females (*MD* = .03, *SE* = .01, *p* = .033).

3.10 Apoptosis of Neuronal and Non-Neuronal Cells in the PVN

Caspase-3^+ cells were not detected in the PVN of any groups at any of the time-points.

3.11 Sex and Treatment Differences in Whole-Brain and Regional BBB Disruption

3.11.1 Regional. ^{14}C-sucrose brain/serum ratios in cerebellar, hippocampal, hypothalamic, and PFC tissue 24 h post-treatment failed to identify any significant interactions and main effects (all $p > .05$) (see Fig. 10A-D).

3.11.2 Whole-brain. ^{14}C-sucrose whole-brain/serum ratios demonstrated a significant time x sex x treatment interaction ($F_{(1, 56)} = 4.55$, $p = .037$, $\eta_p^2 = .075$) and a significant main effect of treatment ($F_{(1, 56)} = 5.75$, $p = .020$, $\eta_p^2 = .093$) (see Fig. 10E and F). LPS-treated females had significantly higher ^{14}C-sucrose whole-brain/serum ratios 24 h post-treatment than their saline-treated and male counterparts (*MD* = 10.09, *SE* = 3.61, *p* = .007 and *MD* = 11.01, *SE* = 3.61, *p* = .003, respectively). ^{14}C-sucrose whole brain/serum ratios were similar across groups one week post-treatment (all $p > .05$).

Discussion

Pubertal CD-1 mice displayed sex-specific patterns of LPS-induced changes to BBB permeability, microglia expression and morphology (see Table 4), and caspase-3-dependent apoptosis in brain regions involved in stress and cognition. The acute effects of systemic LPS on the pubertal neuroimmune system were specific to females (i.e., altered microglial morphology in the hippocampus and the mPFC, increased hippocampal apoptosis, and global BBB disruption). Despite symptomatic recovery from LPS, microglia increased in number in the PVN of males and in the hippocampus of females and displayed several morphometric changes in the PVN of both sexes and in the hippocampus of females one week post-treatment. Four weeks post-treatment, LPS-treated males showed apoptosis of mPFC neurons and heightened expression of microglia in the CA3 region of the hippocampus, whereas their female counterparts showed increased microglial expression in the PVN and altered morphology of microglia in the mPFC and in the hippocampus. The pubertal neuroimmune system's response to systemic LPS, especially within microglia, highlight the sex-specific nature of this critical period for sexual maturation and the sex-specific vulnerability of these interconnected brain systems.

Unlike BBB permeability and caspase-3 activity, microglia displayed baseline region-specific sex differences spanning from puberty through adulthood. Young (i.e., six- and seven-week-old) females had lower microglial expression in the hippocampus than age-matched males, though this sex difference disappeared by adulthood (i.e., ten weeks of age). Microglial expression increased temporarily in the PVN of females at seven weeks of age and diverged significantly between the sexes in the mPFC during adulthood. Fractal analyses of young saline-treated mice identified larger hippocampal microglia, structurally simpler and elongated microglia in the mPFC, and smaller, less structurally complex microglia in the PVN among

females relative to age-matched males. Hippocampal microglia remained larger in females during adulthood, and microglia in the hippocampus and the mPFC of adult mice were more elongated in females relative to males. Sex differences in PVN lacunarity among adult controls imply less bushiness/ramification of PVN microglia in males relative to females. These sex differences in microglia are likely secondary to the sex differences in age of pubertal onset and the ensuing developmental changes towards sexual maturity (Schulz et al., 2009; Sisk, 2016; Sisk and Foster, 2004). Similar sex and region differences in microglia during earlier critical periods (Lenz et al., 2013; Schwarz et al., 2012) are thought to arise in part from sex differences in the organizational and activational effects of fluctuating gonadal steroid hormone levels (Crain et al., 2013; Sierra et al., 2008). The reactivation of the hypothalamic-pituitary-gonadal axis at pubertal onset leads to similar changes in circulating levels of androgenic and estrogenic steroids secreted by the testes and the ovaries (Herbison, 2016; Sisk and Foster, 2004). Therefore, it is plausible that sex differences in the influx of androgens, estrogens, and progesterone at pubertal onset influences microglial expression and phenotype during this critical period (Herbison, 2016; Sisk and Foster, 2004).

Systemic LPS was able to alter baseline number and morphology of pubertal microglia in a sex-specific manner. During LPS-induced sickness, females showed an increase in hippocampal cell complexity and increased circularity and elongation of microglia in the mPFC. Pubertal LPS treatment eliminated baseline sex differences in expression and certain morphological features of hippocampal and PVN microglia seen in seven-week-old mice. The LPS-induced changes to microglial morphology in the PVN one week post-treatment point towards a female-specific transition to a more “activated” microglial state despite symptomatic recovery from LPS-induced sickness. Pubertal LPS treatment also removed the sex difference in

CA3 microglial expression during adulthood and increased PVN microglial expression in adult females. Relative to their saline-treated counterparts, adult females treated with LPS during puberty also showed less circularity in mPFC microglia and greater cell complexity and lacunarity in hippocampal microglia.

Aside from baseline sex differences in microglia during this critical period, pubertal females may have been more vulnerable than their male counterparts to LPS-induced changes to microglia because of sex differences in BBB integrity. Systemic LPS disrupts BBB permeability by interfering with the paracellular and transcellular pathways of transport (Banks et al., 2015; Erickson et al., 2018; Nishioku et al., 2009). We found that systemic LPS significantly increased global BBB permeability during sickness in pubertal females but not males. Given that BBB permeability in the hippocampus, cerebellum, hypothalamus, and PFC were grossly intact during LPS-induced sickness in both sexes, this global BBB disruption likely resulted from additive effects of non-significant changes to BBB permeability across brain regions. This increased global BBB permeability is a double-edged sword because infiltrating circulating immune factors (e.g., peripheral leukocytes, pro- and anti-inflammatory cytokines) may assist with wound healing and debris clearance in the CNS but can also lead to maladaptive immune activation, immune-mediated cellular damage and dysfunction, and increases in intracranial pressure (Varatharaj and Galea, 2017). Follow-up research is needed to confirm the functional consequences of this sex-specific effect of pubertal immune challenge on BBB permeability.

Pubertal fluctuations in gonadal steroid hormones likely contributed to the sex-specific responses of the pubertal neuroimmune network to systemic LPS. Circulating androgenic and estrogenic steroids moderate the neuroimmune network's responses via direct and indirect means (Dantzer, 2017; Hampl et al., 2015; Robison et al., 2019). In general, estrogens tend to dilate

cerebrovascular tone, increase cerebral flow, and facilitate immune-supporessing or anti-inflammatory responses in the brain and cerebrovasculature (Brown et al., 2007; Gonzales et al., 2005, 2008; Gottfried-Blackmore et al., 2008; Kipp et al., 2012; Krause et al., 2002; Ospina et al., 2003, 2004; Sunday et al., 2006). Androgens tend to constrict vascular tone and enhance immune responses (i.e., pro-inflammatory effects). Consistent with our findings, the organizational and activational effects of circulating hormones also extend to the activation of molecular cell death pathways during inflammatory events, fostering a female bias in caspase-dependent apoptosis at younger ages (Arai et al., 1996; Liu et al., 2009; Nuñez et al., 2001; Penaloza et al., 2009; Rau et al., 2003). However, recent evidence of estradiol-mediated amplification of microglial, cytokine, and thermoregulatory responses to systemic LPS in pubertal female C57Bl/6 mice suggests age-related hormonal influences on the neuroimmune system's functioning (Velez-Perez, 2020). Therefore, further investigation is warranted to elucidate the role of circulating gonadal steroid hormones in the sex-specific vulnerability and reactivity of the pubertal neuroimmune network.

The functional significance of the LPS-induced changes to microglial structure reported here are unclear. Morphometric analyses revealed sex- and region-dependent changes to microglial morphology following pu- bertal LPS treatment. Among LPS-treated mice, females were more prone to morphometric changes, several of which are consistent with an increase in "activational" state. For example, the increased circularity and elongation of microglial cells in the mPFC of females during LPS-induced sickness point towards a shift in morphology towards the more activated "rod-like" form (Morrison et al., 2017; Taylor et al., 2014). Although fractal analyses captured the subtle LPS-induced changes to microglial structure that categorical, qualitative approaches cannot (e.g., Fernández-Arjona et al., 2017; Morrison et al., 2017; Soltys

et al., 2001), this approach does not allude to the phenotypic polarization of microglia. The duality in microglia-mediated neuroinflammatory responses (i.e., simultaneous secretion of pro-inflammatory and pro-repair mechanisms of inflammation) complicate the functional translation of these morphological changes on the local microenvironment (Giordano et al., 2021; Loane and Kumar, 2016). Given that the magnitude of this duality also differs between the sexes (Crain et al., 2013; Guneykaya et al., 2018; Hanamsagar et al., 2017; Lively et al., 2018; Villa et al., 2018), additional investigations into the kinds of inflammatory responses employed by pubertal microglia are needed to provide functional context to our findings.

Immune-mediated perturbations during stress-sensitive critical pe- riods like puberty have widespread implications for health. Sex differences in stress responses (Oyola and Handa, 2017) during this critical postnatal period may explain sex biases in the pathogenesis of stress-related disorders that appear during puberty or early adulthood but do not originate in the pre-/post-natal periods or during adulthood (Angold and Costello, 2006; Marcotte et al., 2002). Preclinical and clinical models generally show a strong positive relationship between stress burden during puberty and risk for internalizing and externalizing disorders, reactivity to future stressors, and cognitive functioning in young adulthood (e.g., Angold et al., 1998; Byrne et al., 2017; Ellis et al., 2019; Graber, 2013; Hamilton et al., 2014). Our findings add to this literature by highlighting sex differences in the vulnerability of the pubertal neuroimmune response, particularly within microglia, across brain regions involved in stress responses and cognitive functioning. Microglia play critical roles in immune responses, homeostatic process (e.g., debris clearance), the shaping of immature neural networks, and programming of adult behaviours (Lenz and Nelson, 2018; Li and Barres, 2018; Mot- tahedin et al., 2017; Nelson et al., 2019; Wolf et al., 2016). Therefore, the residual effects of LPS-induced sickness on microglial

expression and morphology in the hippocampus, mPFC, and the PVN suggest mechanistic involvement of these cells in the sex-specific outcomes of pubertal LPS on related behaviours (i.e., stress and immune responses [Cai et al., 2016; Girard-Joyal et al., 2015; Sharma et al., 2019]; depression-like behaviours [Murray et al., 2019]; spatial retention errors on hippocampus-dependent tasks [Kolmogorova et al., 2019]; reactivity to novel stressors and expression of anxiety-like behaviours (Murray et al., 2019]).

4.1 Conclusions

This study examined whether the neuroimmune network contributes to the sex-specific outcomes of a systemic immune challenge during the stress-sensitive pubertal period. The female-specific BBB disruption appears to moderate some of the sex-specific effects of pubertal LPS on microglial expression and morphology in brain regions for stress regulation and cognitive functioning. The residual effects of LPS-induced sickness highlight the stress sensitivity of pubertal microglia and the sex- and region-specific nature of this vulnerability. These significant sex differences in the pubertal neuroimmune system may explain some sex biases in stress-related disorders of brain and behaviour that arise during adulthood.

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Declaration of Competing Interest

We have no conflict of interest to declare.

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Table 1.

Morphometric measures of microglia cells.

Dimension	Measure	Unit	Interpretation	Range
Fractal dimension	$\frac{\ln N\varepsilon}{\ln\varepsilon}$	D_B	Measure of cell's contour bounded by endpoints and process lengths	1-2
Circularity	$\frac{4\pi \text{ x cell area}}{\text{cell perimeter}^2}$	Ratio	Roundness	0-1
Span ratio	$\frac{\text{convex hull eclipse longest length}}{\text{convex hull eclipse longest width}}$	Ratio	Cell shape	0-1
Lacunarity	$\frac{\Sigma\Lambda_{(g)}}{N_{(G)}}$	λ	Distribution of gaps or lacuna in the image	>0
Density	$\frac{\text{\# of pixels within cell outline}}{\text{area of convex hull}}$	$\frac{\text{\# of pixels}}{\text{area}}$	Cell size	0-1

Note. N refers to the number of pixels; *ε* refers to box size or scale; $N_{(G)}$ refers to the number of grid origins; $\Lambda_{(g)}$ refers to the calculated mean lacunarity (λ) per image (Karperien et al., 2013).

Table 2.

Caspase-3-dependent apoptosis in the hippocampus.

Cell	Time post-treatment	Males		Females	
		Saline-treated	LPS-treated	Saline-treated	LPS-treated
$Caspase\text{-}3^+$	24 h	.00 (.00)	.0 (.00)	.0 (.00)	3.25 (.80)
	1 week	.00 (.00)	.00 (.00)	.00 (.00)	.00 (.00)
	4 weeks	.00 (.00)	.75 (.49)	.00 (.00)	.00 (.00)
$Caspase\text{-}3^+/NeuN^+$	24 h	.00 (.00)	.0 (.00)	.0 (.00)	1.88 (.64)
	1 week	.00 (.00)	.00 (.00)	.00 (.00)	.00 (.00)
	4 weeks	.00 (.00)	.25 (.16)	.00 (.00)	.00 (.00)
$Caspase\text{-}3^+/NeuN^-$	24 h	.00 (.00)	.00 (.00)	.0 (.00)	.75 (.31)
	1 week	.00 (.00)	.00 (.00)	.00 (.00)	.00 (.00)
	4 weeks	.00 (.00)	.00 (.00)	.00 (.00)	.00 (.00)

Note. Data present group means (± *SEM*) of total caspase-3^+, caspase-3^+/NeuN$^+$ (i.e., neuronal), and caspase-3^+/NeuN$^-$ (i.e., non-neuronal) cells counted in the hippocampus. Asterisks denote significant sex and treatment differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Table 3.

Caspase-3-dependent apoptosis in the mPFC.

Cell	Time post-treatment	Males		Females	
		Saline-treated	LPS-treated	Saline-treated	LPS-treated
Caspase-3^+	24 h	.25 (1.60)	.00 (.00)	.00 (.00)	.00 (.00)
	1 week	.25 (1.60)	.00 (.00)	.00 (.00)	.00 (.00)
	4 weeks	.00 (.00)	.38 (.26)	.00 (.00)	.00 (.00)
Caspase-3^+/NeuN$^+$	24 h	.25 (1.60)	.00 (.00)	.00 (.00)	.00 (.00)
	1 week	.25 (1.60)	.00 (.00)	.00 (.00)	.00 (.00)
	4 weeks	.00 (.00)	.38 (.26)	.00 (.00)	.00 (.00)
Caspase-3^+/NeuN$^-$	24 h	.00 (.00)	.00 (.00)	.00 (.00)	.00 (.00)
	1 week	.00 (.00)	.00 (.00)	.00 (.00)	.00 (.00)
	4 weeks	.00 (.00)	.00 (.00)	.00 (.00)	.00 (.00)

Note. Data present group means (± *SEM*) of total caspase-3^+, caspase-3^+/NeuN$^+$ (i.e., neuronal), and caspase-3^+/NeuN$^-$ (i.e., non-neuronal) cells counted in the mPFC. Asterisks denote significant sex and treatment differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Table 4.

Summary of key findings in microglia cells.

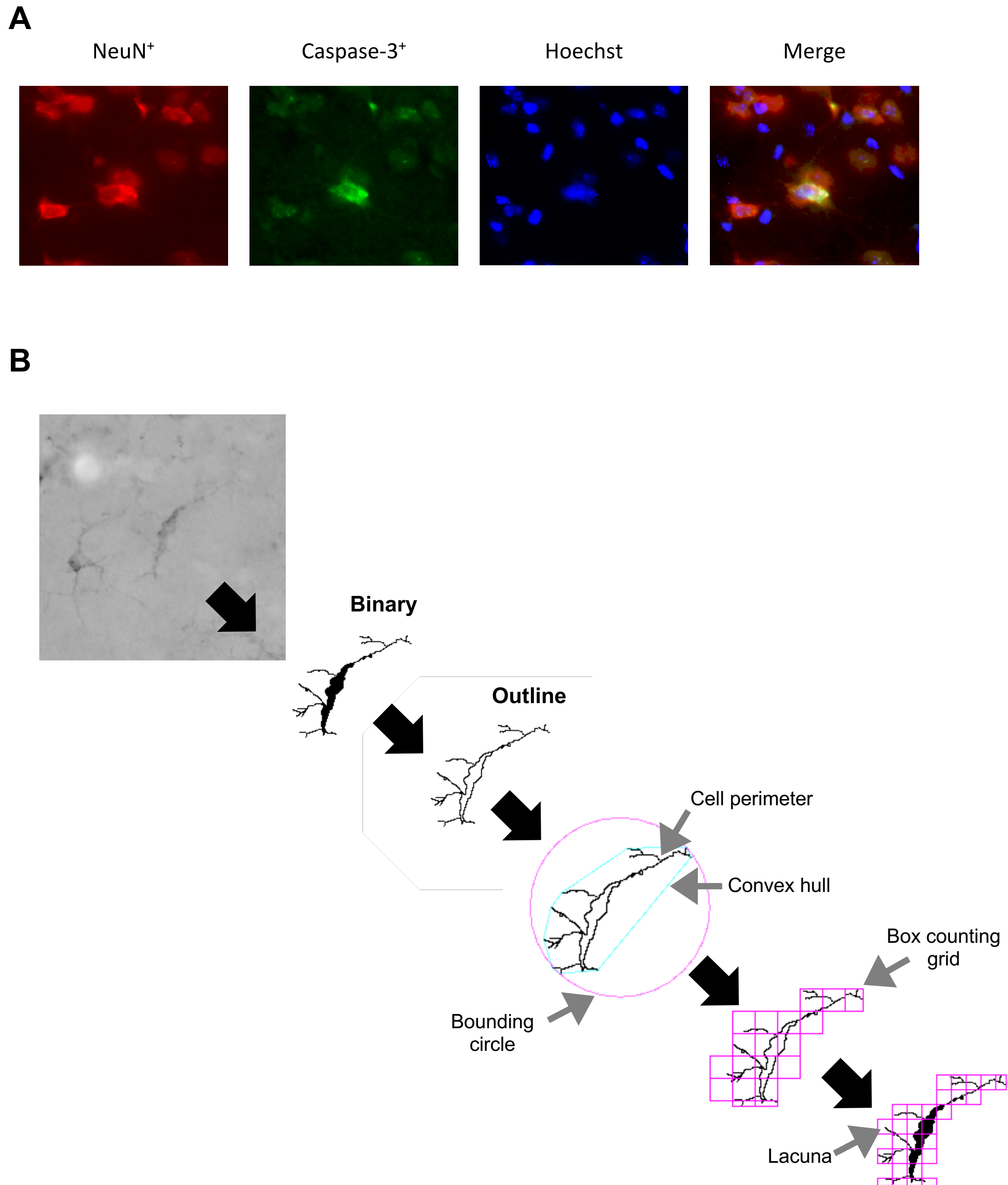
Region	Summary of key findings
Hippocampus	*Counts:* • The baseline male-skewed expression of hippocampal microglia during puberty disappears by early adulthood. Developmental sex differences in microglia numbers were also observed in hippocampal subregions. • Pubertal LPS temporarily increased total microglial numbers in females (i.e., one week post-treatment) and altered microglial numbers in a region- and sex-specific manner. Males were protected from acute LPS-induced changes to microglial expression. *Morphology:* • Among controls, young males showed larger hippocampal microglia relative to their female counterparts. Microglia were more structurally complex and larger in adult females relative to adult males. • Pubertal LPS increased cell complexity in females during sickness and eliminated several baseline morphological sex differences one week post-treatment. During adulthood, LPS-treated females showed greater cell complexity and lacunarity in hippocampal microglia than their saline-treated counterparts. Males were protected from acute and enduring LPS-induced changes to morphology.

mPFC	*Counts:* • Baseline microglial expression diverged during adulthood (i.e., total microglial expression increased in males and decreased in females relative to their younger counterparts). • Pubertal LPS did not impact baseline microglial expression in males and females. *Morphology:* • Several baseline sex differences in mPFC microglia were observed from puberty through adulthood. • Females showed elongation of mPFC microglia during LPS-induced sickness. Pubertal LPS decreased baseline circularity of microglia in adult females.
PVN	*Counts:* • Baseline microglial expression increased temporarily in the PVN of females at seven weeks of age. • Pubertal LPS temporarily increased microglial expression in males at seven weeks of age. LPS-treated females showed an increased in PVN microglial expression in adulthood. *Morphology:* • Young female controls showed smaller, less structurally complex microglia in the PVN relative to their male counterparts. Adult males showed greater lacunarity (i.e., reduced bushiness/ramification) in PVN microglia than adult females.

	• Pubertal LPS temporarily altered morphology in males and females. These LPS-induced changes one week post-treatment suggest a female-specific transition to a more "activated" microglial state despite symptomatic recovery from LPS-induced sickness.

Figure 1.

Apoptotic cells and morphometric analyses of microglial cells.

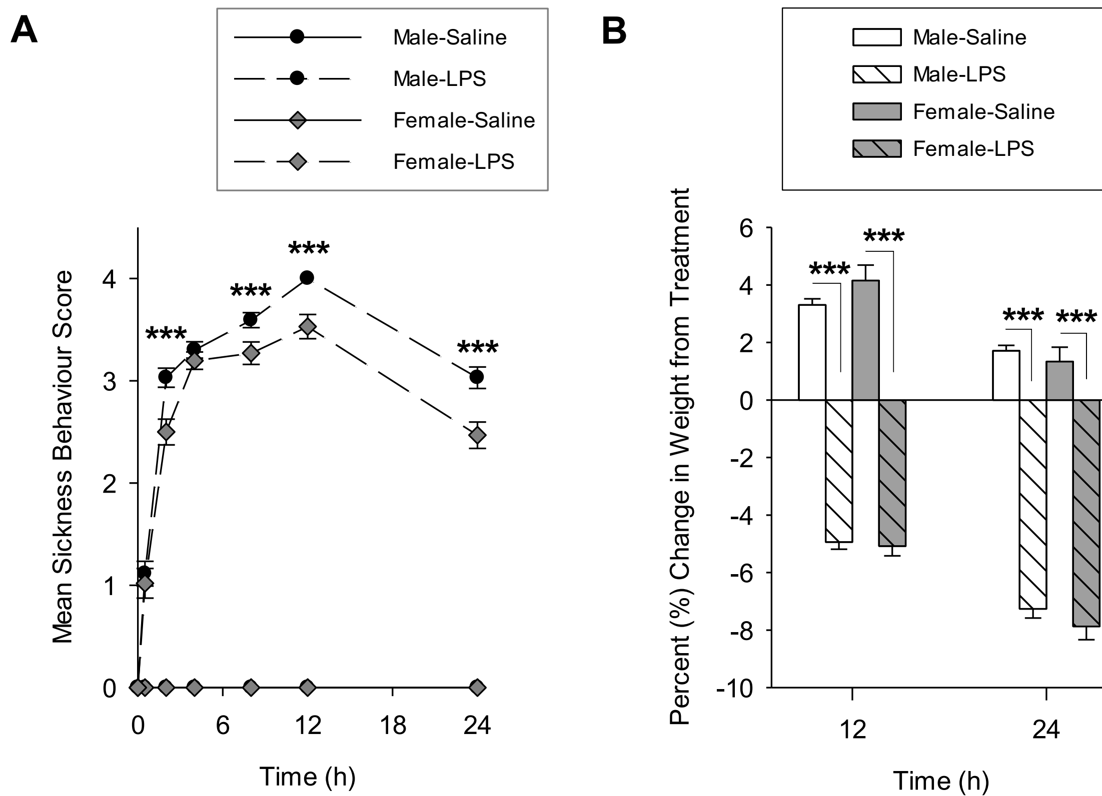


Note. Caspase-3$^+$/NeuN$^+$ cells (i.e., apoptotic neurons) showed identical co-labelling with Hoechst (**A**). Caspase-3$^+$ cells without this colocalization of Hoechst with nearby NeuN$^+$ cells

were considered non-neuronal (i.e., caspase-3^{+}/NeuN^{-} cells). To analyze morphometric features of the randomly selected Iba1^{+} cells, the original photomicrographs were made binary and then converted to outlines in Fiji for fractal analyses using box plot protocol with the FracLac plugin (**B**) (Karperien et al., 1999-2013; Morrison et al., 2017).

Figure 2.

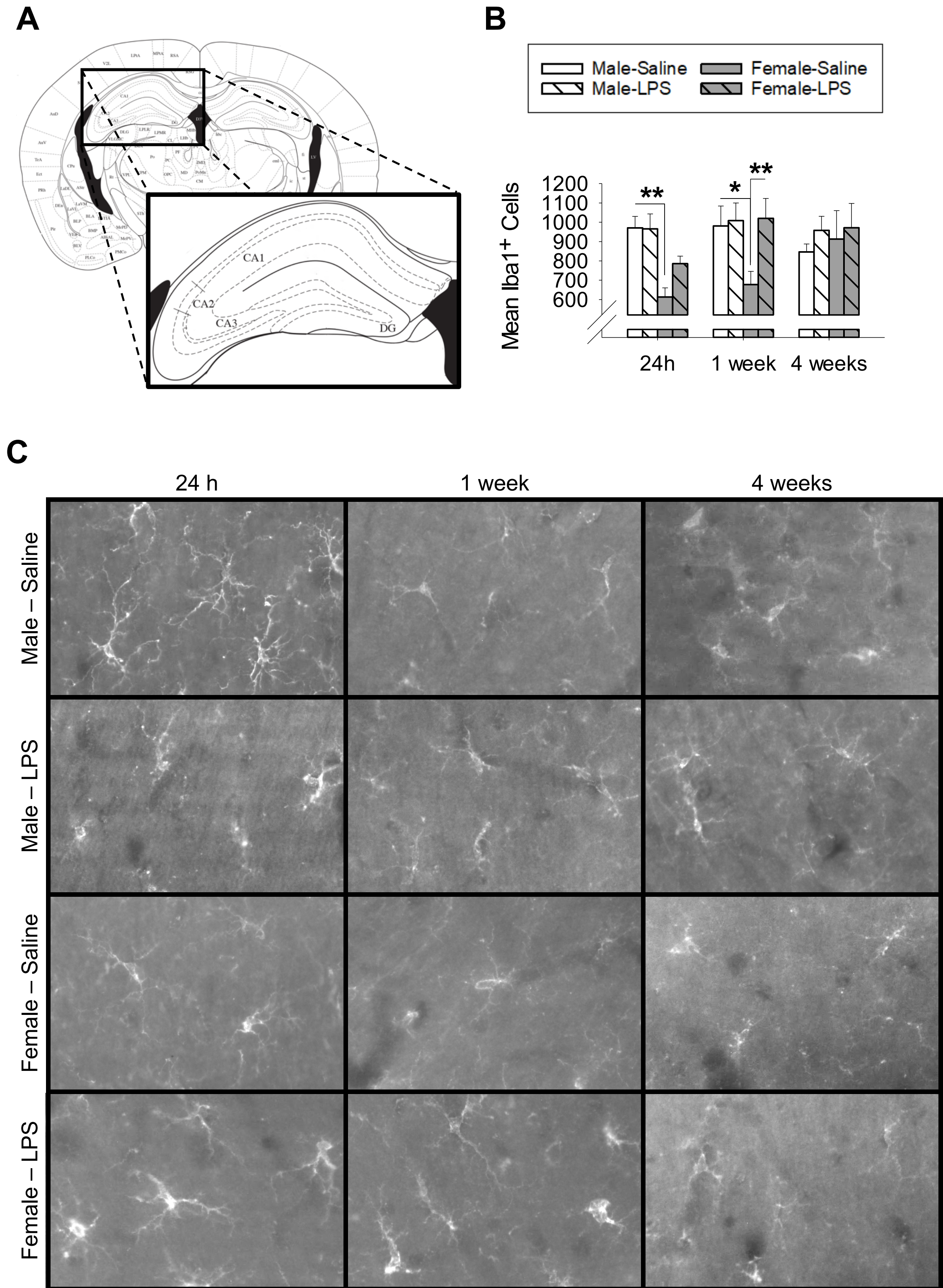
Mean sickness scores and changes in body weight of six-week-old male and female CD-1 mice following treatment.



Note. Sickness behaviours were examined .5, 2, 4, 8, 12, and 24 h after treatment with LPS (1.5 mg/kg body weight, *ip*) or .9% sterile saline (LPS-matched volume, *ip*). Body weights were measured at time of treatment and 12 and 24 h post-treatment to examine percent change in body weight from time of treatment. Data presented as mean (± *SEM*) sickness scores (**A**) and percent (%) change in body weight (**B**). Asterisks denote significant sex differences (**A**) and treatment differences (**B**) (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 3.

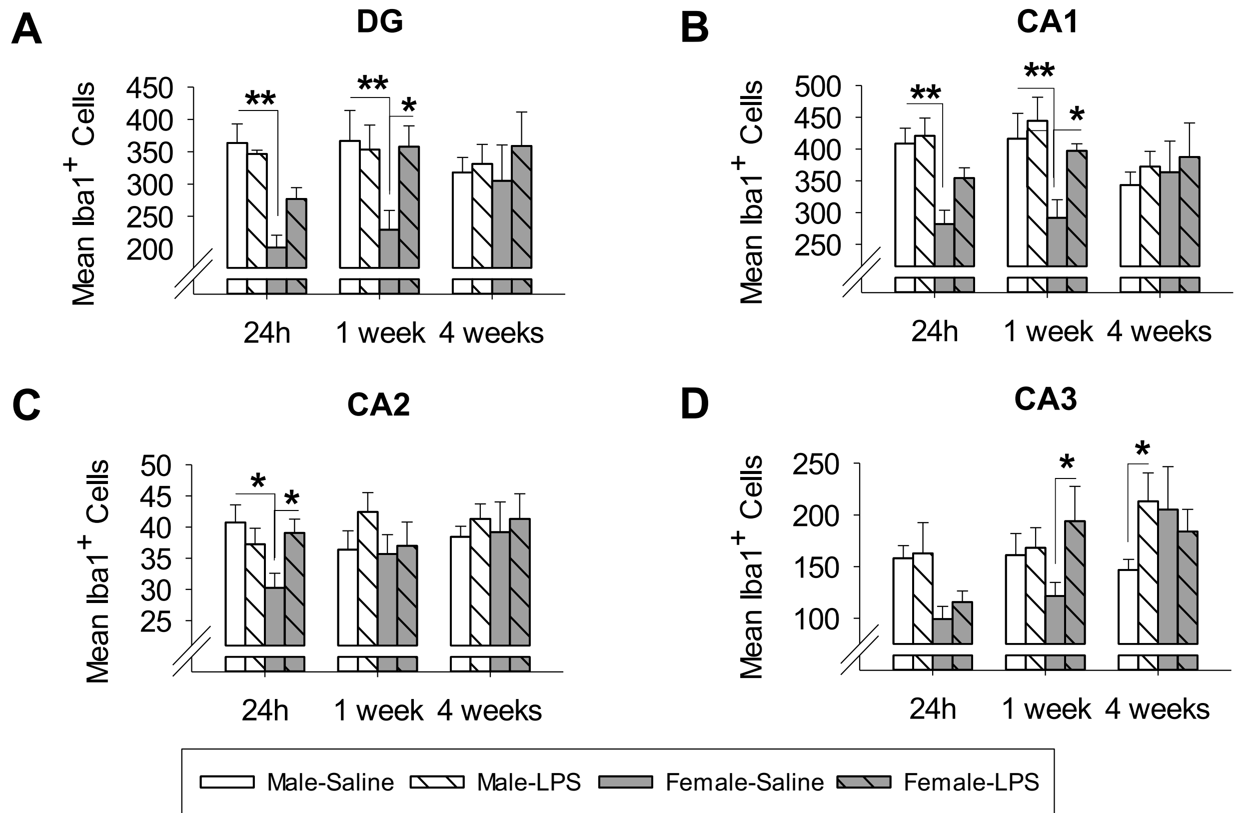
Microglial expression in the hippocampus.



Note. Schematic drawing depicting the region of interest in the dorsal hippocampus and its subregions (i.e., bregma: -2.18 mm) (Franklin & Paxinos, 2007) (**A**). Data presented as mean (± *SEM*) total Iba1$^+$ cells counted in the whole hippocampus (**B**). Photomicrographs of hippocampal Iba1$^+$ cells from the experimental groups from the three time-points (i.e., 24 h, one week, and four weeks post-treatment) (**C**). Asterisks depict significant sex and treatment differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 4.

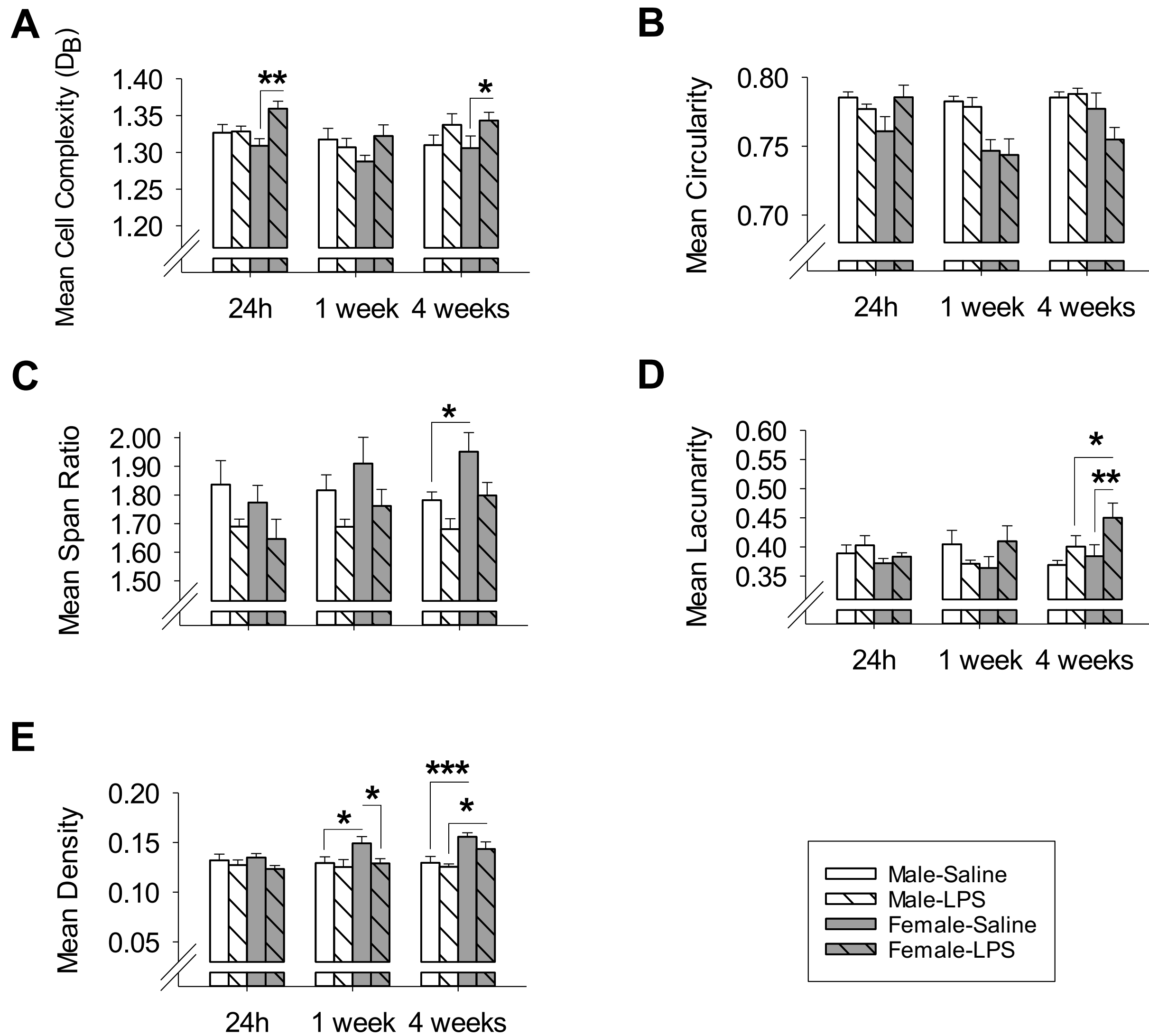
Microglial expression in the hippocampal subregions.



Note. Mean (± *SEM*) total Iba1^{+} cells counted in dentate gyrus (**A**), cornus ammonis (CA) 1 (**B**), CA2 (**C**), and CA3 (**D**) 24 h, one week, and four weeks post-treatment. Asterisks depict significant sex and treatment differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 5.

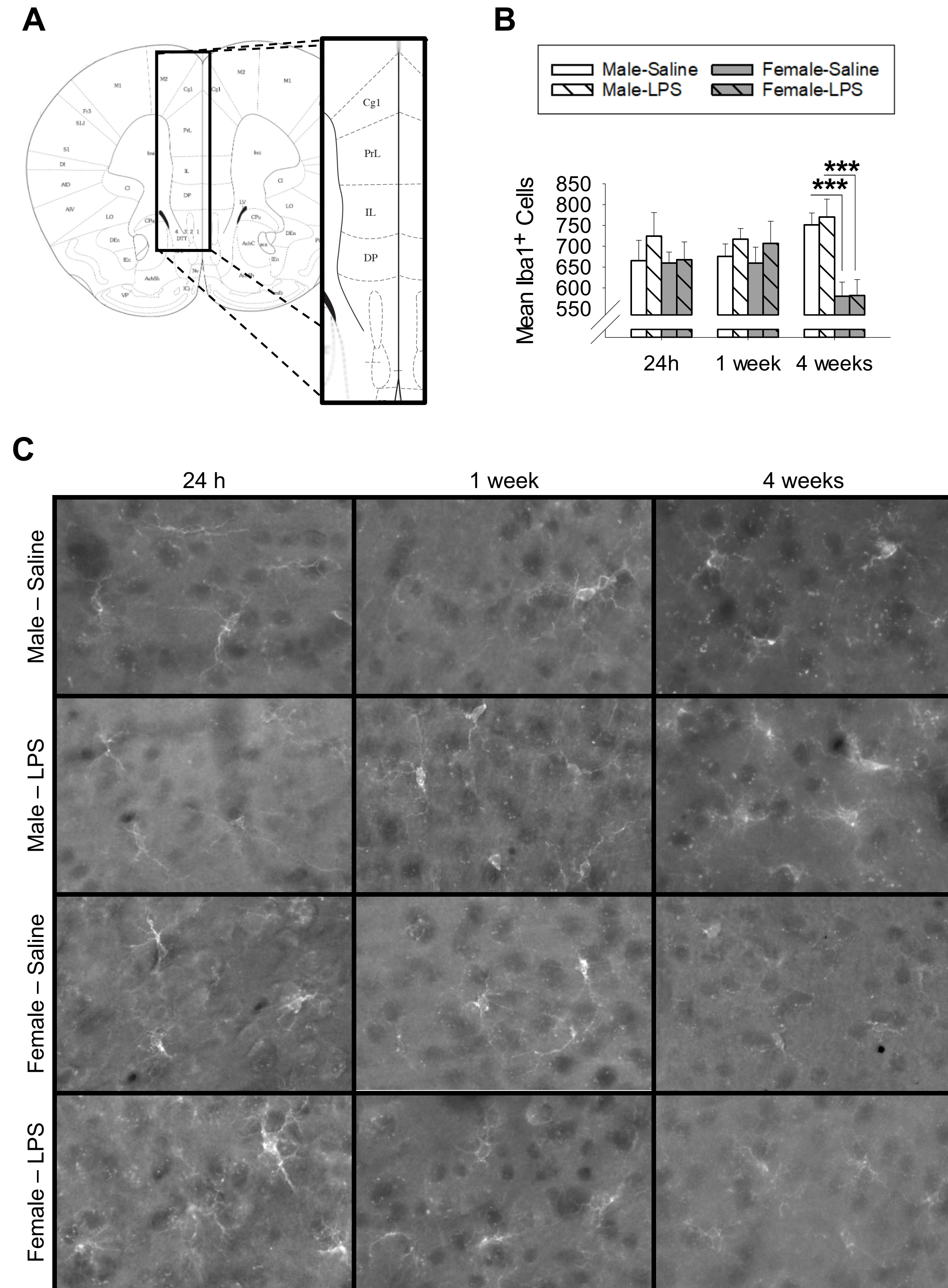
Sex and treatment differences in microglial morphology in the hippocampus.



Note. The figures depict group means (± *SEM*) in fractal analyses (i.e., fractal dimension [**A**], circularity [**B**], span ratio [**C**], lacunarity [**D**], and density [**E**]) of hippocampal microglia 24 h, one week, and four weeks post-treatment. Asterisks denote significant sex and treatment differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 6.

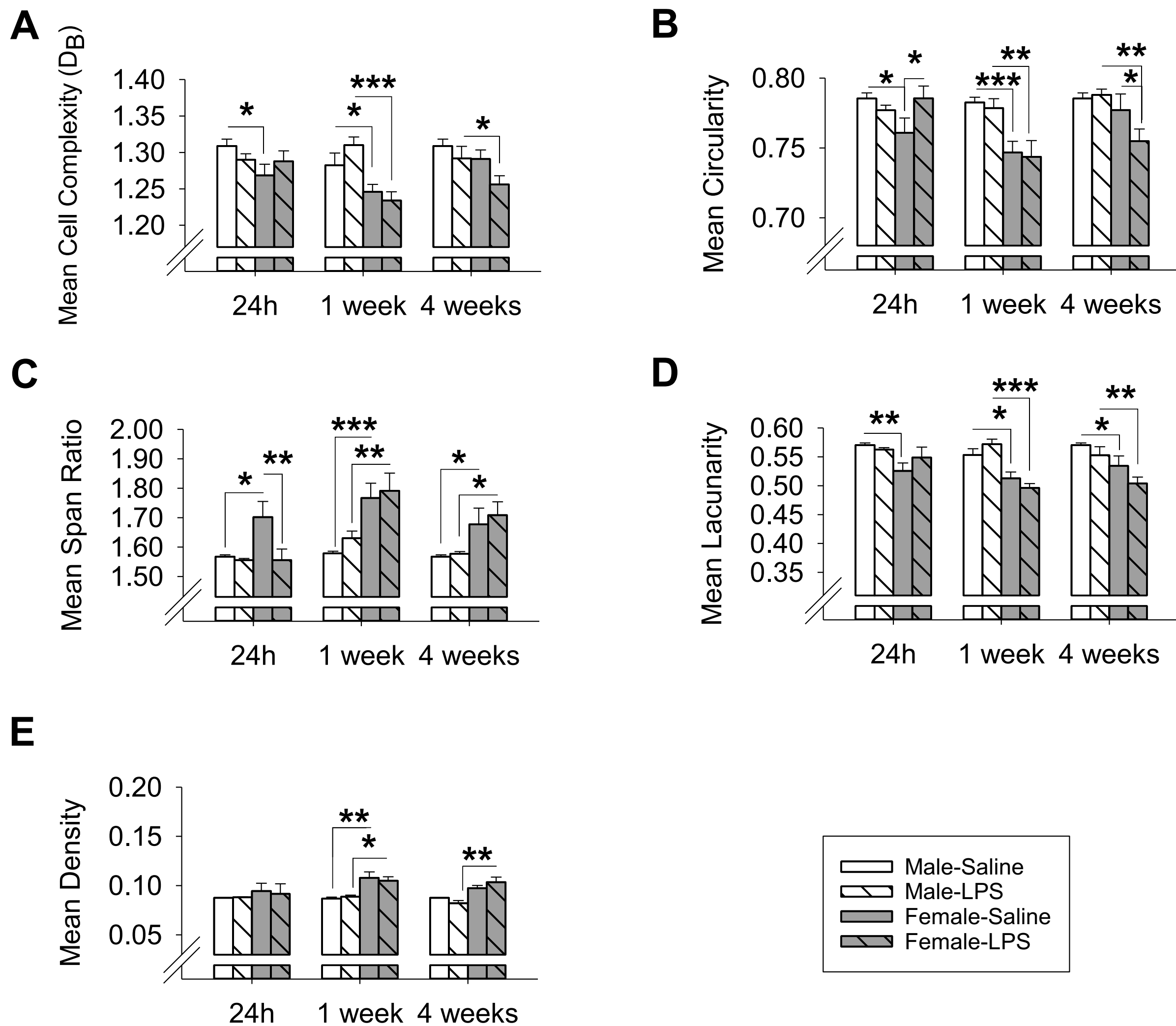
Microglial expression in the mPFC.



Note. Schematic drawing depicting the region of interest in the (i.e., bregma: +1.78 mm) (Franklin & Paxinos, 2007) (**A**). Data presented as mean (± *SEM*) total Iba1$^+$ cells counted in the mPFC (**B**). Photomicrographs of Iba1$^+$ cells in the mPFC from the experimental groups from the three time-points (i.e., 24 h, one week, and four weeks post-treatment) (**C**). Asterisks depict significant sex and treatment differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 7.

Sex and treatment differences in microglial morphology in the mPFC.

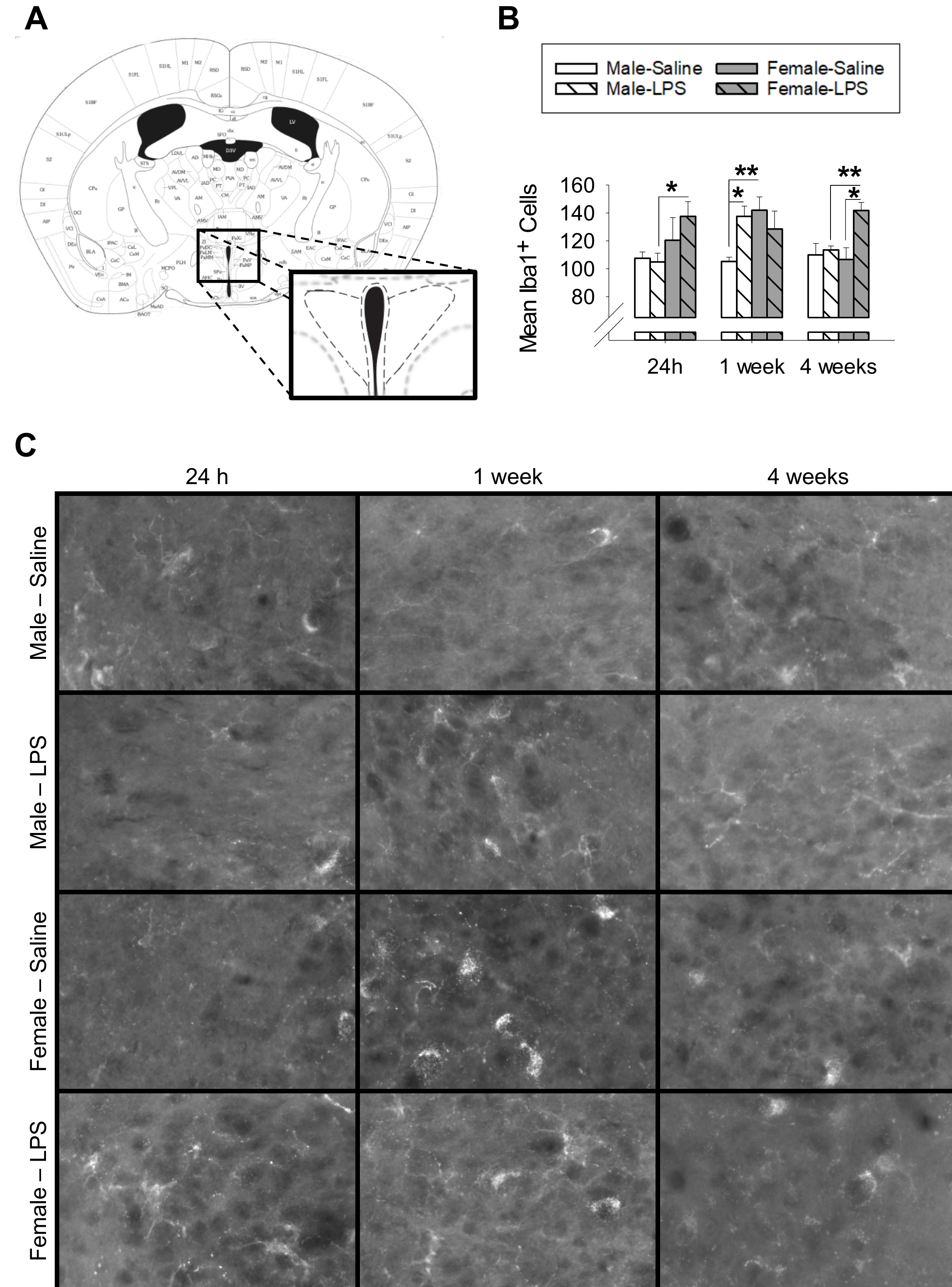


Note. The figures depict group means (± *SEM*) in fractal analyses (i.e., fractal dimension [**A**], circularity [**B**], span ratio [**C**], lacunarity [**D**], and density [**E**]) of microglia in the mPFC 24 h, one week, and four weeks post-treatment. Asterisks denote significant sex and treatment differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 8.

Microglial expression in the PVN.

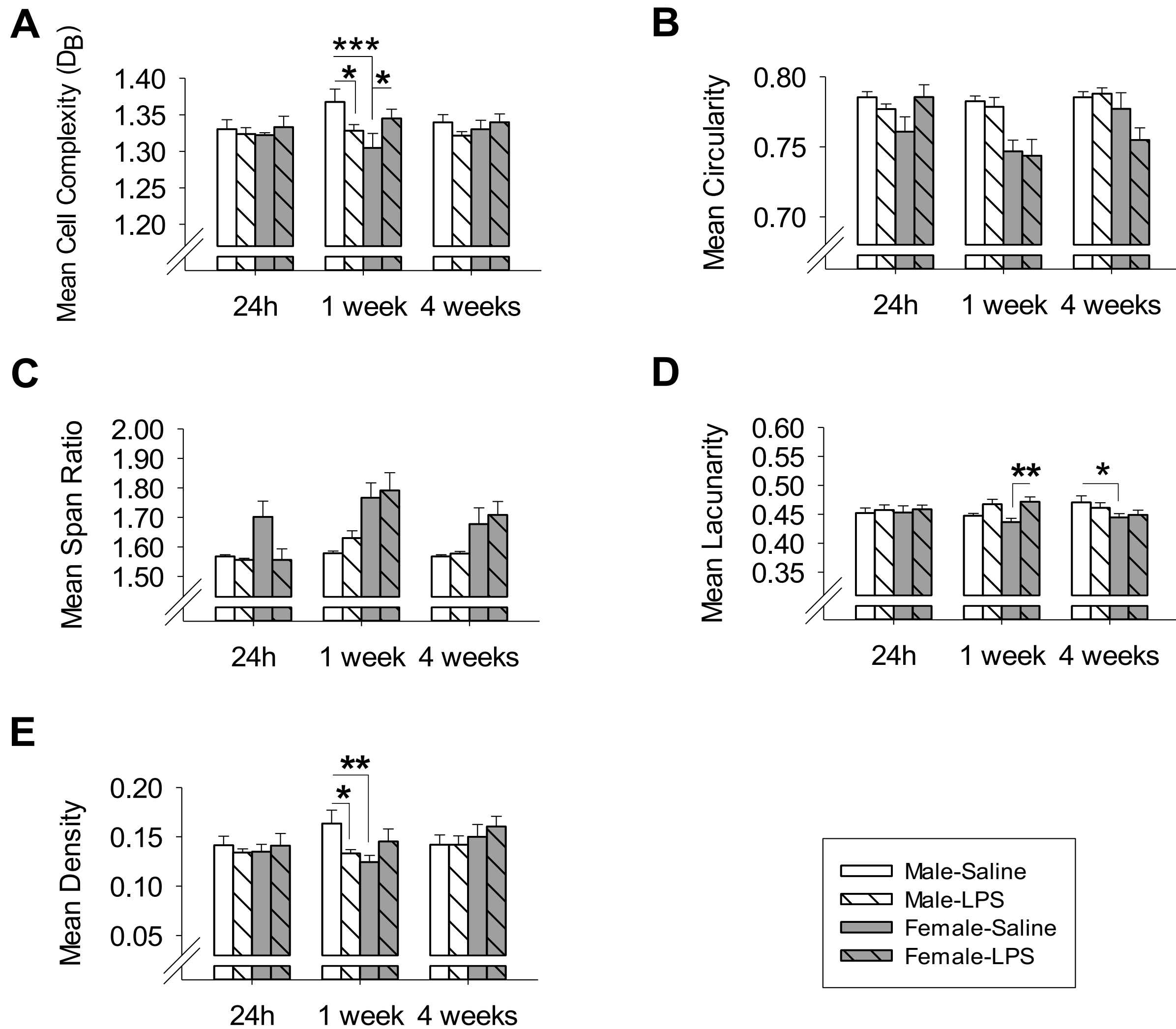
Figure 8.



Note. Schematic drawing depicting the region of interest in the (i.e., bregma: –0.82 mm) (Franklin & Paxinos, 2007) (**A**). Data presented as mean (± *SEM*) total Iba1$^+$ cells counted in the PVN (**B**). Photomicrographs of Iba1$^+$ cells in the PVN from the experimental groups from the three time-points (i.e., 24 h, one week, and four weeks post-treatment) (**C**). Asterisks depict significant sex and treatment differences (* = p < .05, ** = p < .01, *** = p < .001).

Figure 9.

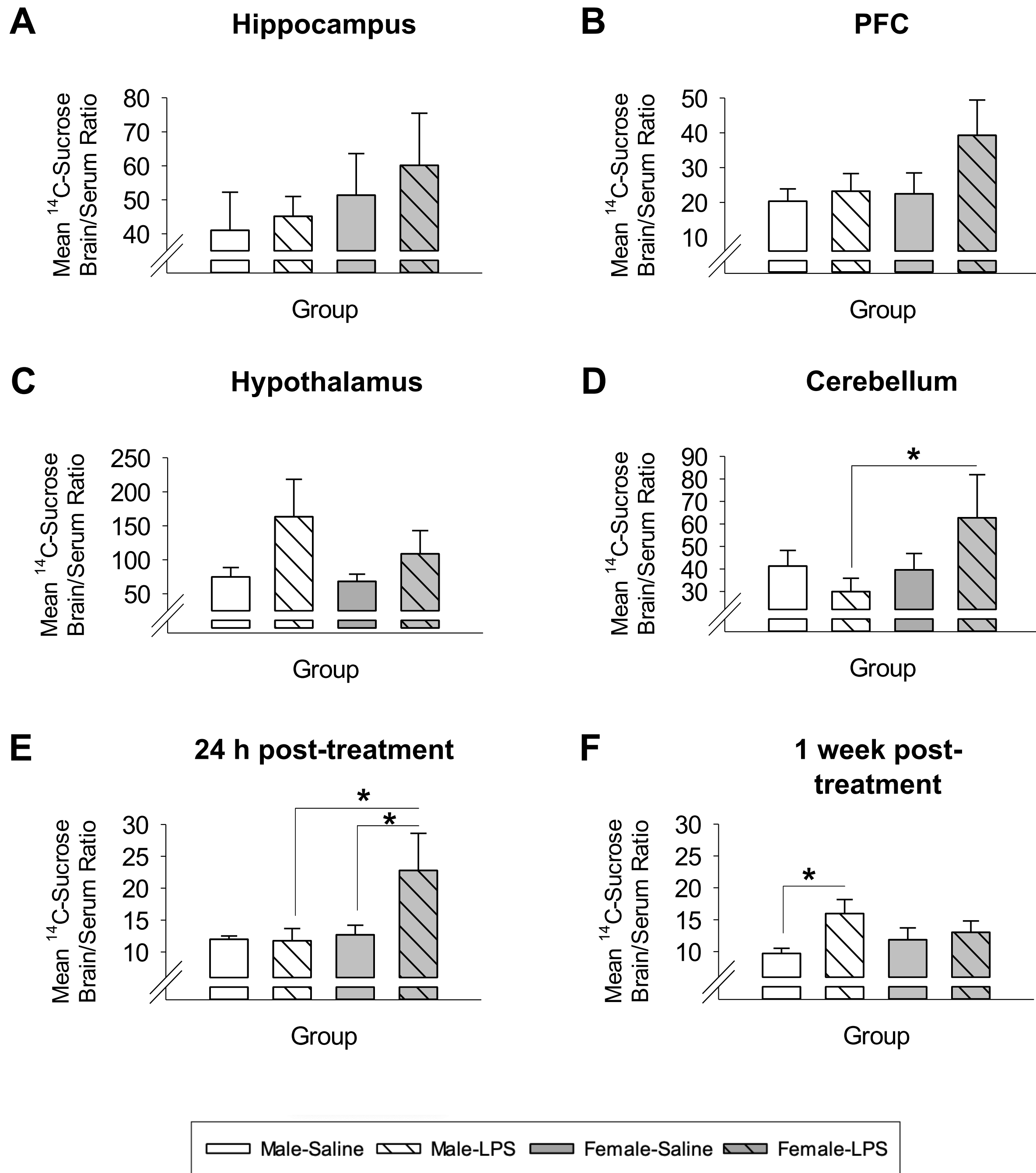
Sex and treatment differences in microglial morphology in the PVN.



Note. The figures depict group means (± *SEM*) in fractal analyses (i.e., fractal dimension [**A**], circularity [**B**], span ratio [**C**], lacunarity [**D**], and density [**E**]) of microglia in the PVN 24 h, one week, and four weeks post-treatment. Asterisks denote significant sex and treatment differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 10.

Sex and treatment differences in global and regional BBB permeability.



Note. Data presented as mean (± *SEM*) ^{14}C-sucrose whole brain/serum (μL/g) ratios in the hippocampus (**A**), PFC (**B**), hypothalamus (**C**), cerebellum (**D**) 24 h post-treatment and in the

whole-brain 24 h (**E**) and one week (**F**) post-treatment. Asterisks denote significant pairwise comparisons (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Study 3: Pubertal LPS Treatment Selectively Alters Baseline PSD-95 Expression in Male CD-1 Mice.

Abstract

Puberty includes a highly stress-sensitive period with significant sex differences in the neurophysiological and behavioural outcomes of a peripheral immune challenge. Sex differences in the pubertal neuroimmune network's responses to systemic LPS (Kolmogorova et al., 2021) may explain some of these enduring sex-specific outcomes of a pubertal immune challenge. However, the functional implications of these sex-specific neuroimmune responses on the local microenvironment are unclear. Western blots were used to examine treatment- and sex-related changes in the baseline expression of regulatory proteins in inflammation (NFκB), cell death (AIF), oxidative stress (SOD-1), and synaptic plasticity (PSD-95) following symptomatic recovery (i.e., one week post-treatment) from pubertal immune challenge. Across the four examined brain regions (i.e., hippocampus, PFC, hypothalamus, and cerebellum), only baseline PSD-95 levels were altered one week post-treatment by the pubertal LPS treatment. Unlike their female counterparts, seven-week-old males showed increased PSD-95 expression in the hippocampus ($p < .05$). Baseline AIF, SOD-1, and NFκB levels in both sexes were unaffected by treatment (all $p > .05$), which suggests appropriate resolution of NFκB-mediated immune responses to pubertal LPS without stimulating AIF-mediated apoptosis and oxidative stress. We also report a significant male-biased sex difference in PSD-95 levels in the PFC and in cerebellar expression of SOD-1 during puberty (all $p < .05$). These findings highlight the sex-specific vulnerability of the pubertal hippocampus to systemic LPS and suggest that a pubertal immune challenge expedites neurodevelopment in the hippocampus in a sex-specific manner (Ismail & Blaustein, 2013; Kolmogorova at al., 2019).

Keywords: lipopolysaccharide, SOD-1, AIF, NFκB, PSD-95, Western blot

Introduction

Puberty includes a period of heightened stress sensitivity during which exposure to a systemic immune challenge alters behaviour and underlying neurocircuitry in a sex-dependent manner (Blaustein et al., 2016; Holder and Blaustein, 2014; Kane & Ismail, 2017). In CD-1 mice, the most robust and enduring effects of a systemic immune challenge (i.e., lipopolysaccharide [LPS], 1.5 mg/kg body weight, intraperitoneal [*ip*]) occur at six weeks of age (Laroche et al., 2009 a, b). Sex differences in the central stress response system moderate responses to pubertal LPS (Cai et al., 2016; Erickson et al., 2018; Girard-Joyal et al., 2015; Sharma et al., 2018). Permanent sex-specific outcomes of pubertal immune challenge are observed in behavioural responsiveness to gonadal hormones (Ismail et al., 2013; Laroche et al., 2009 a, b; Olesen et al., 2011), stress and immune responses (Murray et al., 2019; Sharma et al., 2019), depression-like and anxiety-like behaviours (Murray et al., 2019, 2020), spatial learning and hippocampal cell development (Kolmogorova et al., 2019), and dopamine-sensitive behaviours (Girard-Joyal and Ismail, 2017). Region-specific sex differences in microglial responses to pubertal LPS, particularly after symptomatic recovery (Kolmogorova et al., 2021) may explain some of the sex-specific outcomes of pubertal immune challenge.

Healthy neuroimmune responses follow a transient course of activation that culminates in the resolution of inflammation and healing of damaged tissue (DiSabato et al., 2016; Frank et al., 2019; Yong et al., 2019). Immune threats (e.g., debris, bacteria) transition "resting" surveillant (M0 phenotype) microglia towards the "classic" pro-inflammatory (M1) and the "alternative" anti-inflammatory (M2a, b, and c) phenotypes (Boche et al., 2013; Colton, 2009; Jurga et al., 2020). The M1 phenotype serves as the first line of defence of the innate immune system, releasing pro-inflammatory mediators (e.g., tumor-necrosis factor [TNF]-α and interleukin [IL]-

1β) to purge pathogens, polarize T-cells towards mounting an adaptive immune response, and trigger the healing process. The M2 phenotype instead stimulates cellular processes associated with tissue repair and regeneration (M2a), immunomodulatory activity (M2b), and anti-inflammatory actions and debris scavenging (M2c). Age-related sex differences in microglial density, structure, and activity (e.g., Crain et al., 2013; Lawson et al., 1990; Lively et al., 2018; Penaloza et al., 2009; Schwarz et al., 2012; Yanguas-Casás et al., 2020) influence the nature of microglial responses produced by the coordinated effort of cell signalling pathways, gene expression, and epigenetic mechanisms involved in microglial polarization (for reviews see Askew & Gomez-Nicola, 2018; Bordt et al., 2020; Tan et al., 2020; Villa et al., 2019).

The nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) family of transcription factors regulates the initial M1 polarization to inflammatory stimuli (Baker et al., 2011; Kaltschmidt and Kaltschmidt, 2009; Mémet, 2006). NFκB protein complexes exist in almost all nucleated mammalian cells, most commonly as the p50/p65 heterodimer. NFκB induction occurs via classical/canonical (i.e., regulation of acute and chronic immune responses) and alternative/non-canonical (i.e., supplementary signalling axis in the adaptive immune system) pathways within minutes of stressor exposure and resolves within an hour thereafter (Berghe et al., 2006; Doyle and O'Neill, 2006; Shih et al., 2015). Excessive inflammatory stimuli and mechanistic failures in pro- and anti-inflammatory responses, particularly during critical periods, propel adaptive inflammatory processes towards cytotoxic states of unresolved inflammation (Block et al., 2007; Brown and Neher, 2010; Cherry et al., 2014). For example, repeated or chronic stress prolong inflammatory responses by increasing the magnitude of NFκB induction and delaying its resolution (Bekhbat et al., 2019; Pace et al., 2006). Maladaptive immune responses can initiate apoptotic cell death (i.e., cell shrinkage, DNA fragmentation,

chromatin condensation, and membrane cell death) via caspase-dependent routes in young females and caspase-independent routes (i.e., nuclear translocation of apoptosis-inducing factor [AIF]) in young males (Jog & Caricchio, 2013; Liu et al., 2018; for reviews see Blomgren et al., 2007; Elmore, 2007; Ortona et al., 2014).

The extensive neuroplasticity of the pubertal brain renders the ongoing remodelling and reorganization of neurocircuitry vulnerable to maladaptive microglia-mediated inflammation (Delpech et al., 2015; John & Kaffman, 2018). Similarities in the behavioural outcomes of pubertal immune challenge and post-synaptic density (PSD)-95 protein dysfunction warrant further investigation of this synaptic scaffolding protein's mechanistic involvement in the enduring effects of pubertal LPS. PSD-95 is the major scaffolding protein of the excitatory PSD and is highly abundant in the hippocampus, cerebral cortex, and striatum (Funke et al., 2005; Nikonenko et al., 2008). PSD-95 is key to synaptic plasticity, glutamatergic transmission, and dendritic spine morphogenesis (Chen et al., 2011, 2015; Chowdhury et al., 2017; De Roo et al., 2008; Ehrlich et al., 2007; Jeong et al., 2019). Stress-induced changes in PSD-95 expression during critical periods permanently alters synaptic structure and function in various neural systems (e.g., Coley & Gao, 2019; Hermes et al., 2011). As with pubertal LPS treatment, PSD-95 dysfunction precipitates anxiety-like and depression-like behaviours and impairments in learning and memory (Feyder et al., 2010; Feyissa et al., 2009; Leuba et al., 2008; Migaud et al., 1998; Proctor et al., 2010; Stein et al., 2003). However, the involvement of PSD-95 in the effects of pubertal immune challenge has not been examined.

The study was designed to provide functional insight into the microglial responses persisting past symptomatic recovery from LPS-induced sickness during puberty (Kolmogorova et al., 2021). This work examined sex and treatment differences one week post-treatment in the

expression of regulatory proteins in inflammation (NFκB), apoptosis (AIF), oxidative stress (SOD-1), and synaptic plasticity (PSD-95) across brain regions involved in stress responses (i.e., hypothalamus) and cognition (i.e., hippocampus, prefrontal cortex, cerebellum). Maladaptive microglial responses were expected to increase NFκB-mediated inflammation and trigger oxidative stress and apoptosis. Given the underrepresentation of females in this field, an additional goal of this study was to investigate sex differences in LPS-induced changes in NFκB-related inflammation and PSD-95 expression. LPS-induced changes in AIF were expected to be skewed towards males.

Methods

Animals

Three-week-old mice shipped from Charles River Laboratories (Saint-Constant, Québec) were housed in sex-specific colony rooms maintained under standard conditions (24 ± 2 °C; relative humidity of 40 ± 5 %) on a reversed 14 h:10 h light/dark cycle (lights off at 10:00 a.m.). Dusk and dawn were induced gradually over one hour. All mice were housed three per cage in polycarbonate Lexan cages (17 x 28 x 12 cm [width x length x height]). All cages were bedded with Teklad Corn Cob bedding (Envigo, Madison, WI, US; cat: 7097; .25 in. diameter) and included a cardboard refuge hut (Ketchum Manufacturing, Inc., Brockville, ON, Canada) and one square piece of Nestlet (Ancare Corp., Bellmore, NY, USA). Food (Envigo, Madison, WI, US; T2018 – Teklad Global Diets® 18% rodent) and water were available *ad libitum*. The Animal Care Committee of the University of Ottawa approved all experimental procedures.

Lipopolysaccharide Treatment

Mice in the experimental group were treated with LPS (from *Escherichia coli* serotype O26:B6; L#3755; Sigma Chemical Co., St. Louis, MO, USA; 1.5 mg/kg body weight, *ip*; $n = 32$)

diluted at a concentration of .2 mg/mL in .9 % sterile saline (Kolmogorova et al., 2017). This LPS dose induces sickness for approximately 48 h and has sex-specific outcomes on immune responses (Cai et al., 2016) and reproductive and non-reproductive behaviours (Blaustein et al., 2016; Kane & Ismail, 2017). Control mice received .9 % sterile saline (LPS-matched volume, *ip*; $n = 32$). All treatments were performed towards the end of the light phase.

Sickness Behaviour Monitoring

Sickness behaviour responses (i.e., lethargy, piloerection, ptosis, and huddling behaviour) were assessed by two trained raters .5, 2, 4, 8, 12, 24, and 48 h after treatment (Kolmogorova et al., 2017). Both raters were blind to treatment condition. The average rating (minimum: 0 = no sickness behaviours; maximum: 4 = all four sickness behaviours) at each time-point was used for analyses.

Treatment-Related Changes in Body Mass

Body weights (g) were measured at the time of injection and at 12, 24, and 48 h post-injection. Treatment-induced changes in body weight were examined as percent change in body weight from the time of injection (Kolmogorova et al., 2017).

Brain Tissue Collection

All mice were deeply anesthetized with sodium pentobarbitol (500 mg/kg body weight, *ip*) one week after LPS or saline exposure ($n = 8$ / group). Upon confirmation of deep anesthesia, mice were decapitated and their brains were excised. Hippocampal, cerebellar, hypothalamic, and PFC tissue were then dissected and flash frozen with liquid nitrogen. All tissue samples were stored at –80 °C.

Quantitative Western Blot Analyses

The dissected hippocampal, cerebellar, hypothalamic, and PFC tissues were first

homogenized in radioimmunoprecipitation assay buffer (RIPA; 150 mM sodium chloride / 50 mM Tris / 1% Triton X-100 / .5% sodium deoxycholate / .1% sodium dodecyl sulfate) containing the protease inhibitors Roche PhosSTOP™ (Millipore Sigma; cat: 04906837001) and Roche cOmplete™ ULTRA Tablets EDTA-free (Millipore Sigma; cat: 05892791001). The homogenates were incubated on ice for 10 min, then centrifuged at 4 °C at 19,000 g for 20 min, and the supernatants collected to assay total protein concentrations using the Pierce™ Bicinchoninic Acid Assay (BCA) protein assay kit (Thermo Fisher Scientific). Protein (20 μg) was mixed with Laemmli sample buffer and then heated to 95 °C for 5 min. Samples were electrophoresed on an 12% polyacrylamide gel (TGX Stain-Free™ FastCast™ Acrylamide Kit; Bio-Rad; cat: 1610185) using Mini-PROTEAN® 3 Dodeca™ Cell system (Bio-Rad). The gel image, to be used for internal loading normalization, was then collected with ChemiDoc™ XRS+ System (Bio-Rad). The separated proteins were transferred onto .2 μm nitrocellulose membranes with Trans-Blot® Electrophoretic Transfer Cell system (Bio-Rad). The nitrocellulose membrane was blocked for 1 h at room temperature in a blocking solution containing 5% skim milk in TBST buffer (Tris-buffered saline [TBS] / 20 mM Tris-base / 137 mM NaCl / .1% Tween-20). The nitrocellulose membranes were then incubated overnight at 4 °C in a solution of 5 % milk / TBST containing either rabbit anti-NFκB p65 (1/1000; Cell Signalling Technology; cat: 8242), rabbit anti-AIF (1/1000; Abcam; cat: ab32516), mouse anti-PSD-95 (1/1500; Millipore; cat:MAB1596), or rabbit anti-SOD1/Cu-Zn SOD (1/3000; Novus Biologicals; cat: NBP2-24915SS). Following three 10-min washes with TBST, the membranes were incubated for 1 h at room temperature with relevant goat anti-mouse Alexa Fluor 680 (1/10,000; Molecular Probes, Cat: A21058) and goat anti-rabbit IRDye® 800CW secondary antibodies (LiCor Biosciences; cat: 925-32211) in a 5% skim milk / TBST solution. After a final

set of TBST washes (three x 10 min) the membranes were scanned using a Li-Cor Biosciences Odyssey® infrared imaging system. Protein levels on the membranes were quantified using Li-Cor Biosciences Odyssey® software. Data are presented as mean (± *SEM*) fold change in normalized band intensity values relative to the pooled sample.

Experimental Design

Treatment with either LPS or saline control occurred at six weeks of age. Treatment-induced changes in body weight and behavioural signs of sickness were monitored at various time points post-injection. Hippocampal, cerebellar, hypothalamic, and PFC tissues were collected one week after treatment to correspond with symptomatic recovery from LPS-induced sickness. Quantitative Western blots were used to examine sex and treatment differences in the expression of NFκB, AIF, SOD-1, and PSD-95 proteins in the dissected brain regions.

Statistical Analyses

Extreme statistical outliers (i.e., cases that exceeded the 3 x interquartile range) were adjusted by winsorization to the next highest value. A three-way (sex x treatment x time) mixed-design analysis of variance (ANOVA) was used to assess sickness behaviours and weight change. *F*-values that violated Mauchly's test of sphericity (i.e., $\varepsilon_{\text{Greenhouse-Geisser}} < .75$) were adjusted with the Greenhouse-Geisser correction. All other parameters were examined with a two-way (sex x treatment) between-subjects ANOVA. Significant main effects and interactions were followed by Bonferroni-corrected pairwise analyses to protect against Type 1 error (Abdi, 2007). Effect sizes were estimated using partial eta-squared (η^2). Statistical analyses were performed using IBM® SPSS® (version 27.0.0) statistical software. The criterion for statistical significance was set to $p < .05$. Interactions trending towards statistical significance ($.05 < p < .10$) were also reported.

Results

Treatment- and Sex-Dependent Effects on Sickness-Related Parameters

Sickness Behaviours. As expected, sickness behaviour responses showed a significant time x sex x treatment interaction ($F_{(2.55, 71.44)} = 5.51$, $p = .003$, $\eta_p^2 = .164$) and main effects of treatment ($F_{(1, 28)} = 2743.55$, $p < .001$, $\eta_p^2 = .990$) and sex ($F_{(1, 28)} = 29.51$, $p < .001$, $\eta_p^2 = .513$). Sickness behaviour responses to pubertal LPS differed significantly across time ($F_{(2.55, 71.44)} = 102.38$, $p < .001$, $\eta_p^2 = .785$). LPS-treated females showed significantly more sickness behaviours than their saline-treated (i.e., controls) counterparts .5, 2, 4, 8, 12, and 24 h post-treatment (all $p < .001$). On the other hand, LPS-treated males showed significantly more sickness behaviours relative to control males 2, 4, 8, 12, 24, and 48 h after treatment (all $p < .001$). Among LPS-treated mice, males showed significantly more sickness behaviours than females at 2, 8, 12, 24, and 48 h after treatment (mean difference [*MD*] = .44, standard error [*SE*] = .17; *MD* = .94, *SE* = .16; *MD* = .56, *SE* = .12; *MD* = 1.06, *SE* = .26; and, *MD* = .88, *SE* = .06, respectively; all $p \leq .015$) (see Figure 1).

Body Weight. Percent change in body weight from treatment showed significant time x treatment ($F_{(3, 84)} = 20.51$, $p < .001$, $\eta_p^2 = .423$) and sex x treatment interactions (($F_{(1, 28)} = 4.42$, $p = .045$, $\eta_p^2 = .136$) and significant main effects of time ($F_{(3, 84)} = 19.72$, $p < .001$, $\eta_p^2 = .413$) and treatment ($F_{(1, 28)} = 89.39$, $p < .001$, $\eta_p^2 = .761$). Control males showed significantly greater weight gain than their female counterparts 24 h post-treatment (*MD* = 2.96, *SE* = 1.32, $p = .033$). Unlike their saline-treated counterparts, both LPS-treated males and females had significant weight loss 12, 24, and 48 h post-treatment (all $p \leq .001$) (see Figure 2).

Hippocampal Protein Expression

PSD-95 levels one week post-treatment revealed a significant sex x treatment interaction

($F_{(1, 28)}$ = 8.69, p = .006, η_p^2 = .237) and a significant main effect of treatment ($F_{(1, 28)}$ = 5.38, p = .028, η_p^2 = .161). The main effect of sex was not statistically significant (p > .05). Baseline PSD-95 levels were similar in males and females (p > .05). Although pubertal LPS did not affect PSD-95 expression in females (p > .05), LPS-treated males showed significantly higher PSD-95 expression one week post-treatment relative to their saline-treated and female counterparts (*MD* = 2.35, *SE* = .63, p = .001; and *MD* = 2.05, *SE* = .63, p = .003, respectively). Hippocampal expression of AIF, NFκB, and SOD-1 proteins was similar across groups one week post-treatment (all p > .05) (see Figure 3).

Protein Expression in the PFC

PSD-95 expression in the PFC one week post-treatment was overall significantly higher in males relative to females ($F_{(1, 28)}$ = 1.34, p = .014, η_p^2 = .511). The main effect of treatment and the sex x treatment interaction for PSD-95 expression were not statistically significant (both p > .05). Analyses of NFκB, AIF, and SOD-1 expression in the PFC failed to reach statistical significance (all p > .05) (see Figure 4).

Cerebellar Protein Expression

SOD-1 expression in the cerebellum one week post-treatment was overall significantly higher among males relative to females ($F_{(1, 28)}$ = 5.28, p = .029, η_p^2 = .159). The main effect of treatment and the sex x treatment interaction for SOD-1 expression did not reach statistical significance (p > .05). Cerebellar expression of NFκB, AIF, and PSD-95 proteins was similar across groups one week post-treatment (all p > .05) (see Figure 5).

Hypothalamic Protein Expression

Hypothalamic expression of NFκB, AIF, SOD-1, and PSD-95 proteins was similar across groups one week post-treatment (all p > .05) (see Figure 6).

Discussion

Sex differences in pubertal stress and immune responses pose unique challenges to the developing brain's sensitivity to a systemic immune stressor. Systemic LPS exposure in CD-1 mice during the stress-sensitive pubertal period (i.e., six weeks of age) alters several reproductive and non-reproductive behaviours and underlying neurocircuitry in a sex-specific manner (e.g., Kolmogorova et al., 2019; Murray et al., 2019, 2020; Sharma et al., 2019). Although sex differences in the neuroimmune system's responses to pubertal LPS (Kolmogorova et al., 2021) explain some of the sex-specific outcomes of a pubertal immune challenge, the functional impact of these sex-specific responses on the local microenvironment are unknown. Therefore, we examined sex differences in treatment-induced changes to baseline expression of regulatory proteins in inflammation (NFκB), apoptotic cell death (AIF), oxidative stress (SOD-1), and synaptic plasticity (PSD-95) following symptomatic recovery from pubertal immune challenge (i.e., one week post-treatment). Across the four brain regions examined, PSD-95 protein expression increased in the hippocampus of LPS-treated males only. AIF, SOD-1, and p65 protein levels were unaffected by pubertal LPS at this timepoint, which implies appropriate resolution of NFκB-mediated inflammatory processes following pubertal LPS challenge without stimulating AIF-mediated apoptosis and oxidative stress.

Region-specific sex differences in structural makeup, neurophysiology, and interconnectivity with neural systems (de Vries & Södersten, 2009; Lenz et al., 2012; Premachandran et al., 2020) may have contributed to the sex- and region-specific effects of pubertal LPS on PSD-95 protein levels. The greater sensitivity of the hippocampus to pubertal LPS relative to the other examined brain regions likely stems from its capacity for neurogenesis, high expression of receptors for stress hormones, and close anatomical and functional links with

the central stress response system (Kim et al., 2015; McEwen et al., 2016; Schwabe, 2016). Consistent with other reports (Mastro et al., 2019; Wang et al., 2018 b), baseline PSD-95 protein expression in the hippocampus did not differ significantly between pubertal males and females. The male-specific sensitivity of PSD-95 protein expression to systemic LPS likely arises from several factors which affect the equilibrium of protein interactions at the PSD, including microglia cells. Under certain conditions, the cascade of cytotoxic inflammatory mediators (e.g., TNF-α, IL-1β, inducible nitic oxide synthase) initiated by microglial responses to systemic LPS damages neurons and interferes with the structure and functioning of neurons and their associated synapses (Han et al., 2017; Sheppard et al., 2019; Song et al., 2019). Sex differences in microglial density, structure, and activity across brain regions (e.g., Crain et al., 2013; Lively et al., 2018; Villa et al., 2018) influence the reactivity and magnitude of microglia-mediated inflammatory cascades, thereby creating significant sex differences in the effectiveness of microglial responses (for review see Nelson et al., 2019). These findings suggest that region-specific sex differences in microglial expression, structure, and functioning contribute to the sex-specific residual effects of pubertal LPS-induced sickness on PSD-95 protein expression.

In addition to highlighting sex and region differences in stress vulnerability, the LPS-induced changes to PSD-95 protein levels suggest that systemic immune challenge expedites certain pubertal neurodevelopmental processes in a sex- and region-specific manner. PSD-95 is a core scaffolding protein in glutamatergic synapses which facilitates glutamatergic transmission, development of glutamatergic synapses, and synaptic plasticity by regulating levels of of α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA)-type and N-methyl-D-aspartic acid (NMDA)-type receptors (Coley & Gao, 2018; de Bartolomeis et al., 2014; Gao et al., 2013). Temporary and enduring PSD-95 imbalances influence synaptic processes via downstream

effects on AMPA receptor retention and activity at the synapse. PSD-95 overexpression diminishes synaptic efficacy and accelerates synaptic maturation in developmentally younger synapses (Béïque & Rodrigo, 2003; Coba et al., 2009; Ehrlich & Malinow, 2004; El-Husseini et al., 2000; Gray et al., 2006; Taft & Turrigiano, 2013). Similar sex-dependent treatment effects on neurodevelopment are observed in microglia, in that early life immune activation promotes the neurodevelopment-related functions of microglia rather than priming these cells towards the cytotoxic "pro-inflammatory" phenotype (Hanamsagar et al., 2017; Matcovitch-Natan et al., 2016; Thion et al., 2018). This immune-induced effect on microglial functioning appears to be limited to males in late adolescence (i.e., 60-day-old mice) and may be related to baseline sex differences in various features of microglia (Lively et al., 2018; Matcovitch-Natan et al., 2016; Villa et al., 2018). In the context of microglial responses to pubertal immune challenge (Kolmogorova et al., 2021), systemic LPS appears to encourage microglia-mediated neurodevelopment of the pubertal hippocampus in males but not females. Further investigation is warranted to determine the duration of LPS-induced changes to PSD-95 expression in males and to identify the nature of pubertal microglial responses to systemic LPS in females.

Taken together, the LPS-induced effects identified in this study offer some additional mechanistic insight into the pathogenesis of enduring outcomes of pubertal immune challenge. Systemic LPS (1.5 mg/kg body weight, *ip*) appears to induce transient sickness without permanently affecting baseline expression of regulatory proteins in inflammation (Sharma et al., 2018). Nevertheless, the developing neurocircuitry is vulnerable to permanent changes to its structure and functioning, as illustrated by persistent microglial responses (Kolmogorova et al., 2021), region-specific programming effects on the peripheral and central immune system (Sharma et al., 2019), and enhanced upregulation of toll-like receptor 4 expression in the

paraventricular nucleus to novel stressors during adulthood (Murray et al., 2019). This study suggests a role for PSD-95 in the pathogenesis of sex-specific outcomes of pubertal immune challenge on hippocampus-dependent behaviours.

This work also identified significant baseline sex differences in SOD-1 expression. SOD-1 expression was significantly higher in the cerebellum of male mice relative to their female counterparts, regardless of treatment. SOD-1 expression did not differ significantly between experimental groups in the hippocampus, hypothalamus, and PFC. SOD-1 is a highly expressed enzyme which belongs to a class of enzymes that catalyze the conversion of superoxide into oxygen and hydrogen peroxide, thus acting as the first line of defense against oxidative stress (Fukai & Ushio-Fukai, 2011; Wang et al., 2018 a). Abnormalities in SOD-1 expression are often observed in human and animal studies of amyotrophic lateral sclerosis, with some studies showing male biases in SOD-1 expression (Alexander et al., 2002; Banci et al., 2008; Frutiger et al., 2008; Martineau et al., 2020; Sau et al., 2007; Tang et al., 2019). Therefore, this baseline region-specific sex difference in SOD-1 protein expression places a sex bias during puberty in the vulnerability of SOD-1 protein expression and its behavioural outcomes.

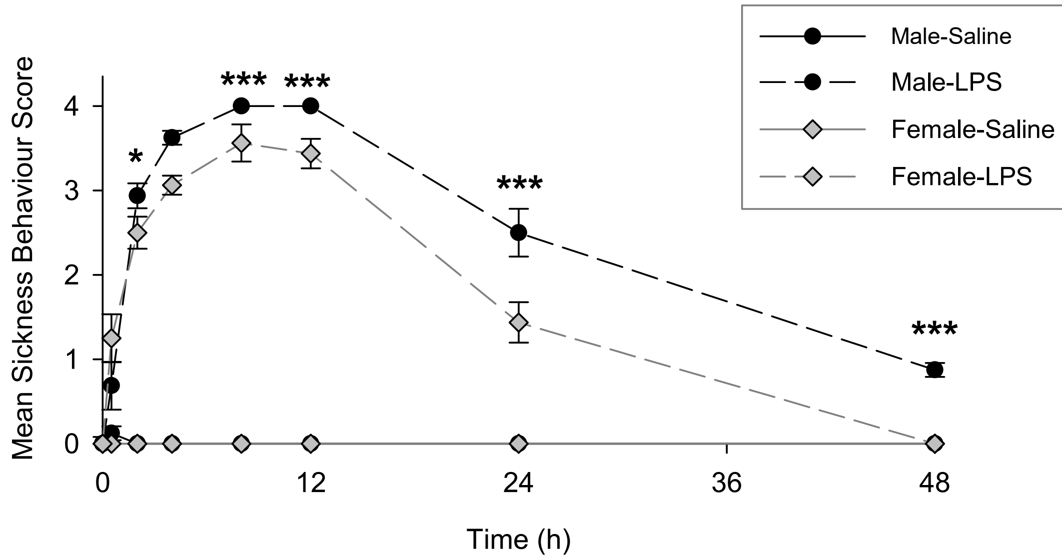
Conclusion

A pubertal immune stressor poses a unique challenge for the developing brain due to inherent sex differences in stress vulnerability. Our findings suggest that a pubertal immune challenge directly impacts the reorganization and remodelling of pubertal neurocircuitry in a sex- and region-specific manner. The significant increase in PSD-95 expression among LPS-treated mice one week post-treatment may expedite pubertal development and thus precipitate the changes in hippocampus-dependent behaviour reported elsewhere. Pubertal LPS treatment was not sufficient to induce persistent cytotoxic inflammatory responses. We also identified a

significant male-biased baseline region-specifc sex differences in synaptic plasticity (hippocampus) and oxidative stress (cerebellum).

Figure 1.

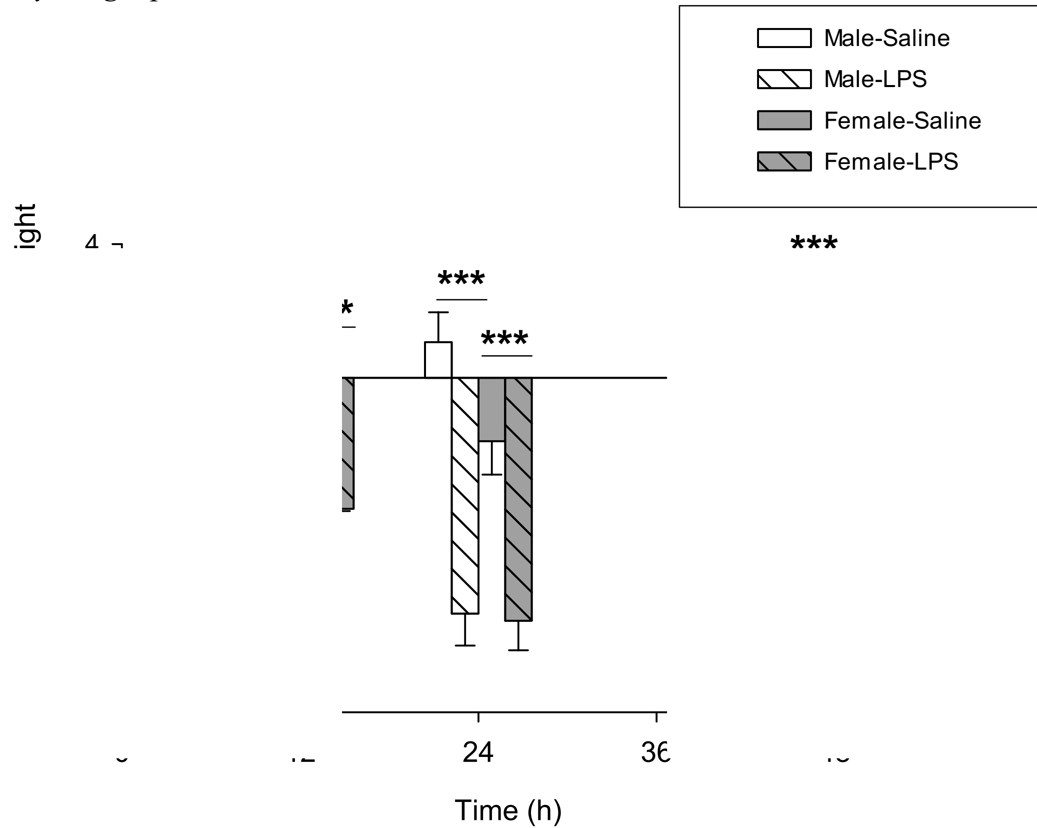
Sickness behaviour responses to systemic LPS in pubertal CD-1 mice.



Note. Sickness behaviours were examined .5, 2, 4, 8, 12, 24, and 48 h after treatment with LPS (1.5 mg/kg body weight, *ip*) or .9% sterile saline (LPS-matched volume, *ip*). Data presented as mean (± *SEM*) sickness scores. Asterisks denote significant sex differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 2.

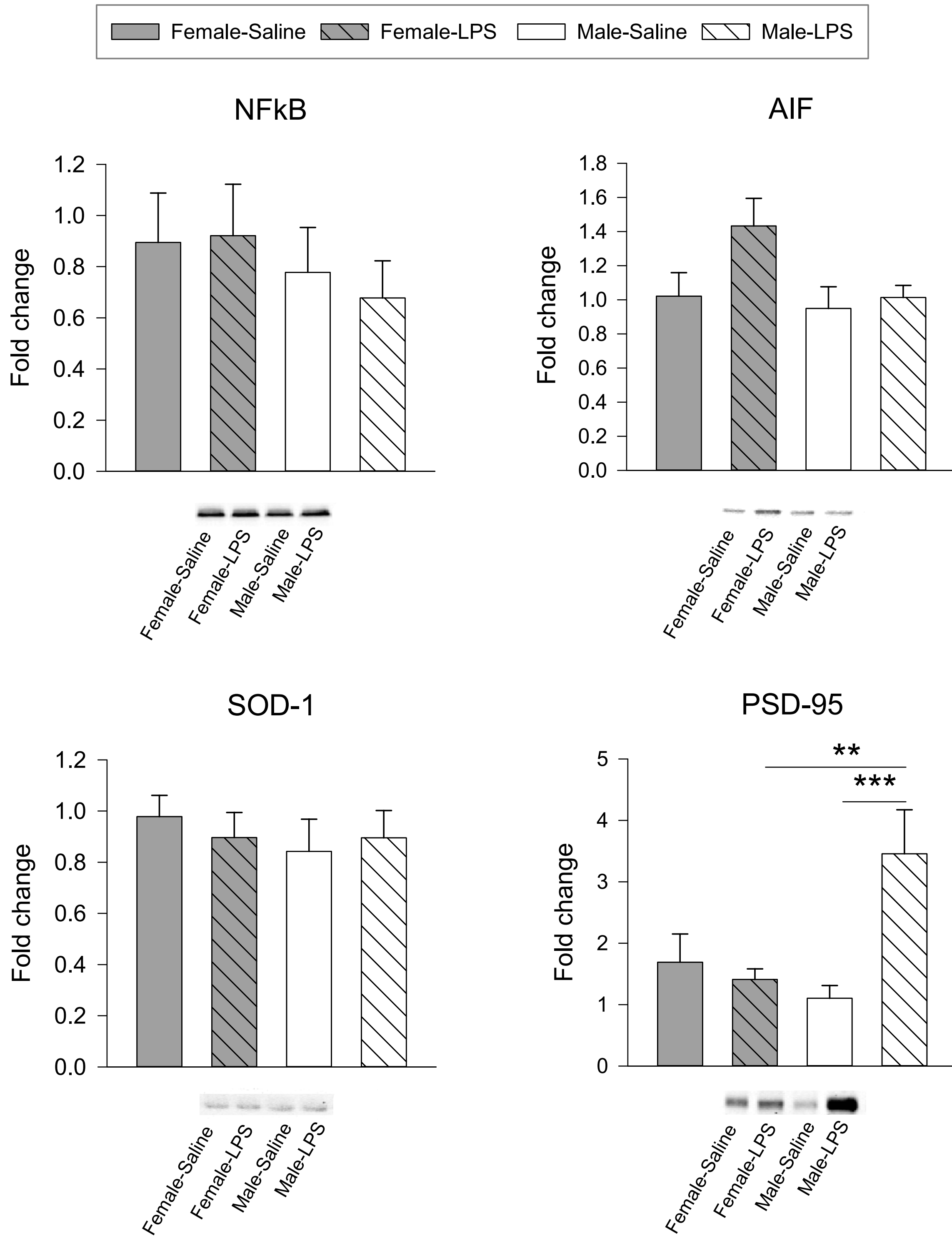
Changes in body weight post-treatment.



Note. Body weights were measured at time of treatment, 12, 24, and 48 h post-treatment to examine percent change in body weight from time of treatment. Data presented as mean (± *SEM*) percent (%) change in body weight. Asterisks denote significant treatment differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 3.

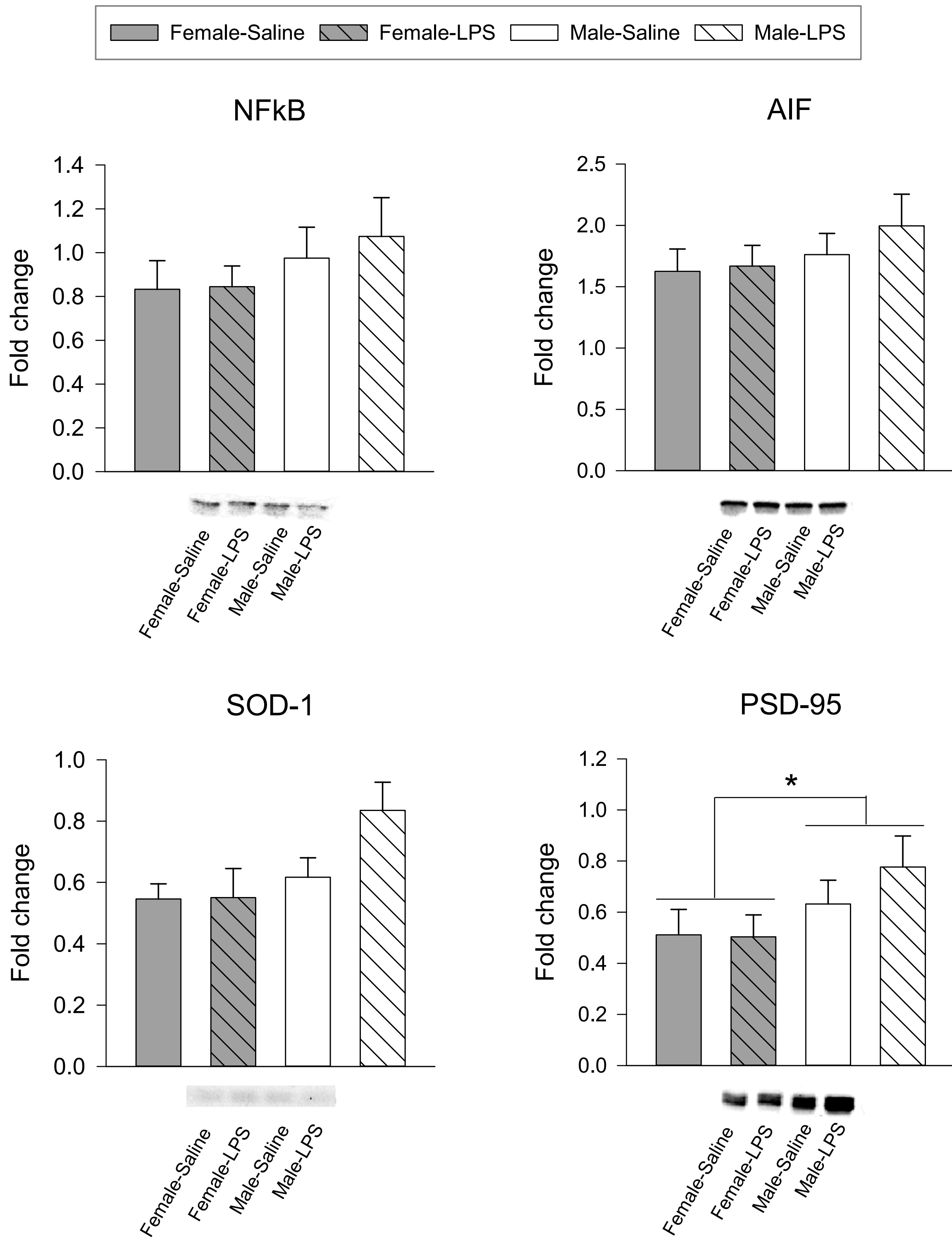
Sex and treatment differences in hippocampal protein expression.



Note. Data in each brain region are presented as mean (± *SEM*) band intensity values, which have been normalized for internal loading and inter-blot variations. Grayscale images of representative Western blots are presented below each bar graph. Asterisks denote significant group differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 4.

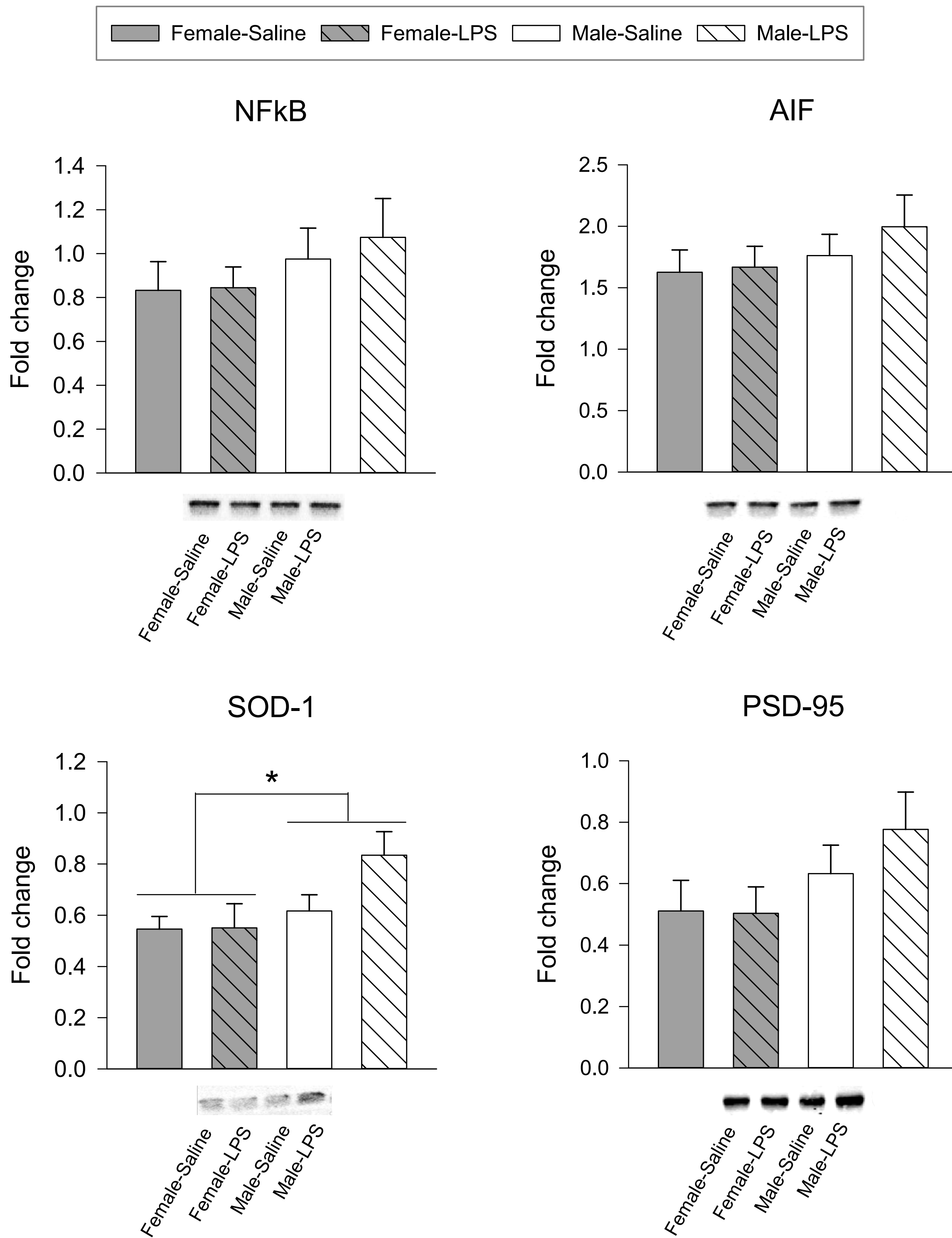
Sex and treatment differences in protein expression in the PFC one week post-treatment.



Note. Data are presented as mean (± *SEM*) band intensity values, which have been normalized for internal loading and inter-blot variations. Grayscale images of representative Western blots are presented below each bar graph. Asterisks denote significant group differences (* = p < .05, ** = p < .01, *** = p < .001).

Figure 5.

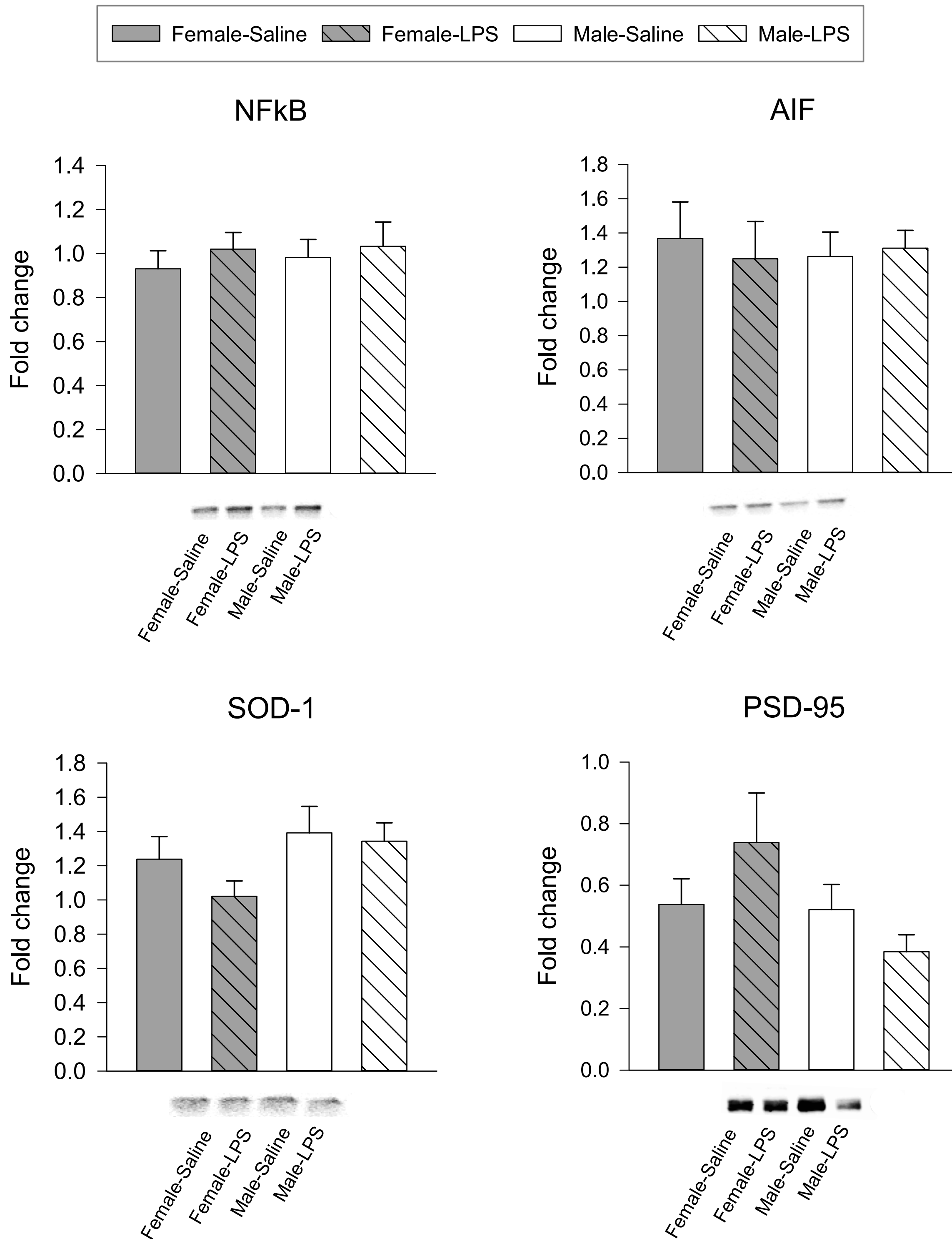
Sex and treatment differences in protein expression in the cerebellum one week post-treatment.



Note. Data are presented as mean (± *SEM*) band intensity values, which have been normalized for internal loading and inter-blot variations. Grayscale images of representative Western blots are presented below each bar graph. Asterisks denote significant group differences (* = $p < .05$, ** = $p < .01$, *** = $p < .001$).

Figure 6.

Sex and treatment differences in protein expression in the hypothalamus one week post-treatment.



Note. Data are presented as mean (± *SEM*) band intensity values, which have been normalized for internal loading and inter-blot variations. Grayscale images of representative Western blots are presented below each bar graph.

General Discussion

Puberty is a critical period for sexual maturation during which the sexually differentiated development of the pubertal brain renders it vulnerable to sex-specific outcomes of a systemic immune stressor (Holder & Blaustein, 2014; Kane & Ismail, 2017; Schulz & Sick, 2016; Sisk & Foster, 2004). In pubertal (i.e., six-week-old) CD-1 mice, intraperitoneal exposure to the bacterial endotoxin lipopolysaccharide (LPS) alters the neuroendocrine and behavioural systems of several reproductive and non-reproductive functions in a sex-specific manner (e.g., Girard-Joyal & Ismail, 2017; Murray et al., 2019, 2020). Converging evidence suggests that sex differences in the effects of a pubertal immune challenge are the product of dynamic interactions between multiple systems, yet much remains to be determined about the specific mechanisms driving the sex-specific sequelae of pubertal immune challenge. One promising explanation for the sex-specific outcomes of pubertal immune challenge may lie in the cascade of neuroinflammatory events that this systemic immune stressor induces.

The overarching goal of this doctoral thesis was to test the hypothesis that sex differences in the enduring cognitive outcomes of pubertal immune challenge arise from sex-specific responses of the pubertal neuroimmune network in underlying neurocircuitry. This thesis focused on hippocampus-dependent learning systems to examine whether previously reported impairments of pubertal immune challenge on hippocampus-dependent cognition in adult female CD-1 mice (Ismail & Blaustein, 2013) extend to their male counterparts. This work first examined whether pubertal LPS treatment alters hippocampus-dependent cognition and hippocampal cell development in a sex-specific manner mediated by circulating gonadal steroid hormones (see Study 1; Kolmogorova et al., 2019). This thesis then investigated sex differences in the pubertal neuroimmune responses to systemic LPS (see Study 2; Kolmogorova et al., 2021)

and the residual effects of pubertal LPS-induced sickness on baseline expression of key proteins in inflammation, oxidative stress, apoptotic cell death, and synaptic transmission (see Study 3). This follow-up work focused on the functionally and structurally interconnected brain regions for cognition (i.e., hippocampus, prefrontal cortex, and the cerebellum) and stress regulation (i.e., hypothalamus).

Adult hippocampus-dependent memory is sensitive to pubertal immune challenge

The dynamic crosstalk between the immune and endocrine systems during the stress-sensitive pubertal period influences hippocampus-dependent cognition in adulthood. Systemic LPS in six-week-old female CD-1 mice impairs social and object recognition memory during adulthood and permanently blocks the cognition-enhancing properties of estradiol (Ismail & Blaustein, 2013). However, sex biases in hippocampus-dependent task performance and the potential variability in performance from cyclical fluctuations in circulating gonadal hormones complicate the translation of these findings to males (Duarte-Guterman et al., 2015; Jonasson, 2005; Mahmoud et al., 2016; Yahi & Galea, 2019).

This work confirms that systemic LPS during the stress-sensitive pubertal period also impairs spatial learning and memory in male and female CD-1 mice during adulthood (see Study 1; Kolmogorova et al., 2019). LPS-treated mice used inaccurate and inefficient searches to locate the escape hole in the Barnes maze (BM) across the four-day acquisition period and showed impaired short-term, but not long-term, retention of the BM escape hole location. Although learning and retention of the escape platform in the Morris water maze (MWM) was similar across treatment groups, LPS-treated mice showed poorer cognitive flexibility than their saline-treated counterparts when learning a repositioned MWM escape platform. In light of hormone-mediated performance discrepancies between these dry-lands and water mazes (for reviews see

Frick et al., 2018; Romeo et al., 2016; ter Horst et al., 2012; Tuscher et al., 2015), high-stress paradigms (i.e., MWM) may mask the cognitive effects of pubertal LPS exposure on hippocampus-dependent spatial memory tasks during adulthood.

The complex dynamics between circulating androgens, estrogens, and progesterone and hippocampus-dependent tasks prompted investigation of gonadal hormone involvement in the LPS-induced effects on adult hippocampus-dependent cognition. Gonadectomy during early adulthood did not consistently moderate the performance of saline-treated and LPS-treated mice in the BM and the MWM (see Study 1; Kolmogorova et al., 2019). Similarly, sex did not significantly influence the relationship between pubertal LPS treatment and hippocampus-dependent cognition during adulthood. Therefore, the task-specific effects of gonadectomy and sex observed here (e.g., gonadectomy-induced impairments in cognitive flexibility in the MWM, female-skewed spatial retention errors in the BM) likely reflect the combined influences of age-related sex differences in motivation, stress reactivity, spatial processing, and the organizational and activational effects of gonadal steroids on hippocampal structure and function (Conrad et al., 2004; Grissom et al., 2013; LaBuda et al., 2002; Lamberty & Gower, 1988; Mishima et al., 1986). Taken together, these findings suggest that males and females are equally vulnerable to the enduring effects of pubertal immune challenge on hippocampus-dependent cognition, which appear to occur independently of circulating gonadal hormones (see Study 1; Kolmogorova et al., 2019).

Adult hippocampal cell development is sensitive to pubertal immune challenge

The enduring cognitive effects of pubertal immune challenge may arise in part from sex differences in LPS-induced changes to learning-associated cellular processes in the hippocampus (see Study 1; Kolmogorova et al., 2019). Pubertal LPS treatment has sex-specific outcomes on

baseline cellular proliferation in the adult (i.e., 17-week-old) cornus ammonis (CA)1 sub-region of the dorsal hippocampus, a region involved in episodic-like memory and the detection of spatial novelty (Barbosa et al., 2012; Drieskens et al., 2017). Ki67^{+} cell expression in the adult CA1 was significantly enhanced among gonadally-intact LPS-treated males relative to their saline-treated and orchiectomized counterparts; these effects of treatment and gonadectomy on cellular proliferation in the CA1 were absent in age-matched females (see Study 1; Kolmogorova et al., 2019). Contrary to our expectations, both sexes were resilient to LPS-induced changes in baseline hippocampal neurogenesis (i.e., DCX^{+} cell expression) and cellular proliferation (i.e., Ki67^{+} cell expression) in the adult (i.e., 17-week-old) SGZ regardless of gonadal status (see Study 1; Kolmogorova et al., 2019). It is possible that the LPS-induced effects on cell development in the SGZ, if any, may have recovered by the time of euthanasia (for reviews see Hueston et al., 2017; Loi et al., 2014; Lucassen et al., 2015).

The sexually dimorphic pubertal brain

Several baseline region-specific sex differences were observed during puberty across interconnected brain regions involved in cognition and stress regulation. Young (i.e., six- and seven-week-old) CD-1 mice display significant sex differences across the hippocampus, prefrontal cortex (PFC), and the hypothalamus in microglial density and morphology (see Study 2; Kolmogorova et al., 2021) and expression of regulatory proteins for synaptic plasticity and oxidative stress (see Study 3). Such baseline sex differences in brain structure, neurophysiology, and interconnectivity with neural systems inherently place certain sex biases in stress sensitivity and outcomes of stress exposure (Brivio et al., 2020; Choleris et al., 2018; Grabowska, 2017; Wellman et al., 2018). Across the brain regions examined in this thesis, the considerable stress vulnerability of the hippocampus likely stems from its propensity for neurogenesis, high

expression of receptors for stress hormones, and close structural and functional links with the central stress response system (McEwen et al., 2016; Osborne et al., 2015).

Innate sex differences in the pubertal neuroimmune system

The phagocytic properties of the brain's resident immune cells, microglia, are key to the shaping of neural circuits during critical periods of neurodevelopment like puberty (Lenz & Nelson, 2018; Paolicelli et al., 2017; Tay et al., 2017; VanRyzin et al., 2018). Developmental sex differences in cell density, phenotype, and function (e.g., Crain et al., 2013; Hanamsagar et al., 2017; Li et al., 2018; Villa et al., 2018) facilitate the sexual differentiation of the brain during early life VanRyzin et al., 2020). Given that microglial changes during puberty have been relatively overlooked compared to earlier critical periods, this work examined baseline sex differences in microglial expression and phenotype across brain regions for cognitive functioning and stress regulation.

Microglia undergo several sex- and region-specific changes to density and morphology spanning from puberty through adulthood (See Study 2; Kolmogorova et al., 2021). Young CD-1 mice show several sex differences in microglial morphology in the hippocampus, medial prefrontal cortex (mPFC), and the paraventricular nucleus (PVN), as well as a male-skewed expression of microglia in the hippocampus. Microglial expression temporarily increases in the mPFC of female mice at seven weeks of age. Although the sex difference in hippocampal microglial density disappears by adulthood (i.e., ten weeks of age), divergences in microglial density in the mPFC among the adult mice result in significantly lower microglial expression among adult females relative to their male counterparts. Several sex and region differences in microglial morphology continue into adulthood. These developmental sex differences in microglial expression and morphology are likely secondary to sex differences in age of pubertal

onset and the organizational and activational effects of the ensuing influx of androgenic and estrogenic steroids secreted by the testes and the ovaries (for reviews see Sierra et al., 2008; Herbison, 2016; Sisk & Foster, 2004; Vigil et al., 2011).

Systemic LPS alters pubertal microglial density and morphology in a sex-specific manner

Microglia, particularly during critical periods of development, are vulnerable to stress-induced changes to expression, structure, and functioning (e.g., Bekhbat et al., 2021; Holder & Blaustein, 2017; Püntener et al., 2012; Qin et al., 2007; Velez-Perez et al., 2020). Immune-mediated perturbations to microglia can dysregulate the homeostatic properties of this cell group (i.e., immune defense, development and maintenance of neurocircuitry) and propel microglia towards cytotoxic inflammatory activity (e.g., Cunningham et al., 2005; Qin et al., 2007). Maladaptive microglia-mediated inflammation is increasingly implicated in the onset and progression of a growing list of brain disorders such as depression, anxiety, and neurodegenerative conditions such Alzheimer's disease and Parkinson's disease (Cunningham, 2013; Madore et al., 2020; Mondelli et al., 2017; Wohleb, 2016). Sex differences in microglia (e.g., Bollinger et al., 2016; Erickson et al., 2018; Liu et al., 2019; Lively et al., 2018) are believed to contribute to sexual dimorphisms in some of these neuroinflammation-mediated conditions (Audet, 2019; Kokkosis & Tsirka, 2020). Therefore, this thesis then examined sex differences in the pubertal microglial responses to systemic LPS.

As expected, systemic LPS during the stress-sensitive pubertal period alters microglial expression and morphology in a sex-specific manner (see Study 2; Kolmogorova et al., 2021). LPS-treated mice show female-specific changes to microglial morphology in the mPFC and the hippocampus during sickness (i.e., 24 h post-treatment). Despite symptomatic recovery from LPS-induced sickness, several treatment effects on microglia persist past LPS-induced sickness.

Microglia in the PVN of LPS-treated females transition to a more “activated” state one week post-treatment. Pubertal LPS also eliminates baseline sex differences in expression and morphometric characteristics of microglia in the hippocampus and the PVN of seven-week-old mice. Adult (i.e., 10-week-old) mice exposed to LPS during puberty show female-specific morphometric changes to microglia in the mPFC and in the hippocampus, as well as elimination of the sex difference in microglial density in the CA3 sub-region of the hippocampus.

The apparent female bias in LPS-induced changes to pubertal microglia may be attributed to baseline microglial sex differences and the temporary female-specific increase in whole-brain BBB permeability during LPS-induced sickness (see Study 2; Kolmogorova et al., 2021). This global BBB disruption appears to result from the additive effects of non-significant LPS-induced increases in BBB permeability across brain regions. Although females also show a small but significant increase in caspase-3-dependent apoptosis of hippocampal neuronal and non-neuronal cells during LPS-induced sickness, the functional implications of this sex-specific increase in BBB permeability are unclear from this work. BBB disruption is a double-edged sword – infiltrating peripheral immune factors (e.g., pro- and anti-inflammatory cytokines, peripheral leukocytes) can assist with wound healing and debris clearance in the brain but also increase intracranial pressure and initiate cytotoxic inflammatory processes that harm surrounding tissues (Varatharaj & Galea, 2017). Additional work is needed to confirm whether this sex-specific treatment effect on BBB permeability confers an advantage or disadvantage to pubertal females in their neuroinflammatory responses to a systemic immune challenge.

Functional outcomes of sex-specific pubertal neuroimmune-neurovascular interactions

Given the extensive neuroplasticity and sex-specific stress sensitivity of the pubertal brain, microglia-mediated inflammatory processes may permanently alter the structure and

functioning of various systems (Nelson & Gabard-Durnam, 2020; Paolicelli & Ferretti, 2017). Pubertal immune challenge appears to expedite certain pubertal neurodevelopmental processes in a sex- and region-specific manner (see Study 3). Pubertal LPS treatment significantly increases baseline hippocampal levels of the synaptic scaffolding protein postsynaptic density (PSD)-95 in males one week post-treatment. In considering the concomitant treatment-induced changes to microglia (Kolmogorova et al., 2021), a pubertal immune challenge may promote microglia-mediated neurodevelopment of the pubertal hippocampus in males. On the other hand, the pubertal LPS treatment is not sufficient to alter baseline expression of regulatory proteins in inflammation, apoptotic cell death, and oxidative stress in brain regions relevant to stress regulation (i.e., hypothalamus) and cognition (i.e., hippocampus, prefrontal cortex, cerebellum).

Limitations and Suggested Future Directions

These findings should be considered in the context of several notable limitations. Although all studies were completed with the ethical use of animals in mind, we cannot underestimate the potential implications the smaller sample sizes may have on the findings (e.g., low statistical power arising from small sample size may overestimate the true effect size of a statistically significant finding) (Button et al., 2013). Second, our understanding of the enduring effects of pubertal LPS treatment on hippocampus-dependent cognition during adulthood (see Study 1; Kolmogorova et al., 2019) is limited by the testing approach. Notably, it is unknown whether there was a carryover effect between the tests. Given the duality in microglia-mediated inflammatory responses (i.e., neurotoxic and neuroprotective functions) (Giordano et al., 2021; Loane & Kumar, 2015), the functional implications of sex-specific microglial responses to pubertal immune challenge are unclear (see Study 2; Kolmogorova et al., 2021). Follow-up work examining the expression profiles of LPS-induced changes to microglia is needed to delineate

phenotypic polarization (i.e., the classical/pro-inflammatory M1 and alternative/repair-related M2 states of microglial activation) (Chhor et al., 2013; Cunha et al., 2016; Jablonski et al., 2015). Likewise, the functional impact of the residual effects of pubertal LPS-induced sickness on hippocampal cell development (see Study 1; Kolmogorova et al., 2019) and PSD-95 expression (see Study 3) is unknown. Future studies can also build upon this work and explore sex and treatment differences in other aspects of cellular processes relevant to hippocampus-dependent cognition (e.g., integration and activation of hippocampal neurons). Lastly, while circulating gonadal hormones are thought to play a role in some of the sex-specific outcomes of pubertal immune stress, this relationship was not tested consistently across studies. Therefore, it is unclear to what degree peripheral gonadal hormones and their cyclical fluctuations influence the effects of pubertal immune challenge on the neuroimmune network (see Study 2; Kolmogorova et al., 2021) and cellular pathways relevant to hippocampus-dependent cognition (see Study 3).

Implications

This dissertation offers a timely investigation into sex differences in the hippocampus-dependent cognitive effects of immune stimulation during the stress-sensitive pubertal period. Given the general under-representation of females in the animal literature, this dissertation offers an important contribution to the field by examining how sex influences the susceptibility towards immune changes in brain and behaviour in the context of a multi-factorial examination of the sexually dimorphic and highly complex neuroimmune response to systemic LPS. The temporary and residual outcomes of pubertal LPS treatment reported here underscore the sex differences in stress coping and resiliency during this critical period of development. The sexually dimorphic stress vulnerability of the pubertal brain to the effects of a systemic immune challenge,

particularly of the hippocampus, is well demonstrated by the LPS-induced changes to pubertal microglia (see Study 2; Kolmogorova et al., 2021). The sex differences in neuroimmune responses during puberty provide a framework for the pathogenesis of sex biases in immune-mediated disorders of the brain and behaviour. In addition to expanding our mechanistic understanding of the sex-specific outcomes of pubertal immune challenge, this doctoral thesis highlights the considerable vulnerability of the pubertal brain to immune-mediated perturbations in the neuroimmune system and hippocampus-dependent cognitive systems. The enduring outcomes of pubertal immune challenge on hippocampus-dependent cognition and relevant brain systems may also help explain the predisposition or exacerbation of immune-mediated disorders of brain and behaviour that emerge during puberty or early adulthood but do not originate in the pre-/post-natal periods or in adulthood.

Summary

This dissertation addresses the important but often overlooked issue of sex differences in the cognitive effects of immune stimulation during critical periods like puberty. Above all else, these novel findings highlight innate sex differences in the pubertal neuroimmune network that appear to precipitate sex biases in immune-mediated disorders of the brain and behaviour during adulthood. Pubertal male and female CD-1 mice show similar vulnerabilities to the enduring effects of systemic LPS on hippocampus-dependent cognition (see study 1; Kolmogorova et al., 2019), whereas the longstanding effects of pubertal LPS on hippocampal cell development are unique to males (see study 1; Kolmogorova et al., 2019). Baseline pubertal sex differences in microglial density and morphology across brain regions for cognition and stress regulation impose innate sex- and region-specific stress vulnerabilities to microglia-mediated inflammatory responses (see study 2; Kolmogorova et al., 2021). Females are uniquely vulnerable to the acute

effects of systemic LPS on the pubertal neuroimmune network (i.e., whole-brain BBB disruption, increased caspase-3-dependent apoptosis in the hippocampus, and changes to microglial density and morphology). The residual effects of LPS-induced sickness on microglial expression and morphology highlight the various sex and region differences in stress sensitivity of these cells. In the context of microglial responses, systemic LPS treatment during puberty appears to accelerate certain neurodevelopmental processes in males but not in females (see studies 2 and 3; Kolmogorova et al., 2021). Increasing evidence of the mediating role of immune stressors in the pathogenesis of sexually dimorphic immune-based disorders of the brain (e.g., depression, Alzheimer's disease) warrant further investigation of the sexually dimorphic stress-vulnerable pubertal period in this body of literature (Bilbo et al., 2011; Tay et al., 2018; Wellman et al., 2018).

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