SEX-SPECIFIC MECHANISMS OF PUBERTAL STRESS-INDUCED INHIBITION OF THE HPG AXIS.

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Abbreviations

ACTH	Adrenocorticotropic Hormone
AP1d	Activator Protein-1
AR	Androgen Receptor
ARC	Arcuate Nucleus
AVP	Arginine Vasopressin
AVPV	Anteroventral Periventricular Nucleus
CA1/2/3/4	Cornu Ammonis
CD4	Cluster of Differentiation
CORT	Corticosterone/Cortisol
CRH	Corticotrophin-Releasing Hormone
E_2	Estradiol
ERα	Estrogen Receptor-alpha
ERβ	Estrogen Receptor-beta
FKBP51	FK506 Binding Protein 51
FSH	Follicle Stimulating Hormone
GABA	Gamma-Aminobutyric Acid
GDX	Gonadectomy
GH	Growth Hormone
GnRH	Gonadotropin-Releasing Hormone
GR	Glucocorticoid Receptor
HPA	Hypothalamic-Pituitary-Adrenal Axis
HPG	Hypothalamic-Pituitary-Gonadal Axis
IFNγ	Interferon Gamma

IL1β	Interleukin 1 beta
IL6	Interleukin 6
IL10	Interleukin 10
IL12	Interleukin 12
Kiss1	Kisspeptin Peptide
Kiss1-/-	Kisspeptin Knockout Mouse
Kiss1R	Kisspeptin Receptor
KP	Kisspeptin Treatment
KNDy	Kisspeptin, Neurokinin B, and Dynorphin Neurons
LH	Luteinizing Hormone
LPS	Lipopolysaccharide
ME	Median Eminence
mPOA	Medial Preoptic Area
mRNA	Messenger Ribonucleic Acid
NF-KB	Nuclear Factor Kappa B
PND	Post-Natal Day
P_4	Progesterone
PR	Progesterone Receptor
PVN	Paraventricular Nucleus
RP3V	Rostral Periventricular Area of the 3rd Ventricle
T	Testosterone
TLR4	Toll-like Receptor 4
TNFα	Tumor Necrosis Factor alpha

Abstract

Puberty is a critical period of development that is characterized by significant remodelling and reorganization of neuronal connections. Additionally, this period is marked by the transition to a fertile state and the development of secondary sex characteristics driven by a surge in gonadal steroid hormones. Puberty is also vulnerable to stress exposure, as pubertal stress during this period results in negative enduring changes to the brain and behavior. Treatment with the bacterial endotoxin lipopolysaccharide (LPS) results in enduring dysfunction of the hypothalamic-pituitary-gonadal (HPG) axis in male and female mice. However, the mechanism underlying this dysfunction was unclear. Thus, this thesis was designed to investigate possible mechanisms through which pubertal stress could alter HPG axis functioning. This work first examined the acute effects of LPS treatment on various components of the HPG axis, such as kisspeptin (Kiss1) and kisspeptin receptor (Kiss1R) expressions in the brain, and luteinizing (LH) and follicle stimulating hormone (FSH) concentrations in the blood (Study 1). The next study examined the enduring effects of LPS treatment on inflammation as well as on Kiss1 and Kiss1R expressions in the brain, and LH and FSH concentrations in the blood (Study 2). The findings showed that Kiss1 and Kiss1R were downregulated in an acute and enduring manner following LPS treatment and are likely responsible for HPG axis downregulation. The final study amalgamates the novel discoveries of the previous findings and hypothesizes that pharmaceutical Kiss1 treatment will prevent adult sexual behavior dysfunction after pubertal LPS treatment. Findings from this study will inform us whether the enduring HPG axis downregulation following pubertal LPS treatment is reversible in adulthood and could provide a viable prospective intervention for fertility complications across species.

Chapter 1: Conceptual and Theoretical Background

1. Pubertal Development

Throughout the lifespan, there are several critical periods of development characterized by major neuronal and behavioral changes (Bronson & Rissman, 1986; Kane & Ismail, 2017; Sisk & Foster, 2004). Critical periods of development are marked by increased neuronal plasticity which shapes the neural and behavioral phenotypes according to genetic predisposition and environmental influences (Nesse & Stearns, 2008; Ruf, 1973). Puberty is one such example of a critical developmental period. The terms puberty and adolescence are often used interchangeably; however, they represent two fundamentally distinct phases of development. Puberty occurs at the beginning of adolescence and involves the transition from a non-reproductive to a reproductive state (Sisk & Foster, 2004). Adolescence refers to the time between the onset of puberty and adulthood, and includes physical, psychological, and emotional development (Boxer et al., 1983; Romeo, 2005; Sisk & Foster, 2004). Puberty and adolescence are both periods of significant physiological and neurological changes (Levitt, 2003; Schulz et al., 2009). These changes are necessary as they establish reproductivity and the transition to adulthood.

Puberty begins earlier in females than in males. In female mice, the onset of puberty is marked by vaginal opening and occurs around postnatal day (PND) 30 (Furness et al., 1970; Kane & Ismail, 2017; Vandenbergh et al., 1972). In male mice, the onset of puberty is marked by preputial separation and occurs around PND 35. Puberty is complete upon sperm production (around PND 50) in males and the first estrus (around PND 60) in females (Kane & Ismail, 2017; Mckinney & Desjardins, 1973). The timing of puberty can vary depending on environmental conditions, such as housing and socioeconomic status, or genetic factors such as, species, strain, or heritable disorders, like central precocious puberty (Antoniazzi & Zamboni, 2004; Corley et

al., 2015;Vandenbergh et al., 1972). Although the timing of puberty is sex-dependent, puberty is initiated by the activation of the hypothalamic pituitary gonadal (HPG) axis in both sexes.

2. The HPG-axis

The HPG-axis is the neuroendocrine system responsible for stimulating the production of sex hormones (McCann & Porter, 1969; Oyola & Handa, 2017). Kisspeptin (Kiss1) initiates HPG axis activity and pubertal development. Kiss1, formerly known as metastin, is a neuropeptide coded by the KISS1 gene, and it is produced in the arcuate nucleus (ARC) and in the anteroventral periventricular nucleus (AVPV) (Lee et al., 1996; Navarro et al., 2009). Kiss1 and its receptor (Kiss1R) are abundantly located within the arcuate nucleus (ARC), the medial preoptic area (mPOA), the anteroventral periventricular nucleus (AVPV), the rostral periventricular nucleus of the 3rd ventricle (RP3V), and in the paraventricular nucleus of the hypothalamus (PVN) (Lehman et al., 2013). Additionally, Kiss1R has also been found in the basal ganglia, amygdala, substantia nigra, and in the hippocampus (Gottsch et al., 2004; Herbison et al., 2010). The density of Kiss1 producing neurons is sexually dimorphic, such that females have greater density of Kiss1 neurons in the AVPV and in the mPOA than males (Clarkson & Herbison, 2006). Kiss1R expression is greatest amongst gonadotrophin-releasing hormone (GnRH) producing neurons, suggesting that GnRH neurons are Kiss1's primary site of action (Lehman et al., 2013). The activity of the HPG axis is maintained by the pulsatile release of GnRH, which is synthesized in the hypothalamus (Yin & Gore, 2010) and contained in highest abundance within the arcuate nucleus and in the mPOA (Clarke & Pompolo, 2005). Axonal projections from GnRH neurons from the median eminence deliver GnRH into the hypophyseal portal system towards the anterior pituitary gland (Burgus et al., 1972; Schally et al., 1971). Upon reception of vascular GnRH, the anterior pituitary gland produces and secretes

the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), into the bloodstream. In males, LH acts on its receptors in the Leydig cells of the testis and controls testosterone (T) production and secretion (Mendis-Handagama, 1997; Roozendaal et al., 1997; Simoni et al., 1999). Increasing concentrations of T in the blood inhibit HPG axis activity by suppressing the production of GnRH, LH and FSH via negative feedback loop (Howles, 2000). FSH, however, carries out its action primarily through the Sertoli cells to initiate spermatogenesis and testicular growth (Creasy & Chapin, 2018; Howles, 2000). In females, a spike in LH induces ovulation and regulates the menstrual cycle, whereas FSH matures the ovarian oocyte into a Graafian follicle, which produces increasing levels of estradiol (E_2) prior to ovulation and increasing levels of progesterone (P_4) following ovulation (Howles, 2000; Kumar & Sait, 2011; Schally et al., 1971). Increasing concentrations of E_2 and P_4 in the blood inhibit HPG axis activity, similarly to T in males, via negative feedback loop (Messinis, 2006; Tilbrook & Clarke, 2001).

Disruptions in Kiss1 signaling impacts reproduction and can lead to infertility and hypogonadism. Kiss1 knockout (Kiss1-/-) male mice show reduced T, LH and FSH concentrations in the blood and experience infertility due to reduced mounting attempts and lordosis quotient and spermatogenesis (Lapatto et al., 2007; Nakamura et al., 2016; Tassigny et al., 2007). Kiss1 -/- male rats displayed less mounting, intromission, and ejaculation than wild type rats (Nakamura et al., 2016). T treatment in adult Kiss1 -/- males only slightly increased the frequency of mounting behavior and had no effect on intromission and ejaculation. Interestingly, pre-pubertal T treatment in Kiss1 -/- male rats restored mounting attempts, although intromission and ejaculation remained low. These findings extend to humans where Kiss1 treatment increased T and LH concentrations in adult men (George et al., 2015). In females, on the other hand,

lordosis quotient was reduced in Kiss1 -/- rats and E_2 treatment failed to resume lordosis quotient to levels seen in wild type rats (Nakamura et al., 2016). Intracerebral infusions of Kiss1 significantly increases LH and FSH concentrations in the blood of female rats, indicating that Kiss1 has direct control over gonadotropin concentrations (Roa et al., 2008). Kiss1 -/- female mice display significantly smaller ovaries and uterus and fail to progress normally through the estrous cycle compared to wild type mice (Nakamura et al., 2016; Tassigny et al., 2007). These findings implicate Kiss1 as integral to HPG axis propagation.

3. Androgens

Androgens are a class of sex hormones that play an important role in the development of the male reproductive system and secondary sexual characteristics (Davey & Grossmann, 2016). The biosynthesis of sex hormones begins with cholesterol, a C27 steroid, aptly named from the number of carbon atoms making up its chemical composition - $C_{27}H_{46}O$ (Chaudhuri et al., 1969). Over a series of enzymatic conformations, cholesterol is converted to pregnenolone, and then to 17α-OH pregnenolone before becoming the C19 androgen, dehydroepiandrosterone, which is a precursor to androstenedione or androstenediol. These two androgens undergo conformation changes to become T, which can be further converted to dihydrotestosterone (Burger, 2002; Gloyna & Wilson, 1969; Miller, 1998). T is a well-known androgen that plays a direct role in affecting pubertal development, including, but not limited to, increased muscle mass and bone density, sexual behavior, secondary sex characteristics, spermatogenesis and HPG axis feedback (Hammes & Levin, 2019). Leydig cells in the testes begin T production after being activated by LH (Shima et al., 2013; Zirkin & Papadopoulos, 2018). Although the testes and ovaries are the central site of production, androgens are also synthesized in the adrenal glands, the liver, and the brain (Cunningham et al., 2012; Zirkin & Papadopoulos, 2018). The effects of

androgens are enacted by binding to the androgen receptor (AR) which is located throughout the body and the brain. The AR translocates into the nucleus to activate the androgen response element, which alters gene expression to enact long-lasting changes such as protein production, or secondary sex characteristics (Cunningham et al., 2012; Zuloaga et al., 2008). AR also resides on the membrane, where it enacts immediate cellular changes, such as the influx of calcium ion into the cellular membrane (Foradori et al., 2008; Solakidi et al., 2005). T is primarily found in males and is produced at a rate of 20x greater than females (Hammes & Levin, 2019). In females, androgens are produced in the ovaries and in the adrenal glands (Burger, 2002). Aside from a precursor to estrogen production, the precise role of female T is currently unclear. However, androgen deficient women show sexual dysfunction, lower body mass, and reduced cognitive function, implying that it may be involved in mediating many distinct physiological functions (Horstman et al., 2012; van Geel et al., 2009). Although androgens contribute to physiological function in females, estrogens are considered the primary sex hormone.

4. Estrogens

Estrogens are a C18 class of sex hormones that develop the female reproductive system and secondary sexual characteristics (Barakat et al., 2016; Jensen, 1962). The biosynthesis of estrogens follows the same pathway from cholesterol as T and dihydrotestosterone, however, they require the presence of an aromatic six carbon ring that other endogenous steroid hormones lack (Cui et al., 2013). The conversion from a non-aromatic to an aromatic steroid is done via the enzyme aromatase (Cui et al., 2013; Wilds & Djerassi, 1946). The estrogens, estrone and E_2, are synthesized from androstenedione and T, respectively. E_2 is the most common of the estrogen hormones (Hamilton et al., 2017). Females produce roughly four times more E_2 compared to males, which are used to drive sexual behavior, the formation of secondary sex characteristics,

folliculogenesis, and the maintenance of the ovarian cycle, and pregnancy (Bordini & Rosenfield, 2011). In both males and females, estrogens are synthesized in the gonads, and also in extragonadal sites such as adipose and bone tissue, the kidneys, and the brain (Blakemore & Naftolin, 2016; Schulster et al., 2016). The gonadal production of estrogens occurs in the ovaries and is carried out via androgen synthesis in thecal cells, followed by aromatase conversion in the granulosa cells before being released into peripheral circulation (Cui et al., 2013; Hillier, 1994). In males, estrogens are produced in the aromatase containing Leydig and Sertoli cells of the testes (Carreau et al., 2011; Dorrington et al., 1978). Male estrogens control spermatogenesis and sperm maturation, bone health and growth, and lipid metabolism (Hammes & Levin, 2019; Hess, 2003). Estrogens exert their actions by binding to estrogen receptor alpha (ERα) and beta (ERβ) located throughout the body, although binding has been experimentally observed at other sites, such as the g-protein regulated receptor-30, and estrogen receptor X (Naftolin et al., 1971; Prossnitz et al., 2007). ERα are located in the uterus, breasts, adipose tissue, the ovarian theca and interstitial cells, and testicular Leydig cells, whereas ERβ are located in the lungs, bladder, granulosa cells of the ovary and prostate (Paterni et al., 2014; Prossnitz et al., 2007). Both receptors are expressed throughout the entire brain (Azcoitia et al., 2011; Wright et al., 2010). Analysis of the two receptors show major overlapping functions, however, the two receptors are distinct from one another (Jia et al., 2015; Morissette et al., 2008). In animal models, ERα knockout mice are infertile, cannot ovulate, show reduced prolactin expression, have immature mammary glands, show reduced bone length, and are obese (Chen et al., 2009). On the other hand, ERβ knockout mice show overlapping phenotypes with ERα knockout mice including reduced fertility, and ovulatory and oocyte production dysfunctions (Curtis Hewitt et al., 2000).

Aggression and depression-like symptoms are elevated in both ERα and ERβ knockout female mice (Faulds et al., 2012; Messinis, 2006; Syed et al., 2007).

5. Progestogens

Progestogens are major class of C21 steroid hormones and the most closely related to cholesterol in steroid hormone synthesis (Conneely et al., 2002; Siiteri et al., 1977). Once cholesterol is transformed to the progestogen pregnenolone, it is enzymatically converted to P_4. Pregnenolone – and its alternative downstream conformation - 17a hydroxy pregneninolone –can be converted to progestogen 17a-hydroxy P_4 (Miller, 1998). P_4 is synthesized in the ovaries, uterus, adrenal and mammary glands, and the placenta during pregnancy, as well as throughout the brain. It is bound throughout the body by P_4 receptor (PR)-A or -B isoforms (Graham & Clarke, 1997). The P_4 receptors require ERα as a coactivator to regulate transcription (Thomas & Gustafsson, 2015). It is difficult to differentiate between the receptors since they share a similar protein structure and are expressed by the same gene, however, PR-A has an inhibitory influence over PR-B, as well as the estrogen receptors (Conneely et al., 2002; Graham & Clarke, 1997). P_4 regulates oogenesis, maintains pregnancy, and mediates sexual responsiveness (Concannon et al., 1975; diZerega & Hodgen, 1981). Along with E_2, P_4 plays a major role in the ovarian cycle. In males, P_4 is synthesized within the testes and the adrenal glands, where it functions to increase sperm cell motility and binding of spermatozoa to the oocyte and decrease sexual behavior (Andersen & Tufik, 2006; Calogero et al., 2000). In addition to their macro role in development, sex hormones and their receptors substantial impact kisspeptin and GnRH neurons regulation of the HPG axis (Moore et al., 2018; Moore et al., 2018).

6. KNDy Hypothesis

The hypothalamic “KNDy” neurons - kisspeptin, neurokinin B, and dynorphin - regulate the pulsatile release of GnRH (Moore et al., 2018). Neurokinin B is a neuropeptide of the tachykinins family that is produced within the hippocampus, bed nucleus of the stria terminalis, amygdala, mPOA and the ARC. On the other hand, dynorphin is an endogenous opioid neuropeptide located in the hippocampus, amygdala, hypothalamus, and spinal cord, and binds to the kappa opioid receptor throughout the central and peripheralαl nervous system (Knoll & Carlezon, 2010). Neurokinin B and dynorphin neurons regulate the HPG axis and GnRH release via a feed forward mechanism on kisspeptin producing neurons (Navarro, 2013; Navarro et al., 2009). More specifically, neurokinin B increases pulse frequency of kisspeptin neurons onto GnRH neurons, while dynorphin inhibits kisspeptin neurons (Navarro, 2013; Navarro et al., 2009). The effects of neurokinin B and dynorphin on kisspeptin neurons are regulated in a sex-dependent manner by sex hormones, as evidenced by the high colocalization of ERα, AR and PR within the KNDy regions (Moore et al., 2019). Females display greater colocalization between KNDy neurons and ERα in the periventricular and medial preoptic nuclei of the hypothalamus. In contrast, male mice displayed reduced KNDy and ERα colocalization in every examined brain region except the PVN (Lehman et al., 2013; Moore et al., 2019). Although information on AR is sparse outside of descriptive colocalization data, it appears that the AR is responsible for increasing dynorphin activity, but not as potently as both the AR and ERα (Ruka et al., 2016). In ovariectomized female mice, GNRH secretion and kisspeptin expression was significantly decreased within hypothalamic KNDy neurons, however, E_2 treatment restores these molecules to levels seen in intact mice (Smith et al., 2005). In male mice, neither sham surgery, GDX or GDX with T replacement alters kisspeptin mRNA expression within the KNDy cells (Dudek et

al., 2017). However, GDX males treated with both E_2 and T show increased dynorphin firing, suggesting that E_2 is a critical component of kisspeptin regulation within both male and female mice (Ruka et al., 2016). Taken together, these results suggest that sex hormones directly mediate KNDy neuronal activity, but in a sex-dependent manner.

7. Organization-Activation Hypothesis

Sex differences in brain and behavior are mediated in part by the HPG axis, as the release of sex hormones organizes and activates neurons in a sex-specific manner and leads to sex-specific behavior (McCarthy, 2016; Phoenix et al., 1959). This notion was initially exemplified in a study where female guinea pigs exhibited masculine characteristics in adulthood after receiving prenatal T treatment (Phoenix et al., 1959). The observation that exposure to sex hormones early in life have such a profound effect later in life led to the formulation of the organizational-activational hypothesis. This hypothesis states that exposure to sex hormones during critical periods of development early in life (i.e. prenatal, perinatal and pubertal periods) permanently organizes tissue to respond to transient activation by sex hormones in adulthood (Arnold, 1985). These effects are not simply limited to physical changes, like the development of sex organs, gamete production, or relative sex hormone levels, but also extend to sexually-differentiated brain circuits and behavior (McCarthy, 2016; McCarthy et al., 2012). For example, in the brain, greater basal numbers of astrocytes are found in the CA1 and dentate gyrus regions of the hippocampus in males compared to females (Kolmogorova et al., 2019). Additionally, LPS-treated pubertal females show poorer spatial memory performance in the Morris water maze in adulthood compared to males (Kolmogorova et al., 2019). Interestingly, female mice treated with LPS do not show alteration in hippocampal cellular proliferation. Male mice, on the other hand, show greater cell proliferation after LPS treatment compared to their saline counterparts

(Kolmogorova et al., 2019). These innate sex-specific physiological and behavioral alterations can in part be explained by the organizational and activational effects of sex hormones. In other words, similar treatment with disparate outcomes between sexes heavily implicates sex hormones as the primary influencing factor, either at the organizational or activational level. Together, these findings exemplify the sexually dimorphic effects of sex hormones on brain, body, and behavior, and support the tenets of the organization-activation hypothesis (Phoenix et al., 1959). Further examples of sexual dimorphism can be identified in HPA axis activation and in the ensuing stress response.

8. The HPA axis

In addition to the HPG axis, the HPA axis also undergoes maturation during puberty. As a major component of the neuroendocrine system, the HPA axis is the primary neural circuitry involved in responding to stress and maintaining homeostasis within the body. Upon stress exposure, the parvocellular neurons in the paraventricular nucleus of the hypothalamus (PVN) initiate the production and release of corticotrophin-releasing hormone (CRH) Heck & Handa, 2019; Herman & Cullinan, 1997). Once CRH travels through the hypophyseal portal system toward the anterior pituitary, adrenocorticotropic hormone (ACTH) is secreted into the bloodstream. The peptide arginine vasopressin (AVP) is released in conjunction with ACTH to enhance the stress response. ACTH travels to the adrenal cortex where it stimulates the release of glucocorticoids, like corticosterone or cortisol in rodents and humans, respectively (Herman & Cullinan, 1997). Glucocorticoids mobilize cellular defense mechanisms for action, operating through negative feedback within the PVN, anterior pituitary, and the hippocampus (Sapolsky et al., 2000; Tasker et al., 2006; Tasker & Herman, 2011).

The intracellular glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) respond to glucocorticoids with either a slow (genomic) or fast (non-genomic) response (Meaney et al., 1989; Sapolsky & Meaney, 1986). The fast response is attributed to quick HPA axis inhibition, and commonly occurs in brain regions that have a high density of GR like the hippocampus and the amygdala, which are primarily involved in fear-memory formation (Goldstein, 1965; LeDoux et al., 1990). Glucocorticoid binding to intracellular GR in the PVN elicits decreases glutamate signalling and increases GABA signaling, resulting in a rapid cessation of CRH-releasing neuron activity (Di et al., 2003). In contrast, the slow feedback response alters the genome of neurons within the anterior pituitary and the PVN. In the slow response, GR are chaperoned into the nucleus by heat shock protein 90 and FKBP51 protein. Genomic changes occur by downregulating transcription of ACTH precursor, pro-opiomelanocortin, and CRH, in the anterior pituitary and PVN, respectively (Gjerstad et al., 2018; Tsai et al., 1988; Zarzer et al., 2013). Following exposure to chronic heterotypic stressors, the density of GR decreases and the HPA axis becomes more sensitive to stress (Herman et al., 1995; Ridder, 2005). Dysfunction ensues as HPA axis activity increases in frequency and intensity upon stress exposure (Bachmann et al., 2005; Juruena et al., 2003). Genetically altered mice expressing less GR than wild-type mice show increased levels of learned helplessness, a symptom of depression-like behavior, and greater CORT levels (Ridder, 2005). It is suggested that HPA axis dysfunction could be linked to a compensatory mechanism, where greater CORT concentration would be necessary to inhibit the HPA axis (Pariante, 2006; Pariante et al., 1995).

The stress-induced decrease in GR expression is especially harmful during critical periods of development where permanent HPA axis dysfunction may occur. For example, maternal separation stress in perinatal mice significantly decreases GR mRNA expression in the

amygdala, hippocampus and PVN in adulthood, and causes enduring anxiety-like behavior (Arnett et al., 2015). In addition to changes in GR expression, the production and release of stress hormones such as CRF and ACTH can also be permanently altered following early-life stress. For example, mice reared in impoverished environments display a downregulation of CRF mRNA expression in the PVN that persists into adulthood. As well, these mice show enduring increases in CORT concentrations and cognitive impairments (Rice et al., 2008). Moreover, maternally separated mice show enduring decreases in hypothalamic ACTH receptors, but greater basal ACTH concentration in adulthood compared to non-separated counterparts (Campana et al., 2021). These findings highlight important developmental changes; however, they do not consider the effects of sex on the HPA axis. Recent published findings indicate that there are sex differences in HPA axis reactivity (Heck & Handa, 2019; McCormick & Mathews, 2007).

9. Sex Differences in the HPA axis

During pubertal development, the influx of sex hormones organizes the HPA axis in a sex-dependent manner and leads to sex differences in HPA axis responsiveness (Romeo, 2005; Sisk & Foster, 2004; Young, 1996). For example, females show greater CORT concentration in the blood following LPS treatment compared to male counterparts (Cai et al., 2016; Girard-Joyal & Ismail, 2017). Chronic variable stress also causes increased sensitization of the CRF pathway in adult females, but not in males. Moreover, exposure to restraint stress increases GR density in the PVN of males, but not of females (Smith et al., 2021). The mechanism through which sex hormones affect the reactivity of the HPA axis is still unclear but may be related to the disparity in the concentrations of androgens and estrogens between males and females.

One way to examine the effect of sex hormones on HPA axis activity is to compare responses of gonadally intact animals to GDX animals. For example, gonadally intact female

mice show greater levels of basal and stress-activated CORT concentrations compared to males, however, this difference disappears in GDX females and GDX females given T (Goel et al., 2014; Spinedi et al., 1992). Adult female and GDX male rats show lower basal and post-LPS treatment GR mRNA expression in the PVN compared to intact male rats indicating that central GR expression may be moderated by sex hormones (Seale et al., 2004).

10. HPA-HPG Axis Interaction

Although sex hormones modulate HPA axis reactivity, the HPG axis only functions optimally during homeostasis (Rivier & Rivest, 1991). The HPG axis is downregulated when the HPA axis is activated in order to redirect and conserve the energy required to ensure survival during stressful experiences (Calogero et al., 2000). In most cases, the allostatic braking of the HPG axis is inconsequential and normal functioning resumes once the stressor has passed (Joseph & Whirledge, 2017; Panagiotakopoulos & Neigh, 2014). However, stressful experiences during critical periods of development run the risk of causing enduring dysfunctions in the HPG axis and in sexual behavior (Ludwig et al., 2019; Romeo, 2005). For example, pregnant dams who received cold-water and ethanol stress gave birth to offspring that showed sexual dysfunction in adulthood (García-Vargas et al., 2019). Moreover, male progeny of dams who received chronic restraint stress two weeks prior to giving birth show reduced peripheral T concentrations, increased testicular cell death, and reduced sperm quality in adulthood compared to non-stressed counterparts (Ward et al., 2003). Females, on the other hand, display lower E_2 concentrations during proestrus, longer estrous cycles, and have fewer pups than non-stressed counterparts (Cárdenas et al., 2013; García-Vargas et al., 2019). Puberty is another critical period of development during which exposure to stress can cause enduring changes in sexual behavior. For example, male and female CD1 mice exposed to shipping stress at 6 weeks of age, during a stress-sensitive pubertal period, display enduring sexual dysfunction that is not observed in mice

exposed to shipping stress at a younger or older age (Laroche et al., 2009b). Similarly, male and female mice exposed to an immune challenge at 6 weeks of age display reduced mount frequency and sexual receptivity, respectively (Laroche et al., 2009a), suggesting that in addition to the HPA axis, the HPG axis and sexual behavior are also modulated by the immune system. Our research in this domain has been extensive, with a particular focus on the timing of puberty-related milestones, such as vaginal opening in females and preputial separation in males.

In our laboratory, separate housing for male and female animals is standard practice to minimize the influence of pheromones on pubertal timing, aligning with Vandenbergh's findings indicating that exposure to pheromones help trigger the onset of puberty (1967). Under our housing condition, females typically begin puberty around 30 days of age, while males begin puberty around 35 days of age (Murray et al., 2023). Six-weeks of age in our mice represents a stress-sensitive period, as exposure to stressors like shipping, LPS, or chronic sleep disruption at that age, causes enduring alterations in reproductive and a numerous non-reproductive behaviors in males and females (Cai et al., 2016; Esposito et al., 2022; Kolmogorova et al., 2019; Kolmogorova & Ismail, 2021; Laroche et al., 2009a, 2009b; Murray et al., 2020, 2023; Sharma et al., 2018, 2019; Smith, 2021). These enduring behavioral changes are limited to stress exposure at 6 weeks of age in mice, as stress exposure at younger or older ages did not cause any enduring change in behavior (Laroche et al., 2009a, 2009b).

11. The Immune System

The immune system is composed of two functional components, the innate and the adaptive immune systems, both responsible for protecting the body against pathogens but differing in the timing of their actions and in their function. The innate immune system is responsible for quickly reacting to pathogens or bodily injury. Cells such as macrophages, granulocytes, and monocytes circulate in the blood stream and identify pathogen-associated

molecular patterns from foreign entities. When a pathogen is consumed by an antigen presenting cell, such as a macrophage, an antigen is displayed on the cells surface with a major histocompatibility complex (Mokhtari & Lachman, 2016). After receiving antigen RNA from the major histocompatibility complex, naïve CD4+ and CD8+ T cell lymphocytes of the adaptive immune system become specialized helper T cells that generate cytokines to destroy infected cells (Hansson et al., 2002). Cytokines are cellular messengers responsible for myriad functions such as, pro- and anti-inflammation, B-cell proliferation, chemoattraction, and apoptosis (Hermus & Sweep, 1990; Turnbull & Rivier, 1995). Unlike the passive nature of the innate immune system, the adaptive immune system actively seeks out pathogens via T and B lymphocytes (Pittman, 2011). Helper T cells secrete chemoattractant cytokines that signal macrophages to the pathogen, whereas B cells release antibodies that neutralize or destroy the pathogen (Hoebe et al., 2004; Kaiko et al., 2008). Activation of the innate and adaptive immune systems cause inflammation through proinflammatory cytokines as an attempt to recruit immune cells to fight the pathogen (Barton, 2008). The bacterial endotoxin lipopolysaccharide (LPS) generates a robust immune response (Cai et al., 2016; Ismail & Blaustein, 2013; Kolmogorova & Ismail, 2021; Murray et al., 2020; Smith et al., 2021). The innate immune system identifies LPS via the highly conserved toll-like receptor 4 with the aid of LPS binding protein myeloid differentiation factor 2 (Gioannini & Weiss, 2007). A cascade of intracellular mechanisms are triggered via the MyD88-dependent pathway which lead to the activation of proinflammatory transcription factors, such as nuclear factor-Kβ (NF-κβ; Jerala, 2007; Lu et al., 2008). NF-κβ-induced gene transcription causes the production and release of proinflammatory cytokines, such as interleukin (IL)-1, IL-2, IL-6, IL-8, IL-12 and tumor necrosis factor α (TNFα; Liu et al., 2017). The influx of proinflammatory cytokines is capable of activating the HPA axis and

triggering a stress response (Cain & Cidlowski, 2017; Dunn, 2007). In mice, IL-1β, IL-6 and TNFα stimulate the anterior pituitary to release ACTH in a similar fashion as CRF causing an increase in glucocorticoids. Immune cell glucocorticoid binding decreases inflammation by inhibiting inflammatory transcription factors NF-κβ, AP-1d and interferon-regulatory factor 3 (Cain & Cidlowski, 2017). Moreover, like the stress response, the immune response is also sexually dimorphic.

12. Sex Differences in Immune Response

Sex differences are ubiquitous in both the innate and adaptive immune system. For example, pubertal male mice display greater concentrations of IL-1β in the blood and greater IL-1β, IL6 and TNFα mRNA expressions in the medial prefrontal cortex (mPFC) and TNFα mRNA expression in the hippocampus two hours following LPS treatment compared to female counterparts (Sharma et al., 2018). However, 8 hours following LPS treatment, IL-1β, IL-6 and TNFα mRNA expression in the mPFC were greater in females compared to male counterparts (Sharma et al., 2019). There are also sex differences in adaptive immune responsivity. For example, GDX male mice show greater phosphatase of CD4+ T cells and IL-12 pro-inflammatory signalling compared to intact mice (Kissick et al., 2014; Klein & Flanagan, 2016). Furthermore, intact males show less resistance to fungal infections compared to GDX males and females. These findings indicate that T potently downregulates the adaptive immune system (Klein, 2000). Moreover, parasitic infection increases humoral T helper cell type 2 in E_2-treated mice, but not in GDX females and T-treated males (Hepworth et al., 2010). This finding complements the previous literature indicating that E_2 upregulates immune activity while T downregulates it (Klein & Flanagan, 2016). Differences in sex hormone concentration between males and females across the lifespan plays a significant role in modulating immune responses to

LPS and other immune challenges. Nevertheless, it is believed that sex hormones are critical for immune system development (Kane & Ismail, 2017).

Along with the HPA axis, puberty is also a critical period for immune system development (Kane & Ismail, 2017). It is likely that the age difference in immune response is linked to the influx of hormones during puberty. Cai and colleagues (2016) showed that LPS-treatment induces more pronounced sickness behavior and body temperature reduction in male mice compared to females. Additionally, adult males show greater sickness behavior and body temperature reduction than pubertal males and pubertal females. Furthermore, TNFα concentration in the blood is greater in adult male mice compared to adult females and pubertal males and females (Cai et al., 2016). Adult female mice also display greater TNFα, IL-12, and IL-1β concentration in the blood compared to pubertal counterparts, as well as pubertal and adult male mice 8 hours following LPS treatment (Sharma et al., 2018). Adult males and females also display greater IL-6 and TNFα mRNA expression in the hippocampus compared to pubertal counterparts. In addition to the age differences in innate immune system response, there are also age differences in the adaptive immune system response. More specifically, adult females display greater immunoglobin variation and enhanced class switching of CD8+ T cells compared to their pre-pubertal counterparts (Lamason et al., 2006). Furthermore, T cell class switching continues into later life, where mice aged 1 year show greater age associated CD11+ B cells, compared to those aged 2-6 months of age. Increases in CD11+ B cells are associated with autoimmune diseases and may explain why these diseases arise more commonly in later life (Rubtsov et al., 2011).

13. Hypothesis Statement

During the critical period of puberty, the interaction between the HPA axis and the HPG axis has the potential to create enduring negative changes to sexual behavior and HPG axis functionality. Specifically, we hypothesize that stress-induced activation of the HPA axis during puberty results in the suppression of Kiss1 and Kiss1R, which are pivotal in initiating puberty and regulating the HPG axis. This suppression, in turn, leads to a cascade of alterations within the HPG axis, including the downregulation of sex hormone receptors such as ERα, AR, as well as disruptions in the levels of gonadotropins like LH and FSH.

Furthermore, we propose that the observed changes in kisspeptin and Kiss1R expression, and subsequent HPG axis dysfunction, are not uniform across developmental stages. We anticipate that puberty represents a stress-sensitive period during which the HPG axis is particularly susceptible to the negative effects of stressors like lipopolysaccharide (LPS), and that these effects may be enduring if the stressor is encountered during this critical developmental window.

Lastly, we hypothesize that therapeutic intervention with kisspeptin during the post-pubertal period can ameliorate the enduring negative effects of pubertal stress exposure on sexual behavior. This restorative effect of kisspeptin treatment suggests its potential as a key mechanism in countering the HPG axis downregulation and sexual dysfunction induced by stress during puberty. This hypothesis underscores the potential of targeted kisspeptin-based interventions as a novel therapeutic approach for stress-induced reproductive disorders.

CHAPTER 2: Pubertal Immune Challenge Suppresses the Hypothalamic-Pituitary-Gonadal Axis in Male and Female Mice

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Abstract

Kisspeptin is a neuropeptide responsible for propagating the hypothalamic-pituitary-gonadal (HPG) axis and initiating puberty. Pubertal exposure to an immune challenge causes enduring sexual behavior dysfunction in males and females, but the mechanism underlying this stress-induced sexual dysfunction remains unknown. Previous findings show that stress exposure can downregulate the HPG axis in adult females. However, it is unclear whether stress induced HPG axis suppression is limited to adult females or also extends to males and to pubertal animal models. The current study was designed to investigate the sex-specific consequences of a pubertal immune challenge on specific components of the HPG axis. Six-week-old pubertal male and female mice were treated with saline or with lipopolysaccharide, a bacterial endotoxin. Expression of hypothalamic Kiss1 and Kiss1R as well as serum concentrations of luteinizing hormone, follicle-stimulating hormone, and growth hormone were examined. Pubertal lipopolysaccharide treatment decreased hypothalamic Kiss1, but not Kiss1R, expression in both males and females. Furthermore, only males showed decreases in circulating luteinizing and follicle-stimulating hormones. These results show that pubertal immune challenge suppresses the HPG axis by inhibiting Kiss1 production and decreasing serum gonadotropin concentrations in pubertal males, but points to a different mechanism in pubertal females.

Keywords: Puberty, Kisspeptin, Sex differences, Lipopolysaccharide, Stress, Hormones

1. Introduction

Kisspeptin (Kiss1) is a neuropeptide produced by neurons located primarily in two hypothalamic nuclei: the anteroventral periventricular nucleus and the arcuate nucleus (Han et al., 2005; Hellier et al., 2018; Irwig et al., 2004). Kiss1 neurons can also be found in the hippocampus and in the medial amygdala (Clarkson et al., 2009). Kiss1 binds to its G-protein activated receptor (Kiss1R) and stimulates the release of gonadotropin-releasing hormone (GnRH) (Terasaka et al., 2013). The increase in the pulsatile secretion of GnRH by Kiss1 activates the hypothalamic-pituitary-gonadal (HPG) axis (Castellano et al., 2005; Han et al., 2005; Hellier et al., 2018; Irwig et al., 2004) and initiates pubertal development (Sisk & Foster, 2004). GnRH is released into the anterior pituitary and triggers luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion into the bloodstream, which in turn initiates reproductive maturation. LH and FSH acting on the gonads stimulate the production and secretion of gonadal steroid hormones, such as androgens and estrogens (Ojeda et al., 1976; Silverman et al., 1987). These hormones permanently reorganize neuronal circuitry, develop secondary sex characteristics, and activate sex-specific behaviors in both males and females (Phoenix et al., 1959; Romeo, 2003; Schulz et al., 2009).

The pubertal period is vulnerable to stress (Maccari et al., 2003; Romeo, 2010; Teicher et al., 2003) as exposure to certain stressors during this period can permanently alter reproductive behavior (Blaustein et al., 2016; McCormick et al., 2010; McCormick & Mathews, 2007). For example, exposure to the stress of shipping from a supplier to the laboratory at six weeks of age, a pubertal stress-sensitive period, reduces sexual receptivity in ovariectomized mice treated with estradiol and progesterone (Blaustein et al., 2016; Laroche et al., 2009a). Although a much smaller effect, pubertal shipping stress also decreases mount frequency in males (Laroche et al., 2009a). However, stress-induced sexual dysfunction has been almost exclusively investigated in

females. The inclusion of both sexes when researching the downstream effects of pubertal stress on the HPG axis will clarify the neural underpinnings that are yet unclear. Like shipping stress, exposure to the bacterial endotoxin lipopolysaccharide (LPS), at 6 weeks of age, also reduces sexual receptivity, suggesting a suppression of HPG axis activity (Laroche et al., 2009a).

Stress exposure activates the HPA axis, resulting in the production of corticotropin-releasing factor (CRF). CRF is released into the hypophyseal portal vasculature where it signals secretion of adrenocorticotropic hormone (ACTH) from the pituitary into the bloodstream. Once ACTH binds to the adrenal cortex, glucocorticoids are produced and released to maintain the stress response through negative-feedback to the paraventricular nucleus (PVN) of the hypothalamus (Kane & Ismail, 2017; Ulrich-Lai & Herman, 2009). HPA-axis mediated HPG axis suppression is well-known (McCormick & Mathews, 2007; Oyola & Handa, 2017). However, stress-induced HPA-axis activation is sexually dimorphic, implying that HPG axis suppression would be different in males and females. To date, findings regarding HPG axis suppression is limited to adult females. For example, corticosterone-containing pellets fed to adult female mice significantly reduce serum LH concentration (Luo et al., 2016). Additionally, CRF administration downregulates GnRH firing in a dose-dependent manner in adult female mice (Phumsatitpong & Moenter, 2017). Furthermore, intracerebroventricular injection of CRF causes a reduction in hypothalamic Kiss1 and Kiss1R mRNA expression in adult female rats (Kinsey-Jones et al., 2009). However, treatment with dexamethasone, a glucocorticoid-agonist, did not change blood FSH concentration in adult female ewes (Phillips et al., 1990). Taken together, these findings provide evidence of stress-induced changes in some parts of the HPG axis of adult females. However, the mechanism underlying the sex-specific effects of stress on

the HPG axis is understudied and necessary for our understanding of HPG-HPA axes interaction (Romeo, 2005).

Therefore, the current study investigated the effect of pubertal LPS treatment on the HPG axis by examining Kiss1 and Kiss1R mRNA expression in the brain and concentrations of LH and FSH in the blood serum of male and female mice. GH concentration is known to increase steadily during puberty, but independently of the HPG axis (Keene et al., 2002; Schally et al., 1971). Thus, GH concentration was also examined to determine if the effects of pubertal stress were limited to HPG axis-dependent hormones or extended generally to other hormones involved in pubertal development. We hypothesized that pubertal LPS treatment would decrease Kiss1 and Kiss1R mRNA expressions in the hypothalamus, hippocampus, and prefrontal cortex (PFC). We also hypothesized that pubertal LPS treatment would decrease LH and FSH concentration in both sexes, but that GH concentration would remain unchanged, as it is not an HPG-axis related sex hormone.

2. Methods

2.1 Animals

Twenty male and 20 female CD-1 mice were delivered from Charles River Laboratories (St-Constant, Canada) at 3 weeks of age. Upon arrival, mice were pair-housed in polycarbonate cages (17cm x 28cm x 12cm [height x width x length]) in either an all-male or an all-female room. Cages were bedded with corn cob (Cat# 7097, Envigo, Mississauga, CA), and included one piece of nestlet (Cat# NES3600, Ancare, Bellmore, USA), and a cardboard hut (Cat# XKA2455, Ketchum, Brockville, CA) for basic enrichment. The rooms were maintained at constant temperature (24 ± 2oC) and humidity (40%), and on a reversed light/dark cycle (lights on between 8:00pm and 10:00am) throughout the experiment. Dusk and dawn were gradually

induced over one hour. Mice were given ad libitum access to food (Envigo, Rodent Chow, Mississauga, Canada) and water. All procedures were approved by the Animal Care Committee of the University of Ottawa.

2.2 Lipopolysaccharide (LPS) Treatment

At 6 weeks of age, male (n = 20) and female mice (n = 20) were treated with sterile saline or LPS at the end of the light phase between 9am and 9:30am. LPS (from E. coli serotype 026:B6; No. L3755; Sigma Chemical Co., St. Louis, USA) was diluted in sterile saline at a concentration of 0.2mg/ml and injected intraperitoneally at a dose of 1.5mg/kg to males (n = 10) and females (n = 10). Saline-treated male (n = 10) and female (n = 10) mice were injected intraperitoneally with an equivalent volume of sterile saline. This dose of LPS has been shown to cause sickness that persists up to 48 hours and to produce enduring effects on reproductive behaviors (Blaustein & Ismail, 2013; Laroche et al., 2009b).

2.3 Euthanasia

Eight hours following saline or LPS treatment, mice were euthanized with an intraperitoneal injection of Euthanyl (prepared from Euthansol; Merck Animal Intervet Canada Corp; Kirkland, Canada). Following confirmation of the absence of reflex, mice were decapitated, and trunk blood was collected. Blood was centrifuged at 10,000g for five minutes and blood serum was collected and stored at -80°C. Brains were extracted from the skull, rapidly frozen using liquid nitrogen, and stored at -80°C.

2.4 Multiplex Assay

Multiplex assays (MPTMAG-49K, Millipore Sigma, Etobicoke, Canada) purchased from Millipore Sigma were used to analyze FSH, LH, and GH concentrations according to instructions from the manufacturer. Briefly, duplicates containing 10 µl of blood serum each were added to

the sample wells. The quality control and standards were added to the appropriate wells according to the manufacturer's protocol. The tray was left to shake for 18 hours overnight at 4°C. 100µl of Drive fluid was added to each well, and the assay was read using MAGPIX software. The sensitivity of the assay was measured in minimum detectable concentration (MinDC) and varied depending on the analyte (LH = 1.9pg/ml, FSH 9.5pg/ml, and GH 1.7pg/ml). A polyclonal rabbit antibody was used to detect LH, and monoclonal mouse antibodies were used to detect FSH and GH. Cross-reactivity between the antibodies was negligible. Intra- and inter-assay coefficients of variability were both < 10%.

2.5 Real-time quantitative Polymerase Chain Reaction

Frozen brains were sliced into 350um thick slices on a Leica CM1900 Microtome. Slices were then mounted onto Fisherbrand Superfrost Plus microscope slides. The hippocampus, PFC, and hypothalamus were bilaterally punched from brain slices based on the Mouse Brain Atlas in Stereotaxic Coordinates (Franklin and Paxinos, 2013). Punched tissue was prepared for messenger RNA (mRNA) extraction using Isol-RNA lysis reagent purchased from Fisher Scientific (Waltham, USA) (Cat. No. 2302700). Genomic DNA was removed from the extracted mRNA, and cDNA was synthesized using Qiagen (Toronto, Canada) QuantiTect Reverse Transcription kit (Cat. No. 205311). β-actin (forward: 5'-GAACCCTAAGGCCAACCGTG-3', reverse: 5'-GGTACGACCAGAGGCATACAGG-3'), Kiss1 (forward: 5'-CTACATCCAGCAGGTCTCGGT-3', reverse: 5'-CCAGATGCTGAGGCTGACA-3'), and Kiss1R (forward: 5'-CTACATCCAGCAGGTCTCGGT-3', reverse: 5'-CCCAGATGCTGAGGCTGACA-3') primers were diluted to a concentration of 0.5uM. All primers were ordered through Integrated DNA Technologies. The relative gene expression was measured using SSoFast EvaGreen Supermix (Cat. No. 1725202, Bio-Rad, Montreal, Canada).

RT-qPCR samples were run in triplicates, and the average of two samples with less than 0.400 standard deviations was used for analysis. The qPCR product was loaded on a 2% agarose gel with ethidium bromide for electrophoresis. The gel was visualized under ultraviolet light to confirm the product size. Bio-Rad CFX Maestro version 3 was used to view RT-qPCR results (http://www.bio-rad.com/en-eh/product/cfx-manager-software). The 2–ΔΔCt method was used to calculate fold change in gene expression, using β-actin as the reference/housekeeping gene. β-actin cycle quantification values did not vary between groups.

2.6 Statistical Analysis

Box plots were used to identify outliers, which were winsorized to the next extreme value in the distribution (Howell, 2011). Based on this criterion, one saline-treated female mouse data point was winsorized in the LH statistical analysis and one LPS-treated male mouse data point was winsorized in the GH statistical analysis. Two-way analyses of variance (ANOVA) (sex x treatment) were used to compare Kiss1 and Kiss1R mRNA expression, as well as LH, FSH and GH concentrations. A Pearson correlation was conducted to analyze correlations between Kiss1 and Kiss1R mRNA expression, and peripheral LH, FSH, and GH concentrations. Partial eta-squared (ηp2) was used to calculate the effect size of main effects and interactions. Bonferroni corrected pairwise comparisons were used when appropriate. Statistical significance was set to $p < 0.05$. Statistical analyses were conducted using IBM SPSS version 21.

3. Results

3.1 *Kisspeptin (Kiss1)*

The ANOVA revealed a main effect of treatment in hypothalamic Kiss1 mRNA expression ($F_{(1, 36)} = 6.25$ $p < 0.05$, $\eta_p^2 = 0.15$). There was no main effect of sex ($F_{(1, 36)} = 0.90$, $p > 0.05$, $\eta_p^2 = 0.02$), or sex x treatment ($F_{(1, 36)} = 1.37$, $p > 0.05$, $\eta_p^2 = 0.04$) interaction for

hypothalamic Kiss1 mRNA expression. LPS-treated mice displayed significantly lower Kiss1 mRNA expression in the hypothalamus compared to saline controls (MD = 0.40, SE = -0.16, $p < 0.05$) (Figure 1). There was no difference in Kiss1 mRNA expression in the hypothalamus between males and females (MD = 0.46, SE = 0.49, $p > 0.05$). There was no main effect of sex ($F_{(1, 36)} = 0.71$, $p > 0.05$, $\eta_p^2 = 0.02$), or treatment ($F_{(1, 36)} = 0.02$, $p > 0.05$, $\eta_p^2 = 0.00$), and no sex x treatment ($F_{(1, 36)} = 0.00$, $p < 0.05$, $\eta_p^2 = 0.00$) interaction on hippocampal Kiss1 mRNA. There was no difference in Kiss1 mRNA expression in the hippocampus between males and females (MD = 0.45, SE = 0.54, $p > 0.05$), or between saline- and LPS-treated mice MD = 0.08, SE = 0.39, $p > 0.05$). Additionally, there were no main effect of sex ($F_{(1, 36)} = 0.28$, $p > 0.05$, $\eta_p^2 = 0.01$), or treatment ($F_{(1, 36)} = 1.41$, $p > 0.05$, $\eta_p^2 = 0.00$), and no sex x treatment interaction on Kiss1 mRNA expression in the PFC ($F_{(1, 36)} = 2.11$, $p > 0.05$, $\eta_p^2 = 0.55$). There was no difference in Kiss1 mRNA in the PFC between male and female (MD = 0.24, SE = 0.51, $p > 0.05$), or between saline- and LPS-treated mice (MD = 0.16, SE = 0.36, $p > 0.05$). A full list of non-significant interactions is provided in Table 1.

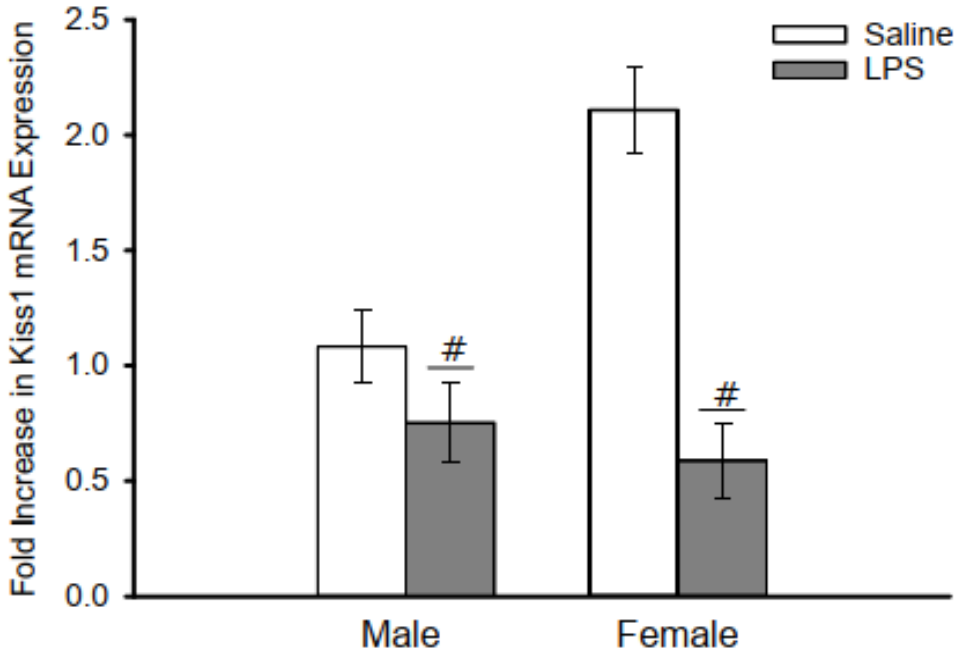


Figure 1.

Title: Hypothalamic Kiss1 mRNA expression

Caption: Mean fold change (±SEM) in Kiss1 mRNA expression in the hypothalamus of 6-week-old male and female mice treated with saline or lipopolysaccharide (LPS). The asterisk (*) denotes significant differences ($p < 0.05$) for the main effect of treatment.

3.2 *Kisspeptin receptor (Kiss1R)*

There was no main effect of sex ($F_{(1, 36)} = 1.11$, $p > 0.05$, $\eta_p^2 = 0.03$), or treatment ($F_{(1, 36)} = 1.20$, $p > 0.05$, $\eta_p^2 = 0.03$), and there was no sex x treatment interaction on hypothalamic Kiss1R mRNA expression ($F_{(1, 36)} = 0.46$, $p > 0.05$, $\eta_p^2 = 0.02$). There was no difference in Kiss1R mRNA in the hypothalamus between males and females (MD = 0.37, SE = 0.35, $p > 0.05$), or between saline- and LPS-treated mice (MD = 0.38, SE = 0.25, $p > 0.05$). There was also no main effect of sex ($F_{(1, 36)} = 0.81$, $p > 0.05$, $\eta_p^2 = 0.02$), or treatment ($F_{(1, 36)} = 0.23$, $p > 0.05$,

$\eta_p^2 = 0.01$), and no sex x treatment interaction on hippocampal Kiss1R mRNA expression ($F_{(1, 36)} = 0.00$, $p > 0.05$, $\eta_p^2 = 0.00$). There was no difference in Kiss1R mRNA in the hippocampus between males and females (MD = 0.08, SE = 0.39, $p > 0.05$), or between saline- and LPS-treated mice (MD = 0.08, SE = 0.39, $p > 0.05$). Finally, there was no main effect of sex ($F_{(1, 36)} = 0.22$, $p > 0.05$, $\eta_p^2 = 0.00$), or treatment ($F_{(1, 36)} = 0.10$, $p > 0.05$, $\eta_p^2 = 0.00$), and no sex x treatment interaction ($F_{(1, 36)} = 0.04$, $p > 0.05$, $\eta_p^2 = 0.00$) on Kiss1R mRNA expression in the PFC. There was no difference in Kiss1R mRNA in the PFC between males and females (MD = 0.24, SE = 0.51, $p > 0.05$), or between saline- and LPS-treated mice (MD = 0.16, SE = 0.36, $p > 0.05$).

Table 1

Table of non-significant individual mean differences (MD) and standard errors (SE)

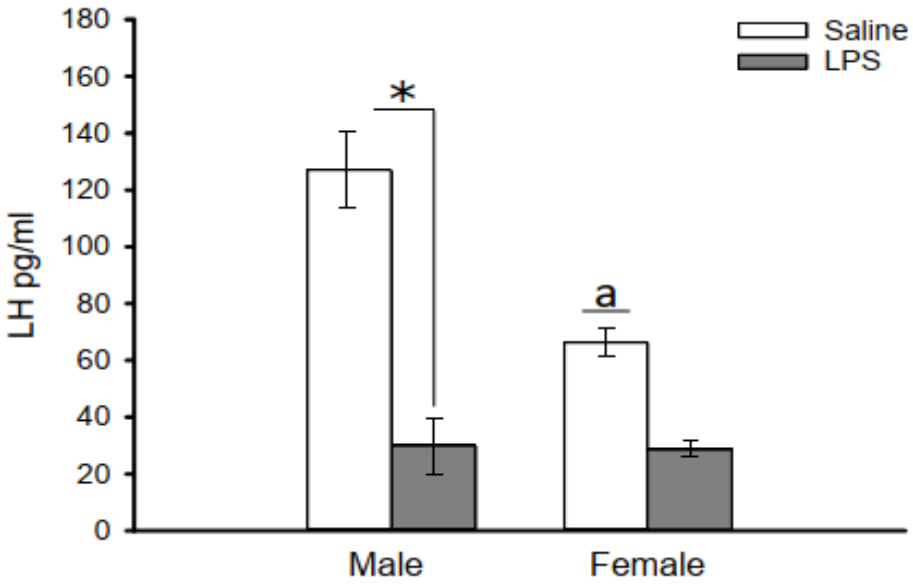
Variable	**Male vs Female**		**Saline vs LPS**	
	MD	***SE***	***MD***	***SE***
Hypothalamic Kiss1				
Saline	1.03	0.48		
LPS	0.11	0.10		
Male			0.41	0.10
Female			1.55	0.50
Hippocampal Kiss1				
Saline	0.46	0.37		
LPS	0.45	0.38		
Male			0.74	0.41
Female			0.81	0.32
Prefrontal Kiss1				
Saline	0.84	0.35		
LPS	0.40	0.24		
Male			1.12	0.38

Female			0.11	0.21
Hypothalamic Kiss1R				
Saline	0.60	0.30		
LPS	0.13	0.16		
Male			0.14	0.13
Female			0.62	0.32
Hippocampal Kiss1R				
Saline	0.50	0.36		
LPS	0.48	0.40		
Male			0.09	0.25
Female			0.08	0.47
Prefrontal Kiss1R				
Saline	0.34	0.34		
LPS	0.14	0.37		
Male			0.26	0.40
Female			0.06	0.31
GH				
Saline	1812.67	634.50		
LPS	1182.13	766.53		
Male			858.94	734.15
Female			2135.86	677.57

3.3 *Luteinizing Hormone (LH)*

There were significant main effects of sex ($F_{(1, 36)} = 5.14$, $p < 0.05$, $\eta_p^2 = 0.13$), and treatment ($F_{(1, 36)} = 22.49$, $p < 0.05$, $\eta_p^2 = 0.38$), and a significant sex x treatment interaction ($F_{(1, 36)} = 4.17$, $p < 0.05$, $\eta_p^2 = 0.10$) on LH serum concentration. Saline-treated males displayed significantly greater concentrations of LH compared to saline-treated female mice (MD = 59.10, SE = 19.40, $p < 0.05$). LPS-treated males had significantly less LH concentrations contrasted with saline-treated male mice (MD = 93.05, SE = 19.40, $p < 0.05$). LPS-treated females did not

significantly differ from saline-treated counterparts (MD = 37.02, SE = 19.40, $p > 0.05$) (Figure 2).



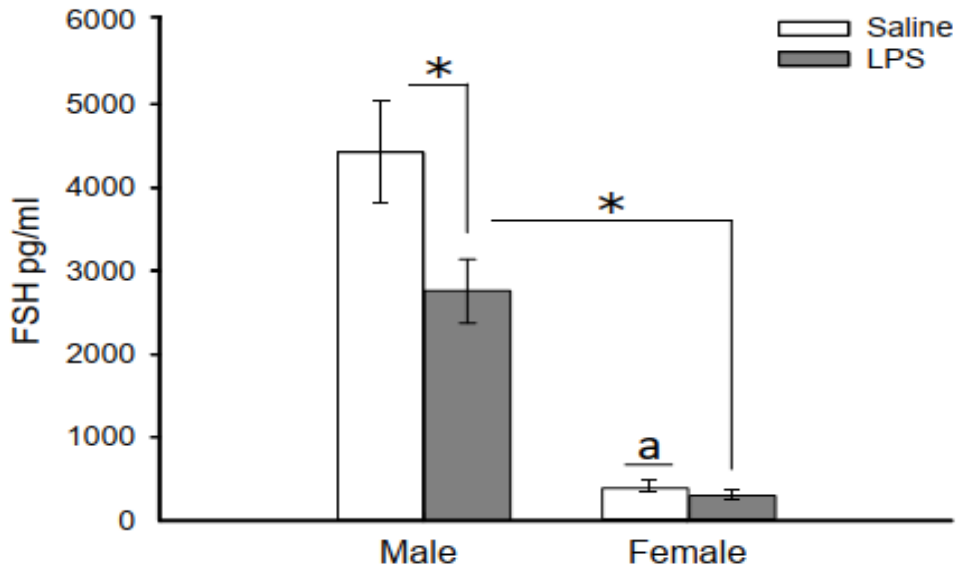
Title: LH concentration

Caption: Mean (±SEM) luteinizing hormone (LH) concentration (pg/ml) in the serum of 6-week-old male and female mice treated with saline or lipopolysaccharide (LPS). The asterisk (*) denotes significant differences ($p < 0.05$). The *a* denotes significant differences between male and female saline groups.

3.4 *Follicle Stimulating Hormone (FSH)*

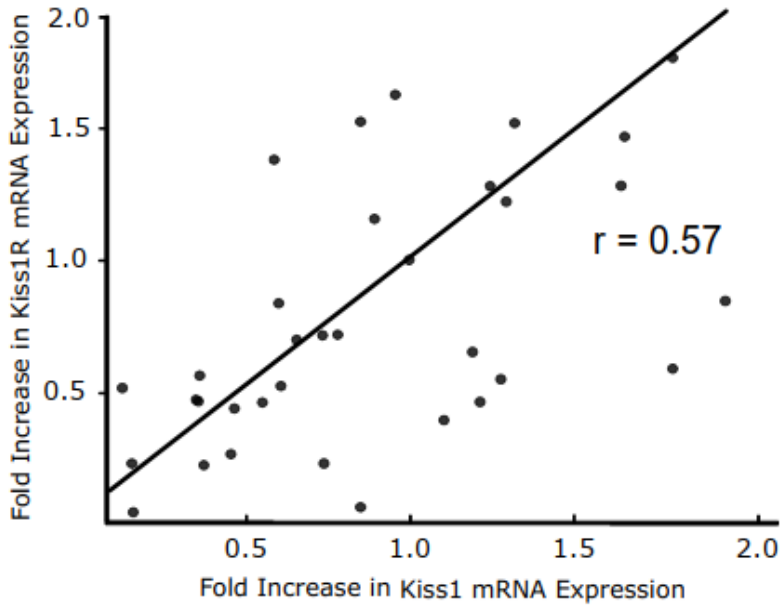
There were significant main effects of sex ($F_{(1, 36)} = 106.84$, $p < 0.05$, $\eta_p^2 = 0.75$), treatment ($F_{(1, 36)} = 6.44$, $p < 0.05$, $\eta_p^2 = 0.15$), and a significant sex x treatment interaction ($F_{(1, 36)} = 5.15$, $p < 0.05$, $\eta_p^2 = 0.13$) on FSH serum concentration. Saline-treated males displayed significantly higher FSH concentration compared to female counterparts (MD = 3945.95, SE = 442.69, $p < 0.05$). LPS-treated male mice displayed significantly lower FSH concentration compared to saline controls (MD = 1504.70, SE = 442.69, $p < 0.05$), but greater FSH

concentration than LPS-treated female counterparts (MD = 2525.24, SE = 442.70, $p < 0.05$) (Figure 3).



Title: FSH concentration

Caption: Mean (±SEM) Follicle stimulating hormone (FSH) concentrations (pg/ml) in the serum of 6-week-old male and female mice treated with saline or lipopolysaccharide (LPS). The asterisk (*) denotes significant differences ($p < 0.05$). The *a* denotes significant differences between male and female saline groups.



Title: Kiss1 and Kiss1R mRNA Expression

Caption: Fold change (±SEM) in Kiss1 mRNA expression positively correlated with Kiss1R mRNA expression in the hypothalamus of 6-week-old male and female mice treated with saline or lipopolysaccharide (LPS). The asterisk (*) denotes a significant positive correlation ($p < 0.05$).

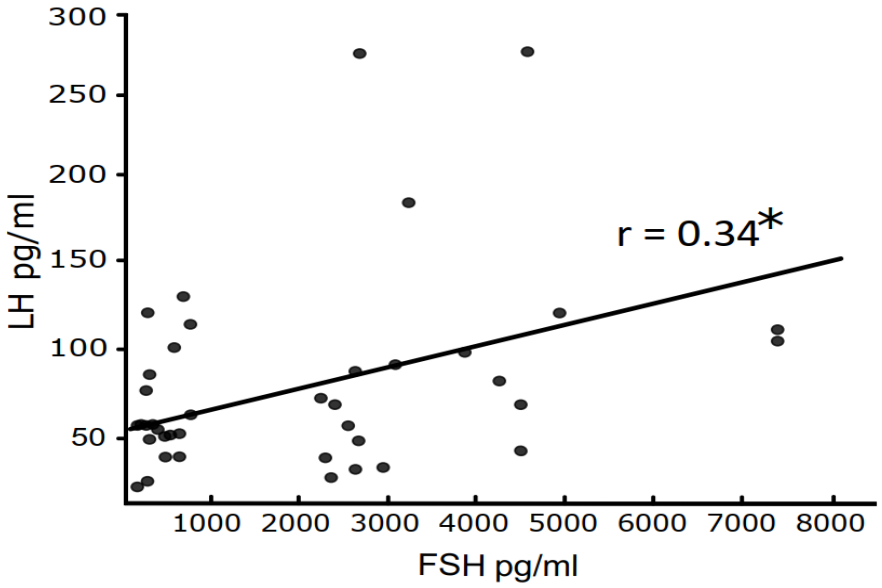
3.5 *Growth Hormone (GH)*

There was no main effect of sex ($F_{(1, 36)} = 0.10$, $p < 0.05$, $\eta_p^2 = 0.00$), or treatment ($F_{(1, 36)} = 0.42$, $p > 0.05$, $\eta_p^2 = 0.01$), and no sex x treatment interaction ($F_{(1, 36)} = 2.23$, $p > 0.05$, $\eta_p^2 = 0.06$). There was no difference in GH concentration between males and females (MD = 3234.60, SE = 313.03, $p > 0.05$), or between saline- and LPS-treated mice (MD = 2353.13, SE = 221.35, $p > 0.05$).

3.6 *Correlation Analysis*

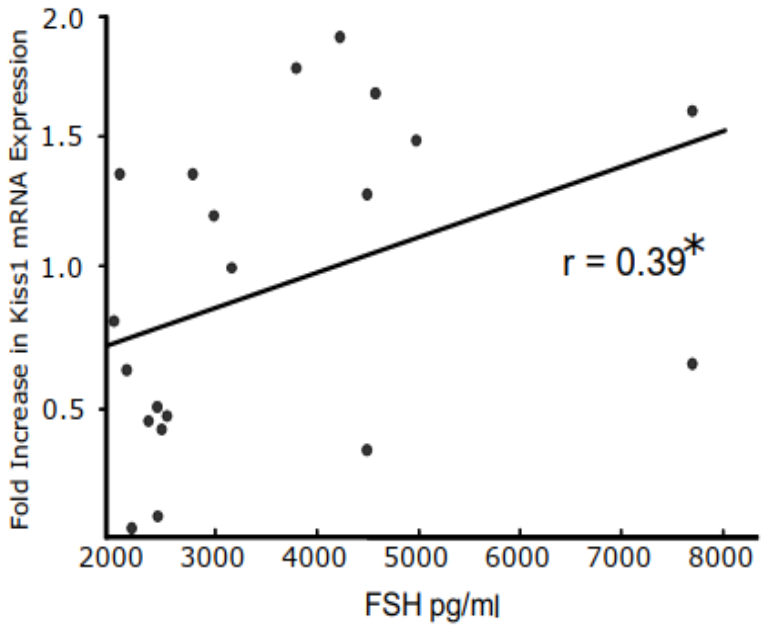
There was a significant positive correlation between hypothalamic Kiss1 and Kiss1R mRNA expression in both males and females, regardless of treatment (r = 0.57, $p < 0.05$) (Figure

4). There was a significant positive correlation between LH and FSH in both males and females, regardless of treatment (r = 0.34, $p < 0.05$) (Figure 5). There was also a significant positive correlation between serum FSH and hypothalamic Kiss1 mRNA expression in male mice treated with either saline or LPS ($r = 0.39$, $p < 0.05$) (Figure 6). No significant correlation was found between hypothalamic Kiss1 and LH ($r = 0.08$, $p > 0.05$), hypothalamic Kiss1R and LH ($r = -0.01$, $p > 0.05$), or hypothalamic Kiss1R and FSH ($r = -0.16$, $p > 0.05$).



Title: LH and FSH Concentration

Caption: Serum Luteinizing Hormone (LH) concentrations (pg/ml) positively correlated with serum Follicle Stimulating Hormone (FSH) concentrations in 6-week-old male and female mice treated with saline or lipopolysaccharide (LPS). The asterisk (*) denotes a significant positive correlation ($p < 0.05$).



Title: Male FSH and Kiss1 mRNA Expression

Caption: Hypothalamic Kiss1 mRNA expression positively correlated with serum Follicle Stimulating Hormone (FSH) concentration (pg/ml) in 6-week-old male mice treated with saline or lipopolysaccharide (LPS). The asterisk (*) denotes a significant positive correlation ($p < 0.05$).

4. Discussion

In the current study, we examined the acute effects of a pubertal immune challenge on HPG axis activity by measuring Kiss1 and Kiss1R mRNA expressions in the prefrontal cortex, hypothalamus and hippocampus, and LH, FSH, and GH concentrations in blood serum. Due to the rapidly changing synaptic connections during pubertal development, exposure to stress and activation of the HPA axis can result in the allostatic downregulation of the HPG axis (Oyola & Handa, 2017; Rivier & Rivest, 1991; Romeo, 2005). The sexually dimorphic stress response and HPA axis activation warrants investigation into whether the stress-induced downregulation of the HPG axis is also sexually dimorphic. Currently, most reports examine the effects of stress on the

HPG axis in females only (Iwasa et al., 2008, 2014). It is therefore critical to further our understanding of the mechanisms underlying the stress-induced downregulation of the HPG axis and to examine whether this downregulation is sex-specific or also extends to males.

Kiss1 is a critical neuropeptide involved in the activation of the HPG axis and initiation of puberty. Our results show that a single LPS injection during puberty reduces hypothalamic Kiss1 mRNA expression in male and female mice. This discovery exemplifies the vulnerability of the HPG axis during pubertal development. Further, it shows that the effects of environmental insults on the HPG axis during puberty are not sex specific. The LPS-induced downregulation of Kiss1 mRNA expression is consistent with previous findings showing that LPS administration significantly decreases hypothalamic Kiss1 expression in adult male (Castellano et al., 2005) and female rats (Iwasa et al., 2008, 2014; Kinsey-Jones et al., 2009). However, our results extend previous findings by showing that LPS-induced decrease in hypothalamic Kiss1 expression is not restricted to adulthood when the HPG axis is fully developed.

Kiss1R is a g-protein regulated-receptor commonly found on GnRH neurons and is responsible for stimulating gonadotropins following Kiss1 binding. We evaluated the effect of LPS treatment on Kiss1R mRNA expression in both male and female pubertal mice. However, contrary to our hypothesis, we found that LPS treatment did not significantly alter Kiss1R mRNA expression. Our hypothesis was based on previous work showing that intravenous treatment of 0.5μg/kg of LPS significantly decreased Kiss1R mRNA expression in the hypothalamus in adult female rats (Kinsey-Jones et al., 2009). C57BL6 adult male mice also showed a decrease in Kiss1R mRNA expression in the hypothalamus after 12, 24 and 48 hours of food deprivation (Luque et al., 2007). Although our results did not support our hypothesis, they are consistent with some previous reports. Iwasa and colleagues (2014) also found a

decrease in Kiss1 but not in Kiss1R expression following intravenous or intraperitoneal injections of LPS in adult female rats (Iwasa et al., 2014). Neonatal LPS treatment also failed to alter Kiss1R mRNA expression in female rats on post-natal days 14 and 42, on the day of vaginal opening, and at 11 weeks of age (Knox et al., 2009) It is unclear why findings are conflicting. Nevertheless, there is culminating evidence that LPS treatment often fails to alter Kiss1R expression (Iwasa et al., 2014; Knox et al., 2009). Downregulation of Kiss1R expression following fasting likely occurs due to the high energetic requirement for reproduction (Iwasa et al., 2010; Luque et al., 2007). LPS treatment may not impact metabolism for a sufficiently long time to induce changes in Kiss1R expression. Our findings suggest that LPS treatment potently downregulates the HPG-axis via decreased Kiss1 production rather than reducing the expression of its g-protein coupled receptor. This function may be beneficial in reducing allostatic load while keeping biological makeup of the HPG axis intact when homeostasis returns. The function of Kiss1R and its relation to the Kiss1 peptide continue to remain elusive prompting further investigation into their complex interconnectivity.

LH is one of the two gonadotropins mediated by the HPG axis and secreted from the anterior pituitary (Raju et al., 2013; Silverman et al., 1987). LH is primarily responsible for stimulating the gonads to produce androgens and estrogens (Raju et al., 2013). We found that pubertal LPS treatment significantly decreased serum LH concentration in males, but not in females. These findings partially support our hypothesis, as pubertal LPS treatment failed to alter LH concentration in both sexes. Our findings are inconsistent with previous work showing that LPS treatment decreases LH concentration in female adult rats (Iwasa et al., 2008, 2014; Li et al., 2006). Other stressors, such as restraint stress (Li et al., 2006; Roozendaal et al., 1997) and food deprivation (Knuth & Friesen, 1983; Matsuzaki et al., 2011) have also been shown to

decrease LH concentration in adult male and female rats. The sex difference in LPS-induced changes in LH concentration in the current study is likely not due to a sex difference in HPA axis activation following LPS treatment. Previous work from our laboratory has shown that LPS treatment induces an increase in corticosterone levels in pubertal and adult male and female mice. While there is a sex difference in corticosterone concentration in adult mice following LPS treatment, no sex difference in corticosterone concentration was found in pubertal mice (Girard-Joyal & Ismail, 2017). Nevertheless, our results are novel and show that the effect of pubertal LPS treatment on LH concentration is limited to males.

FSH is a gonadotropin hormone that works in conjunction with LH to stimulate spermatogenesis and mature ovarian follicles (Ulloa-Aguirre et al., 2018). In the current study, LPS treatment significantly reduced FSH concentration in males, but not in females. These findings partially support our hypothesis that pubertal LPS treatment would downregulate the HPG axis in both males and females. One reason LPS treatment may have failed to cause significant changes in FSH concentration in female mice could be due to a floor effect, as female mice display low baseline FSH concentration prior to adulthood (Michael et al., 1980). Male mice, on the other hand, show significantly greater FSH concentration than age-matched female mice. Studies examining the effect of stress on FSH production primarily utilize female ewes (Bartlewski et al., 2011; Herman et al., 2010). Rodent-based reports are rare, and the findings are inconsistent. For example, in adult female rats, chronic unpredictable mild stress reduces serum estradiol, GnRH, and ovarian follicular counts but increases FSH concentrations (Fu et al., 2018). In adult male rats, acute and chronic immobilization stress reduces LH serum concentrations, but does not affect FSH concentration (Demura et al., 1989). In contrast, chronic restraint stress decreases both LH and FSH serum concentrations in adult male rats (Liberzon et

al., 1999). A previous report employing a bolus injection of LPS did not find any change in FSH serum concentration in adult male rats. Similarly, the frequency and amplitude of FSH release is not altered following LPS treatment (Refojo et al., 1998). LPS-induced decrease in LH and FSH is associated with a decrease in reproductive function. Previous findings show that, in pubertal mice, LPS treatment causes an enduring decrease in sexual receptivity (Ismail et al., 2011; Laroche et al., 2009a). Similarly, exposure to shipping-stress during puberty (at 6-weeks of age) causes an enduring decrease in mounting behavior in males and a decrease in sexual receptivity in females (Laroche et al., 2009b). These findings warrant further investigation into FSH functionality in rodents, especially in mice, to appropriately assess the effects of stress on this component of the HPG-axis. Our divergent findings could also be due to differences in LPS-responsivity between mice and rats. Recent findings show that rats display greater white blood cell concentration than mice following LPS treatment, implying that the rat immune system is more responsive to LPS treatment (Steven et al., 2017). Additionally, mice are more susceptible to LPS-induced sepsis than rats. BALB/c mice experience sepsis at 7mg/kg, but Wistar rats can receive up to 25mg/kg before an adverse reaction (R. C. Thomas et al., 2014). Given the variability in response, caution is advised when interpreting the findings of LPS treatment between rodents, and particular attention must be paid to the LPS dosages between studies.

We also found a positive correlation between Kiss1 mRNA expression and FSH concentration in males but not in females, suggesting that Kiss1 may regulate FSH levels in pubertal males only. In pubertal females, FSH concentration appears to be regulated independently of Kiss1, and it may instead be regulated by estradiol. Ovariectomized adult female wild-type mice show greater FSH concentrations compared to estradiol-treated ovariectomized mice (Glidewell-Kenney et al., 2008; Wersinger et al., 1999). These findings

suggest that FSH concentrations are high when estradiol concentrations are low. Moreover, estrogen receptor alpha (ERα)-knockout mice display similar FSH levels as ovariectomized female mice, implying that estradiol regulates FSH concentration through ERα. However, it is difficult to assess if there is a definitive link between FSH and Kiss1, as there are very few previous reports examining Kiss1 and FSH in rodents. Although pubertal female mice displayed a decrease in Kiss1 mRNA expression following LPS treatment, it is currently unclear why FSH concentration remain unchanged in these females, as prior findings are sparse.

Contrary to our hypothesis, and despite reporting significant group changes, we did not observe a significant correlation between hypothalamic Kiss1 expression and peripheral LH concentration. However, we do not believe that the non-significant finding implies disharmony amongst hypothalamic Kiss1 and LH within the HPG-axis in pubertal mice. A correlation is unlikely because our analysis reports non-significant LH expression changes between LPS- and saline-treated females. Non-significant LH changes between these groups would result in homogeneity, and therefore lack increasing or decreasing data points. Furthermore, we only found a main effect of treatment in hypothalamic Kiss1 mRNA expression. These results suggest that the effects of LPS treatment on hypothalamic Kiss1 mRNA expression in males and females could be less pronounced than the combined main effect. However, we are not justified in analyzing the pairwise comparisons as we did not identify a significant sex and treatment interaction. An investigation into why LH levels are unaffected by LPS treatment in pubertal females will help elucidate the reason for non-correlative results.

We found no difference in GH concentration following LPS treatment regardless of sex. These results suggest that the effects of pubertal immune challenge are limited to hormones directly associated with GnRH and HPG axis activity, like LH and FSH (Schally et al., 1971).

Although GH is instrumental in pubertal development, it does not directly contribute to the regulation of LH and FSH (Keene et al., 2002). While the current results are consistent with our hypothesis, they are not consistent with previous findings showing a decrease in GH concentration following a bolus injection of LPS or exposure to chronic stress in adult male rats and adult mice and rats, respectively (Kasting & Martin, 1982). Moreover, treatment with ether was also found to significantly decrease GH concentration in adult male and female mice (Schindler et al., 1972). Given that these experiments were conducted in adult mice and rats, it is possible that the effect of stress on GH concentration are age-specific and do not extend to younger rodents.

5. Conclusion

The current study shows that pubertal immune challenge causes a downregulation of the HPG axis that is likely related to the suppression of Kiss1 signaling in the hypothalamus. However, the effects of pubertal immune challenge on the gonadotropin hormones is sex specific. Taken together, this study highlights the sensitivity of the pubertal period and the vulnerability of the HPG axis to an immune challenge. Future studies should continue examining the influence of a pubertal stress in both male and female mice. ERα, androgen receptor, and the respective sex hormones should be included as potential, direct, mechanism underlying the effect of stress on pubertal maturation. In addition to central Kiss1 and Kiss1R mRNA expression, the inclusion of GnRH mRNA expression would bolster the findings of this study. Given the direct action of Kiss1 on GnRH stimulation, and the significance of GnRH on HPG axis activation, future experiments should include an analysis of GnRH mRNA expression in the hypothalamus. Additionally, investigating the enduring effects of LPS-treatment would elucidate whether pubertal stress has lasting negative effects on the HPG axis.

References

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Study 1 provided crucial insights into the acute effects of pubertal immune challenges on the hypothalamic-pituitary-gonadal (HPG) axis, focusing on the downregulation of this axis during a period marked by significant neurological and endocrinological developments. It elucidated the roles of key components such as Kiss1, Kiss1R, LH, and FSH in this process, uncovering several intriguing findings. Firstly, the study revealed a reduction in hypothalamic Kiss1 mRNA expression in both male and female mice post-LPS treatment during puberty, suggesting a non-sex-specific vulnerability of the HPG axis to LPS stress However, the study also identified certain sex-specific responses, such as the significant decrease in serum LH and FSH concentrations observed exclusively in male mice following LPS treatment, emphasizing the complexity and sexual dimorphism in stress responses during puberty.

While these findings mark significant strides in understanding the acute impacts of stress on the HPG axis during puberty, they also pave the way for Study 2: an exploration in the enduring effects of stress. Study 2 aims to delve deeper into the enduring consequences of pubertal immune stress on the HPG axis, expanding the scope of this thesis to include the expressions of AR and ERα, as well as the concentrations of LH, and FSH. The hypothesis driving this study is multifaceted, predicting not only the downregulation of Kiss1, Kiss1r, AR, and ERα expressions post LPS treatment. Most crucially, this research seeks to distinguish the enduring effects of pubertal LPS treatment and the effects of adulthood LPS treatment. In doing so, this study aims to fill the gaps left by the previous research, providing a more comprehensive understanding of the long-term consequences of pubertal stress on the HPG axis and how these effects are modulated by sex and age.

CHAPTER 3: Effect of Kisspeptin Treatment on Lipopolysaccharide-Induced Suppression of Sexual Behavior in Mice

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Abstract

Pubertal stress causes enduring sexual behavior dysfunction in males and females, but the underlying mechanism remains unknown. These changes may arise from pubertal programming of the hypothalamic-pituitary-gonadal axis. Previous findings show that stress exposure downregulates the hypothalamic-pituitary-gonadal axis, particularly through the reduction of the neuropeptide kisspeptin (Kiss1) and its receptor (Kiss1R). Although acute changes in *Kiss1* and *Kiss1r* genes have been observed following pubertal immune stress, it is unclear whether immune stress-induced downregulation of *Kiss1* and *Kiss1r* persists beyond puberty. The current study investigated the enduring sex-specific consequences of lipopolysaccharide on the expression of *Kiss1* and *Kiss1r* in 160 pubertal or adult mice at multiple time points. Six-week and 10-week-old male and female mice were treated with either saline or with lipopolysaccharide. Mice were euthanized either 8 hours or 4 weeks following treatment. Results revealed that lipopolysaccharide treatment decreases hypothalamic *Kiss1* and *Kiss1r* in both pubertal and adult mice within 8 hours of treatment. The decreased hypothalamic *Kiss1* expression persists 4 weeks later only in male and female mice treated with lipopolysaccharide during puberty. Unlike females, males show significantly greater decreases in hypothalamic *Kiss1r* expression following lipopolysaccharide treatment in puberty and in adulthood. Our findings highlight the sex- and age-dependent vulnerability of the hypothalamic-pituitary-gonadal axis to immune stress, providing a better understanding of the mechanisms implicated in allostatic shift during immune stress. Finally, our findings also show the effects of immune stress on various components of the hypothalamic-pituitary-gonadal axis, which could have implications for sexual and fertility-related dysfunctions.

Keywords: Kisspeptin, development, stress, reproduction, hormones, sex differences

1.1 Introduction

Puberty is a critical period of development characterized by rapid neuronal reorganization and remodeling (Levitt, 2003). Puberty also marks the advent of secondary sexual differentiation and the transition from a non-reproductive to a reproductive state (Ojeda et al., 1976). Sexual dimorphisms occur from the organizational and activational effects of sex hormones such as estradiol, progesterone, and testosterone, that permanently shape or transiently activate the brain and behavior (Phoenix et al., 1959; Schulz et al., 2009). The complex nature of sex hormone-mediated neuronal reorganizing and remodeling potentiates its vulnerability to stress during puberty (Teicher et al., 2003). In mice, treatment with the bacterial endotoxin lipopolysaccharide (LPS) at 6 weeks of age, during the pubertal stress sensitive period, causes long-lasting impairments to sexual behavior (Ismail et al., 2011; Laroche et al., 2009a), cognition (Kolmogorova & Ismail, 2021), and elicits affective disorders, like anxiety-like and depression-like behaviors (Murack et al., 2021; Murray et al., 2019, 2020) that are still present in adulthood (10 weeks of age). However, these enduring changes occur only when mice are exposed to the stressor during the pubertal stress-sensitive period (6 weeks of age), as exposure to LPS earlier or later than 6 weeks of age does not cause enduring consequences on behavior (Laroche et al., 2009a).

The hypothalamic-pituitary-adrenal (HPA) axis responds to stressors through increased cellular metabolism, inflammation, and reduced non-essential functions, like reproduction and digestion (Turnbull & Rivier, 1995). The HPA axis exerts these effects via glucocorticoids, like cortisol or corticosterone (CORT), in humans and rodents, respectively (Herman et al., 1989). The stress response begins in the hypothalamus where neurons producing corticotropin-releasing factor (CRF) in the paraventricular nucleus (PVN) secrete CRF into the pituitary portal system (Herman et al., 1989) to stimulate the anterior pituitary gland to secrete adrenocorticotropic

hormone (ACTH) into the bloodstream. ACTH triggers glucocorticoid release from the adrenal cortex that circulates through the bloodstream and provides negative feedback to the PVN (Herman et al., 1995). While this system is functional from birth, it undergoes developmental and sexual differentiation under the influence of the newly abundant sex hormones during puberty. These differentiations of the HPA axis cause sex- and age-specific changes to the brain and behavior (Kane & Ismail, 2017; Malendowicz, 1974; Smith et al, 2021). For example, LPS-treated adult female mice display greater plasma CORT concentrations than adult males (Girard-Joyal et al., 2015). Additionally, adult male and female LPS-treated mice show greater CORT concentrations than their pubertal counterparts (Ismail et al., 2011; Laroche et al., 2009a, 2009b). Moreover, HPA axis activation during puberty can disrupt sexual behavior in an enduring manner (Ismail et al., 2011; Laroche et al., 2009b). Shipping stress and LPS treatment at 6 weeks of age reduces sexual receptivity in adult female mice, as well as the number of mount attempts in adult males (Ismail et al., 2011; Laroche t al., 2009a, 2009b). It is well-known that the hypothalamic-pituitary-gonadal (HPG) axis is downregulated following HPA axis activation as an allostatic measure to increase survival during stress (Bambino & Hsueh, 1981) however, the mechanism is elusive.

The HPG axis is a neuroendocrine system responsible for driving sexual and reproductive maturation via estrogen and androgen production. The neuropeptide kisspeptin regulates gonadotropin releasing hormone (GnRH) neurons (Han et al., 2005), which stimulate the production and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary (Howles, 2000). These hormones bind to receptors on the gonads, stimulating androgen and estrogen synthesis, which provides negative feedback to hypothalamic kisspeptin and GnRH neurons (Moore et al., 2019). Pubertal LPS treatment decreases

hypothalamic *Kiss1* and *Kiss1r* mRNA expressions in male and female mice (Smith et al., 2021). LPS-treated adult male and female rats also show *Kiss1* and *Kiss1r* downregulation (Knox et al., 2009). Furthermore, LPS has been shown to effectively downregulate not only kisspeptin, but also GnRH and LH in rodents (X. F. Li et al., 2007; Magata et al., 2022; Walker et al., 2011). However, it is unclear whether these effects are long-lasting and if other components of the HPG axis are also downregulated.

This study examined the acute and enduring effects of pubertal immune stress on the HPG axis. We examined *Kiss1* and *Kiss1r* mRNA, as well as androgen receptor (AR) and estrogen receptor alpha (ERα), expressions in the hypothalamus. Plasma LH, FSH, interleukin (IL)-6 and tumor necrosis factor alpha (TNFα) were also analyzed. We hypothesized that *Kiss1*, *Kiss1r*, AR, and ERα expressions would be downregulated in the hypothalamus, whereas IL-6 and TNFα concentrations would be upregulated in the plasma of LPS-treated mice. Lastly, we predicted that LPS-induced downregulation of the HPG axis will be long-lasting in mice treated during puberty, whereas it will be short-lasting in mice treated during adulthood. We report that LPS did result in persistent downregulation only in pubertal mice, with particularly greater downregulation in male mice, reflecting the sex-dependent and age-dependent impacts of stress on the HPG axis.

2.1 Experimental Procedures

2.1.1 Animals

80 male and 80 female CD-1 mice arrived at 3-weeks of age from Charles River Laboratory. Upon arrival, mice were housed in all-male or all-female rooms in cages of either 2 or 3 mice. The polycarbonate cages (17 cm x 28 cm x 12 cm [height x width x length]) were lined with corn cob bedding, one cardboard hut (CAT# XKA2455, Ketchum, Brockville, CA), and one piece of nestlet (Ct# NES3600, Ancare, Bellmore, USA). Room temperature and

humidity were kept constant (24 +/- 2 °C and 40%, respectively). Mice had *ad libitum* access to food (Teklad Global Diets, Envigo) and water throughout the experiment. A reversed light/dark cycle from 8:00pm to 10:00am was maintained. Dusk and dawn were induced gradually over a period of one hour. The Animal Care Committee of the University of Ottawa approved all procedures.

2.1.2 Lipopolysaccharide (LPS) treatment

Mice were treated with either sterile saline or LPS at 6 (pubertal stress sensitive period) or 10 weeks of age (adulthood). Lyophilized LPS (from E. coli serotype 026:B6; No. L3755; Sigma Chemical Co., St. Louis, USA) was dissolved in sterile saline at a concentration of 0.2 mg/ml. Sterile saline or 1.5 mg/kg LPS was injected intraperitoneally at the end of the light phase between 9am and 9:30am. This dose was selected because it induces a sickness response and causes enduring impairments to brain and behavior when administered at 6 weeks of age (Ismail & Blaustein, 2013; Laroche et al., 2009a). Pubertal onset was determined through vaginal opening in females and scrotal size in males, since preputial descent is not clearly observable in male mice. Previous findings from our laboratory have shown that females display vaginal opening on average at 30 days of age and begin estrous cycling around 60 days of age and scrotal measurement reaches adult size around 65 days of age, suggesting that 6-week-old mice are still pubertal, while 10-week-old mice have reached adulthood (Murray et al. 2023)

2.1.3 Euthanasia and tissue collection

Regardless of the condition, all mice were euthanized between 5pm and 6pm (seven to eight hours after the onset of the dark phase) with an intraperitoneal injection of 500mg/kg intraperitoneal injection of Euthanyl (sodium pentobarbital USP; Cat# 11940222, Bimeda-MTC Animal Health Inc, Quebec, Canada). Following the injection, we confirmed the absence of a

pedal withdrawal reflex and collected trunk blood and extracted the brain. Trunk blood was collected into Microvette CB 300 K2E blood extraction tubes (Sarstedt AG & Co, Nümbrecht, Germany) that were coated with an anti-coagulant, EDTA. Tubes were temporarily kept in the fridge at 4 °C. Blood samples were then centrifuged at 1000×*g* at 20°C for 15 min. Plasma was extracted and stored in aliquots at -80 °C.]. The brain was extracted from the skull and immersed into liquid nitrogen for 10 seconds. Once flash frozen, the brain was packaged in tinfoil and stored at -80°C. Frozen brains were sliced into 300µm sections using a Leica Cryostat. The slices were mounted onto Fisherbrand Superfrost Plus microscope slides and immediately returned to the -80°C freezer. The entire hypothalamus was removed from the brain slices and placed into RNA-free 1.5ml Eppendorf tubes before being returned to the -80°C freezer. The samples were divided for processing with either PCR or Western Blot.

2.1.4 Sickness behavior

Sickness behavior was monitored in all mice 2, 4, 8, and adult mice at 24- and 48-hours following treatment. Symptoms of sickness behavior, including lethargy (reduced locomotion), huddling (curled body posture), ptosis (drooping eyelids), and piloerection (erection of fur), were assessed by two raters that were blind to treatment conditions. A score was assigned to each mouse following each assessment, corresponding to the number of symptoms displayed (one symptom = 1, two symptoms = 2, three symptoms = 3, four symptoms = 4 (Kolmogorova et al., 2017). Sickness scores were averaged from the two raters prior to statistical analysis.

2.1.5 Real-time quantitative polymerase chain reaction (RT-qPCR)

Messenger RNA was extracted from frozen hypothalamic tissue using Isol-RNA lysis Reagent (Cat. No. 2302700, Fisher Scientific, Waltham, USA). After RNA was extracted from each sample, complementary DNA synthesis was performed using QuantiTect Reverse

Transcription kit (Cat. No. 205311, Qiagen, Toronto, Canada), which included a gDNA removal step. β-actin (Accession No. NM007393; forward (F): GAACCCTAAGGCCAACCGTG, reverse (R): GGTACGACCAGAGGCATACAGG), *Kiss1* (Accession No. NM178260; F: AATGCCTGGGAAAAGGAATCG, R: CATGGCGACGACCTACGAG), and *Kiss1r* (Accession No. NM053244; F: CTACATCCAGCAGGTCTCGGT, R: CCCAGATGCTGAGGCTGACA) primers were all obtained from Integrated DNA Technologies. Each primer was diluted to a final concentration of 0.5 uM for use in real-time qPCR with SSoFast EvaGreen Supermix (Cat. No. 1725202, Bio-Rad, Montreal, Canada), 10ul reaction mix was used following the manufacture's instruction. Annealing temperature optimization and evaluation of qPCR amplification efficiency were performed using pooled cDNA samples. Primer specificity and efficiency were assessed by conducting qPCR reactions with serially diluted cDNA using the standard curve method. Only primers that exhibited an efficiency between 97-103%, produced no primer-dimer artifacts and correct amplicon size, as confirmed by visualizing amplicons on 2% agarose gels, were utilized in this study. All primers were run at an enzyme activation temperature of 95°C for 30 seconds, a denaturation temperature of 95°C for 5 seconds, and an annealing temperature of 67.5°C, 60.5°C, and 63°C for B-actin, *Kiss1* and *Kiss1r*, respectively. A melt curve spanning from 65°C to 95°C was used, all primers produce a single melting curve peak at 83.0°C, 85.0°C, and 88.0°C for B-actin, *Kiss1* and *Kiss1r*, respectively. The results of the real-time qPCR reaction were viewed using Bio-Rad CFX Maestro version 3. Three technical replicates were performed for each sample, and the mean of two samples with standard deviations less than 0.400 was used for analysis. β-actin served as the reference gene when comparing gene expression between groups. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative changes in gene expression where the saline-treated acute condition acts as

the standardized control for corresponding age and sex conditions. No significant variance in β-actin cycle quantification values existed between experimental groups.

2.1.6 Multiplex immunoassay

The plasma concentrations of LH, FSH (Millipore Cat# MPTMAG-49K; RRID:AB_2811194), IL-6, and TNFα (Millipore Cat# MAGE1MAG-25K; RRID:AB_2921329), were analyzed using a MILLIPLEX® multiplex immunoassay. Blood plasma was removed from the -80°C storage freezer, thawed, and diluted two-fold using the assay buffer. We added plasma matrix, standards, plasma samples, quality controls and multiplexing magnetic beads to the wells. The plate was then covered and left to incubate overnight on a horizontal shaker at 2-8°C. After washing, detection antibodies were added. The plate was incubated and streptavidin-phycoerythrin was added prior to a 30-minute incubation. The plate was washed, the beads were resuspended, and analytes were quantified with a MAGPIX system. The intra-assay coefficients of variance were <10% for IL-6 and TNFα and <15% for LH and FSH. The inter-assay coefficients of variance were <20% for all analytes. Percent recovery is 98% (IL-6) 106% (TNFα) and 96% (LH and FSH). There was no or negligible cross-reactivity between the antibodies. Assay sensitivity, measured in minimum detectable concentration, was 1.8 pg/mL for IL-6, 1.7 pg/mL for TNFα, 4.92 pg/mL for LH, and 9.5 pg/mL for FSH. All samples evaluated were above the detection limit.

2.1.7 Western blot

The dissected tissues were homogenized in radioimmunoprecipitation assay buffer (RIPA; 150 mM sodium chloride / 50mM Tris / 1% Triton X-100 / 0.5% sodium deoxycholate / 0.1% sodium dodecyl sulfate) containing phosphatase inhibitor (Roche PhosSTOP; Millipore Sigma; cat: 04906837001) and protein inhibitor (Roche cOmplete [KS15] ULTRA; 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 were collected to assay total protein concentrations using the Pierce Bicinchoninic Acid Assay (BCA) protein assay kit (Thermo Fisher Scientific). 30 ug of extracted protein was mixed with Laemmli sample buffer and then heated to 95°C for 5 min. Samples were electrophoresed on 12% polyacrylamide gels in duplicate (TGX Stain-Free FastCast Acrylamide Kit;Bio-Rad; cat: 1610185) using Mini-PROTEAN 3 Dodeca Cell system (Bio-Rad). A pooled protein sampler was loaded to each of the gels to account for inter-gel variation. The gel image was acquired with ChemiDoc™ XRS+ System (Bio-Rad) and it was used for total protein normalization (TPN) quantification instead of running a housekeeping protein as a reference as TPN as is less susceptible to interference from the signal intensity and systematic experimental skewing of the housekeeping protein (Eaton et al., 2013; Fosang & Colbran, 2015). The separated proteins were transferred onto 0.2µm nitrocellulose membranes with Trans-Blot® Electrophoretic Transfer Cell system (Bio-Rad) and the membranes for ERα were incubated in 4% paraformaldehyde. All membranes were blocked in 5% skim milk in phosphate buffered saline (PBS) for one hour and then incubated for 36 hours at 4°C in a solution of 5% milk/TBST (Tris-buffered saline [TBS] / 20 mM Tris-base / 137 mM NaCl/0.1% Tween-20) containing either rabbit anti-AR (1/750; (Millipore Cat# 06-680; RRID:AB_310214) or rabbit anti-ERα (1/350; Abcam Cat# ab32063; RRID:AB_732249). Following three 10-min washes with TBST, the membranes were incubated for 1 h at room temperature with goat anti-rabbit IRDye 800 secondary antibodies (1/10,000; LI-COR Biosciences Cat# 925-3221; RRID:AB_2651127) in a 5% skim milk / TBST solution. After a final set of TBST washes (3 x 10 min), the membranes were scanned using a Li-Cor Biosciences Odyssey infrared imaging system. TPN was conducted using Li-Cor Image Studio (V5.2) where

total protein intensity was measured and used to normalize target protein intensity. To control for inter-gel variation, the ratio of the normalized protein intensity between each sample to their intra-gel pooled sample was then calculated and presented as a fold change (mean of duplicate ± SEM), which was used for relative protein abundance comparison between groups. All replicates used for data analysis showed a coefficient of variation of less than 10%.

2.1.8 Procedure

At 6 (pubertal) or 10 (adult) weeks of age, male and female mice (n = 160) received either an injection of saline or LPS. Mice were monitored for sickness behavior over the next 48 hours. One cohort of mice was euthanized 8 hours (acute) following treatment (24- and 48-hour sickness behavior not recorded in these mice), while the other cohort was euthanized 4 weeks (enduring) following saline or LPS treatment. Brains were extracted and trunk blood was collected for analysis. See Figure 1 for the timeline (Fig. 1).

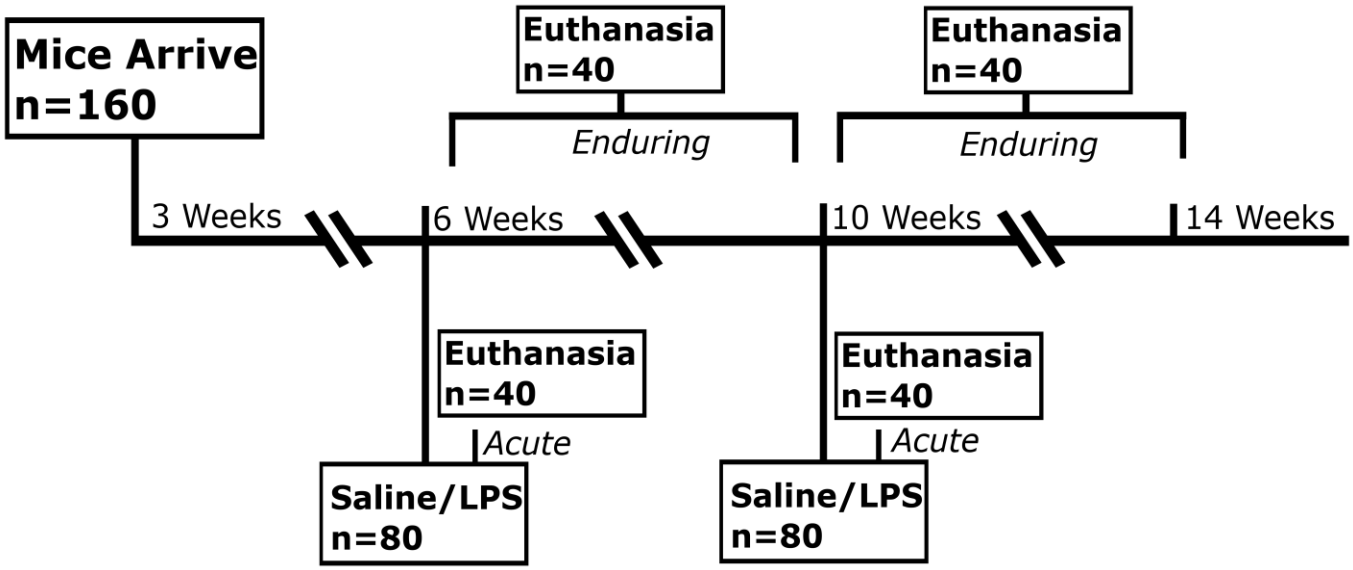

Fig. 1. Experimental timeline. Experimental timeline of saline- and LPS-treated, pubertal and adult male and female mice.

2.1.9 Statistical analysis

A power analysis using G*Power (V3.1) determined our sample sizes were sufficiently powered ($\beta > 0.80$). Statistical analyses were performed using IBM SPSS (V23). All data were initially screened for outliers using box plots. Outliers were winsorized to the nearest extreme value in the distribution. No more than one data point per group was winsorized. A four-way mixed analysis of variance (ANOVA) was used to examine the within-subject effect of time (2, 4, 6, 8, 24 and 48 hours), and the between-subject effects of sex (male or female), treatment (saline or LPS), and age (pubertal or adult) on sickness behavior. The Greenhouse-Geisser correction was applied to all *F*-values that violated Mauchly's test of sphericity for mixed ANOVA (< 0.75). A four-way fixed-effect ANOVA was used to examine the effect of sex (male or female), treatment (saline or LPS), age (pubertal or adult), and time (acute or enduring) to examine *Kiss1*, *Kiss1r*, AR, and ERα expressions in the brain, as well as LH, FSH, TNFα, and IL-6 concentrations in the blood plasma. Partial eta-squared (η_p^2) was used to calculate the effect size of all significant main effects and interactions (Richardson, 2011). Significant main effects and interactions are reported in the results section. Significant interactions were followed up with pairwise comparisons using a Bonferroni correction. A threshold of $p < 0.05$ was set for statistical significance.

3.1 Results

3.1.1 Sickness behavior

Compared to pubertal mice, adult LPS-treated female mice displayed greater sickness behaviors at 2 (mean difference [*MD*] = 1.16, standard error [*SE*] = 0.19, $p < 0.05$) hours

following treatment. However, this difference was reversed at 6 hours and 8 hours (*MD* = 0.37, *SE* = 0.20, $p < 0.05$; *MD* = 0.45, *SE* = 0.09, respectively post-treatment). Adult LPS-treated males showed greater sickness behavior than pubertal mice at 8 (*MD* = 0.25, *SE* = 0.09, $p < 0.05$) and 48 hours (*MD* = 0.90, *SE* = 0.12, $p < 0.05$) post-treatment. Both adult LPS-treated male and female mice showed greater sickness behavior than pubertal counterparts at 24 hours (*MD* = 0.65, *SE* = 0.12, $p < 0.05$; *MD* = 0.36, *SE* = 0.18, $p < 0.05$, respectively). Pubertal LPS-treated female mice showed greater sickness behavior than males at 4 (*MD* = 0.91, *SE* = 0.2, $p < 0.05$), 6 (*MD* = 0.32, *SE* = 0.16, $p < 0.05$) and 8 hours post-treatment (*MD* = 0.07, *SE* = 0.12, $p < 0.05$). Adult LPS-treated females showed greater sickness behavior than males at 2 (*MD* = 0.40, *SE* = 0.18, $p < 0.05$), and 4 hours post-treatment (*MD* = 0.60, *SE* = 0.19, $p < 0.05$), whereas adult LPS-treated males showed greater sickness behavior than females at 8 (*MD* = 0.45, *SE* = 0.09, $p < 0.05$) and 48 hours post-treatment (*MD* = 0.70, *SE* = 0.12, $p < 0.05$) (Fig. 2).

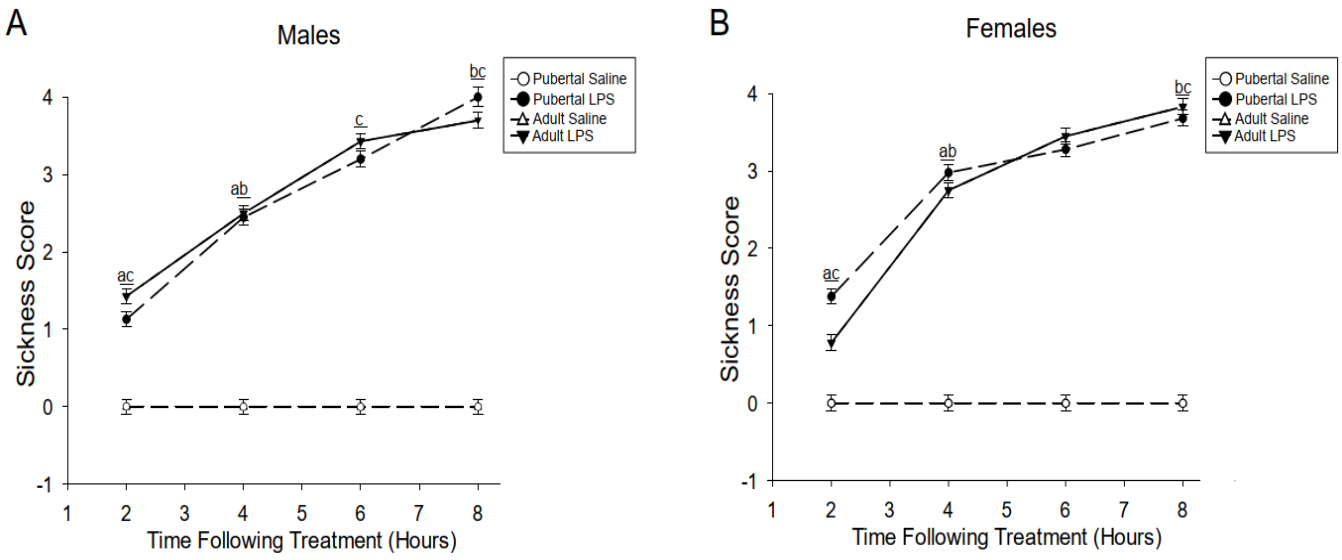

Fig. 2. Sickness behavior symptoms. Mean (±SEM) sickness scores of male (A) and female (B), mice treated with saline or lipopolysaccharide (LPS). The asterisk (*) denotes significant

differences between LPS-treated pubertal and adult groups. The *a* denotes significant differences between male and female pubertal mice. The *b* denotes significant differences between male and female adult mice.

3.1.2 Interleukin-6 (IL-6) concentration

LPS-treated mice displayed significantly greater concentrations of IL-6 compared to saline-treated mice ($F_{1,144} = 62.20$, $p < 0.05$, $\eta_p^2 = 0.30$). Adult mice also displayed greater concentrations of IL-6 compared to pubertal mice ($F_{1,144} = 19.51$, $p < 0.05$, $\eta_p^2 = 0.12$). Pubertal and adult LPS-treated mice showed significantly greater IL-6 concentration 8 hours post-treatment compared to their saline-treated counterparts (*MD* = 3813.77, *SE* = 1107.21, $p < 0.05$; *MD* = 13674.26, *SE* = 1107.21, $p < 0.05$, respectively). Additionally, LPS-treated adult mice showed increased IL-6 concentration 8 hours post-treatment compared to pubertal counterparts (*MD* = 9843.15, *SE* = 782.91, $p < 0.05$). Finally, LPS-treated pubertal and adult mice displayed greater IL-6 concentration at 8 hours post-treatment than at 4 weeks post-treatment (*MD* = 3870.51, *SE* = 782.91, $p < 0.05$; *MD* = 13748.21, *SE* = 1107.21, $p < 0.05$) (Fig. 3 (A, B)).

3.1.3 Tumor necrosis factor alpha (TNFα) concentration

The four-way ANOVA revealed significantly greater TNFα concentrations in LPS-treated mice compared to saline-treated counterparts($F_{1,144} = 139.02$, $p < 0.05$, $\eta_p^2 = 0.49$). The pairwise comparisons showed that LPS-treated pubertal and adult mice displayed significantly greater TNFα concentration 8 hours post-treatment compared to saline-treated counterparts (*MD* = 47.56, *SE* = 5.25, $p < 0.05$; *MD* = 81.88, *SE* = 5.25, $p < 0.05$, respectively). Furthermore, LPS-treated adults displayed significantly greater TNFα concentration 8 hours post-treatment compared to pubertal mice (*MD* = 32.59, *SE* = 5.25, $p < 0.05$). Both LPS-treated pubertal and

adult mice displayed significantly greater TNFα concentration 8 hours post-treatment than at 4 weeks post-treatment (*MD* = 47.05, *SE* = 5.25, $p < 0.05$; *MD* = 84.87, *SE* = 5.25, $p < 0.05$, respectively) (Fig. 3 (C, D)).

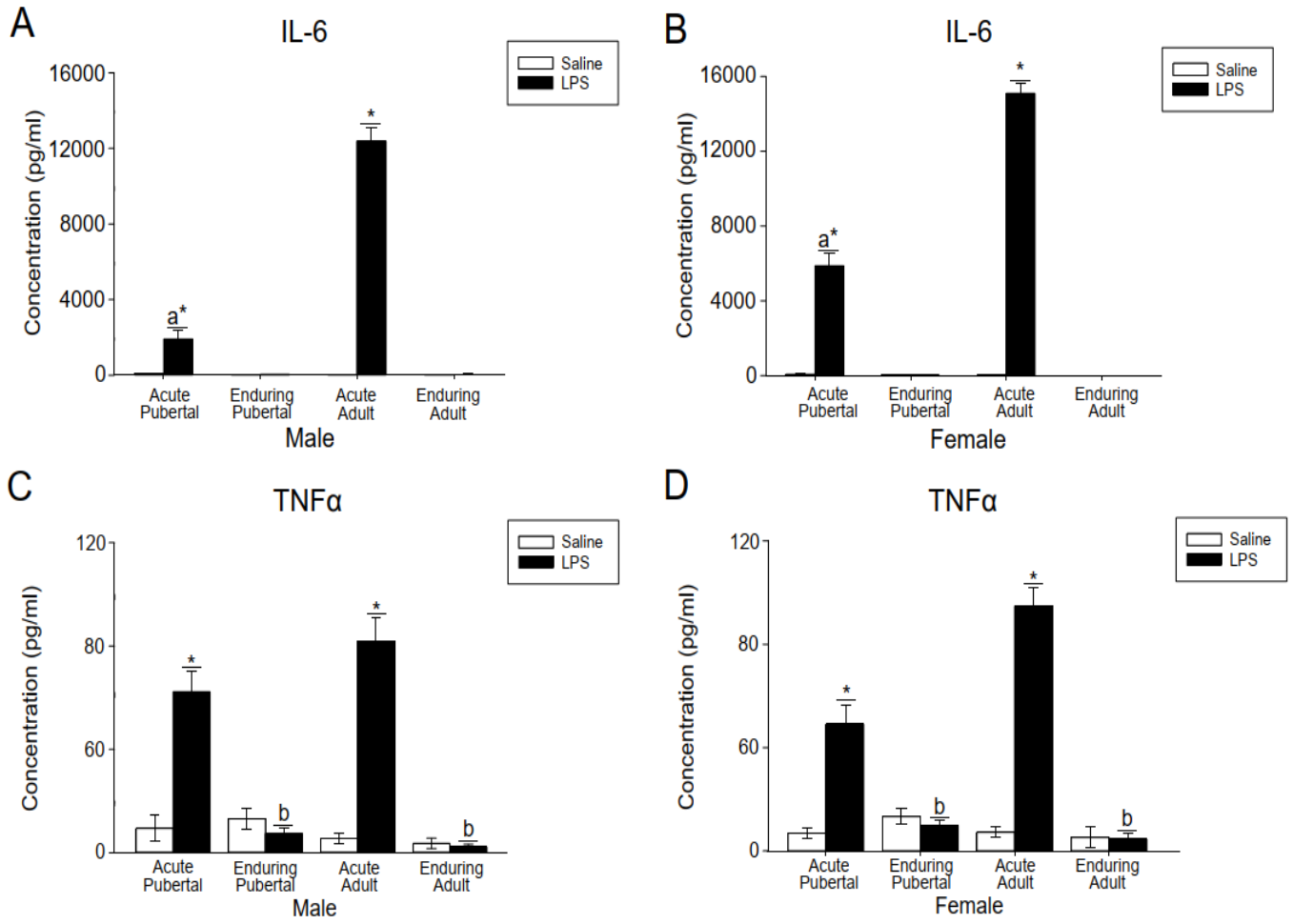

Fig. 3. Cytokine concentration. Mean (±SEM) acute and enduring interleukin-6 (IL-6) concentration (pg/ml) in plasma of male (A) and female (B) and tumor necrosis factor alpha (TNFα) concentration (pg/ml) in male (C) and female (D) pubertal and adult mice treated with either saline or lipopolysaccharide (LPS). The asterisk (*) denotes significant differences between saline and LPS groups ($p < 0.05$). The *a* denotes significant differences between acute

LPS-treated pubertal and adult mice. The *b* denotes significant differences between enduring LPS-treated pubertal and adult groups.

3.1.4 Hypothalamic kisspeptin (*Kiss1*) mRNA

The four-way ANOVA revealed significantly less hypothalamic *Kiss1* mRNA expression in LPS-treated mice compared to saline-treated mice ($F_{1,63} = 16.64$, $p < 0.05$, $\eta_p^2 = 0.21$). Moreover, adult mice displayed greater *Kiss1* mRNA expression compared to pubertal mice ($F_{1,63} = 9.70$, $p < 0.05$, $\eta_p^2 = 0.13$). Pubertal LPS-treated mice showed significantly less *Kiss1* mRNA expression in the hypothalamus compared to saline-treated (*MD* = 0.63, *SE* = 0.12, $p < 0.05$) and adult counterparts (*MD* = 0.55, *SE* = 0.12, $p < 0.05$). LPS-treated mice showed significantly less *Kiss1* mRNA expression 8 hours post-treatment compared to 4 weeks post-treatment (*MD* = 0.36, *SE* = 0.12, $p < 0.05$). LPS-treated pubertal mice displayed significantly less *Kiss1* mRNA expression than adult counterparts 4 weeks following LPS treatment (*MD* = 0.44, *SE* = 0.12, $p < 0.05$). Finally, LPS-treated adult mice showed significantly less *Kiss1* mRNA expression 8 hours after treatment compared to 4 weeks after treatment (*MD* = 0.31, *SE* = 0.12, $p < 0.05$) (Fig. 4 (A, B)).

3.1.5 Hypothalamic kisspeptin receptor (*Kiss1r*) mRNA

The four-way ANOVA revealed significantly greater hypothalamic *Kiss1r* mRNA expression in females compared to males. ($F_{1,63} = 11.43$, $p < 0.05$, $\eta_p^2 = 0.15$ Regardless of age and time following treatment, LPS-treated males displayed less *Kiss1r* mRNA expression compared to females (*MD* = 0.52, *SE* = 0.11, $p < 0.05$) and saline-treated male counterparts (*MD* = 0.39, *SE* = 0.11, $p < 0.05$) (Fig. 4 (C, D)).

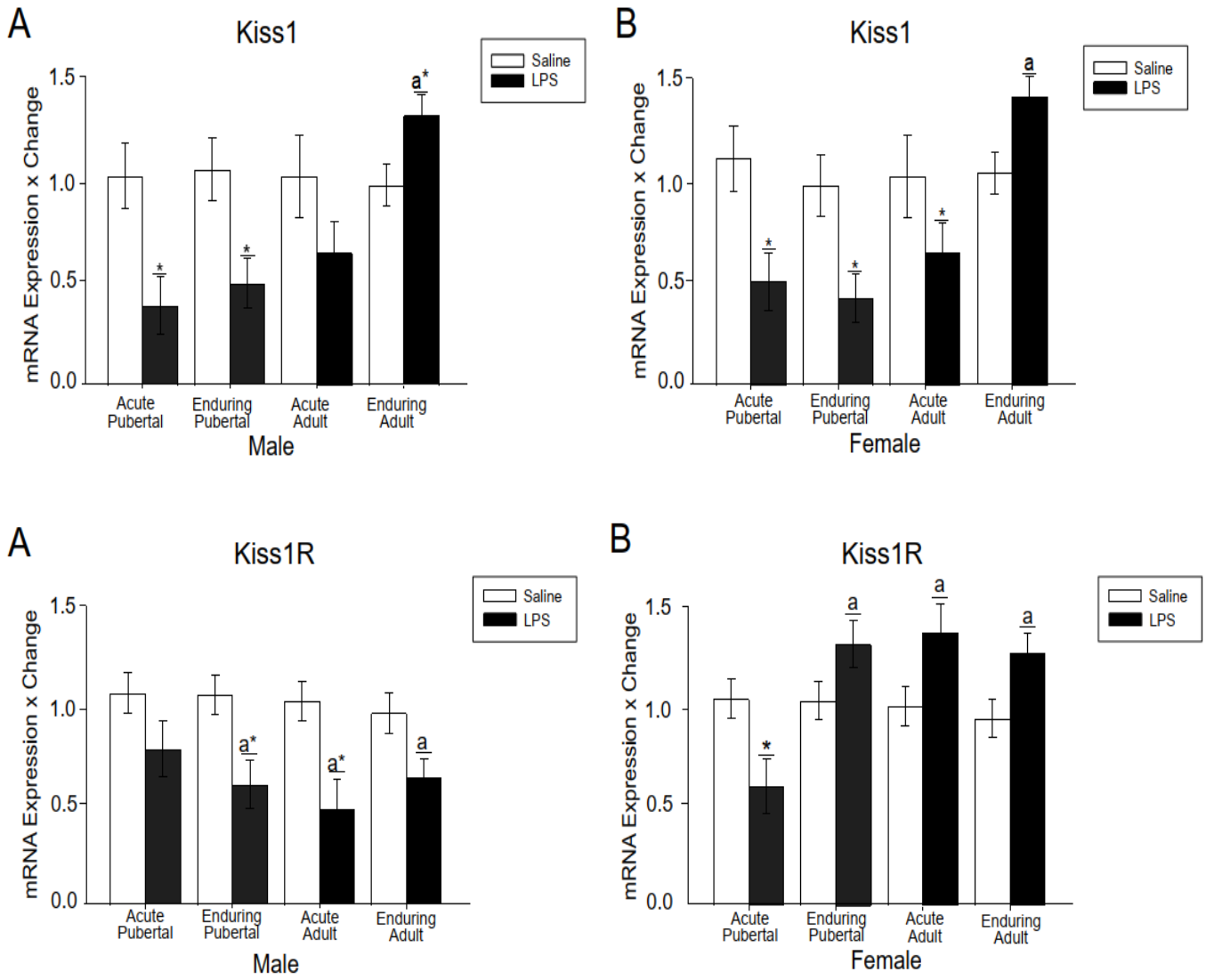

Fig. 4. Hypothalamic *Kiss1* and *Kiss1r* mRNA expression. Mean (±SEM) fold change in acute and enduring hypothalamic *Kiss1* mRNA expression in male (A) and female (B) and *Kiss1r* mRNA expression in male (C) and female (D) mice treated with either saline or lipopolysaccharide (LPS). The asterisk (*) denotes significant differences between saline and LPS groups ($p < 0.05$). The *a* denotes significant differences between enduring LPS-treated pubertal and adult groups. The *b* denotes significant differences between acute LPS-treated adult and enduring groups. The *c* denotes significant differences between males and females.

3.1.6 Luteinizing hormone (LH) concentration

The ANOVA revealed significantly lower LH concentrations in LPS-treated mice compared to saline-treated mice ($F_{1,144} = 20.08$, $p < 0.05$, $\eta_p^2 = 0.12$). Additionally, mice euthanized 4 weeks post-treatment showed greater LH concentrations compared to mice euthanized 8 hours post-treatment ($F_{1,144} = 7.75$, $p < 0.05$, $\eta_p^2 = 0.05$). Regardless of sex and age, acute LPS-treated mice displayed significantly lower LH plasma concentration compared to saline-treated mice (*MD* = 74.05, *SE* = 12.46, $p < 0.05$). Additionally, acute LPS-treated mice displayed significantly lower LH plasma concentrations compared to LPS-treated mice 4 weeks post-treatment (*MD* = 59.10, *SE* = 12.47, $p < 0.05$) (Fig. 5).

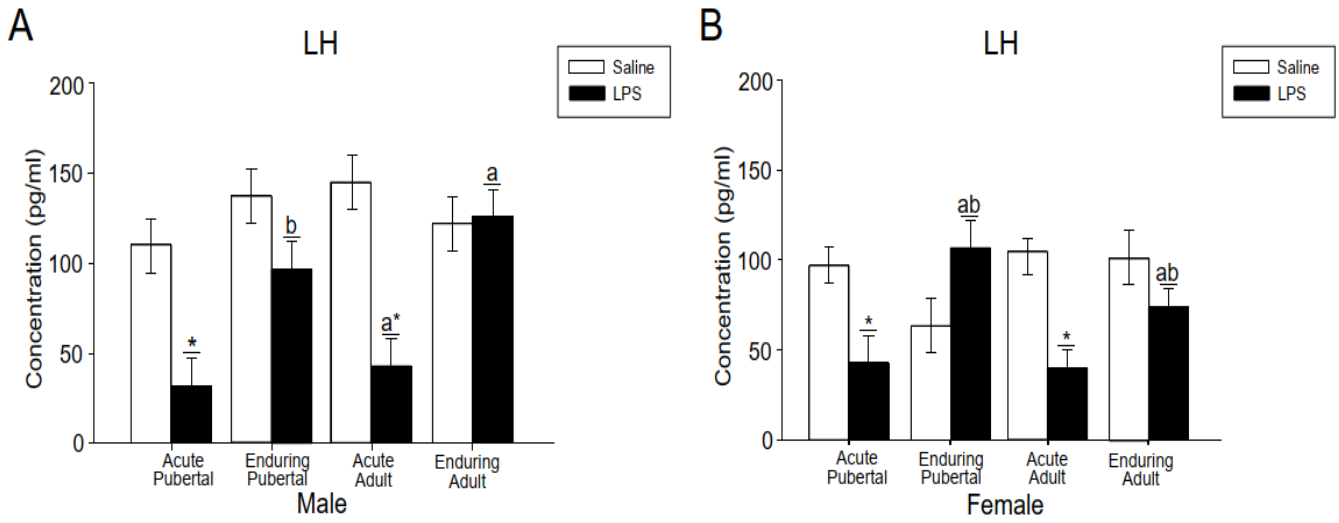

Fig. 5. LH concentration. Mean (±SEM) acute and enduring luteinizing hormone (LH) concentration (pg/ml) in the plasma of male (A) and female (B) pubertal and adult mice treated with saline or LPS. The asterisk (*) denotes significant differences between saline and LPS groups ($p < 0.05$). The *a* denotes significant differences between acute and enduring LPS-treated groups.

3.1.7 Follicle stimulating hormone (FSH) concentration

The four-way ANOVA revealed significantly greater FSH concentrations in males compared to females ($F_{1,144} = 315.27$, $p < 0.05$, $\eta_p^2 = 0.69$) and greater FSH concentrations in pubertal mice compared to adult mice ($F_{1,144} = 57.66$, $p < 0.05$, $\eta_p^2 = 0.29$).Pubertal and adult males showed significantly greater FSH concentrations than their female counterparts (*MD* = 6938.77, *SE* = 393.66, $p < 0.05$; *MD* = 2946.30, *SE* = 393.66, $p < 0.05$, respectively). Additionally, pubertal males had significantly greater FSH concentration than adult counterparts (*MD* = 4109.89, *SE* = 393.66, $p < 0.05$; Fig. 6).

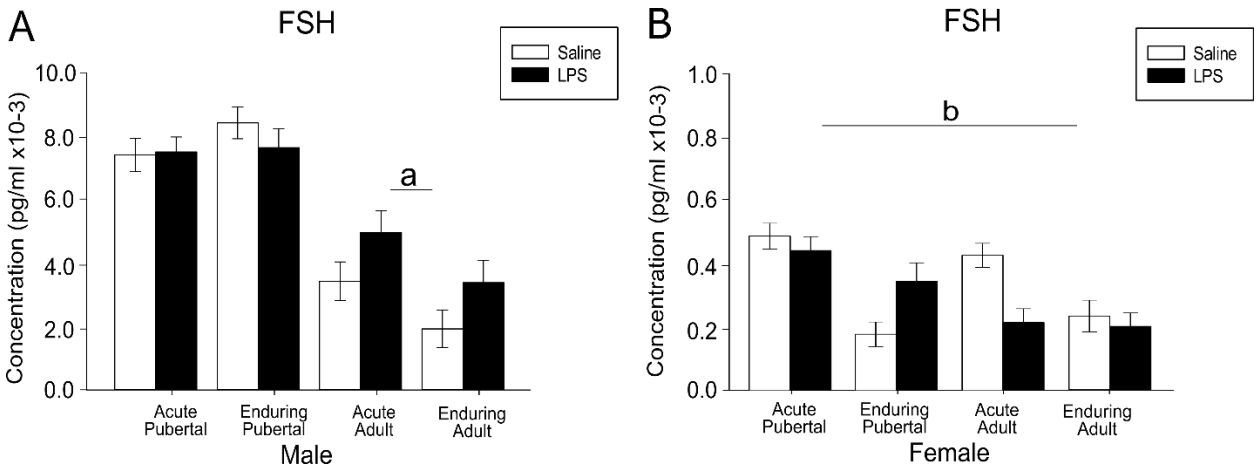

Fig. 6. FSH concentration. Mean (±SEM) acute and enduring follicle stimulating hormone (FSH) concentration (pg/ml) in the blood plasma of male (A) and female (B) pubertal and adult mice treated with saline or LPS. The *a* denotes significant differences between pubertal and adult males. The *b* denotes significant differences between males and females.

3.1.8 Hypothalamic androgen receptor (AR) protein expression

The four-way ANOVA revealed significantly less AR expression in LPS-treated mice compared to saline-treated mice ($F_{1,60} = 4.52$, $p < 0.05$, $\eta_p^2 = 0.07$).LPS-treated males showed reduced AR expression compared to females and saline-treated male counterparts (*MD* = 0.22, *SE* = 0.08, $p < 0.05$; *MD* = 0.24, *SE* = 0.08, $p < 0.05$, respectively; Fig 7).

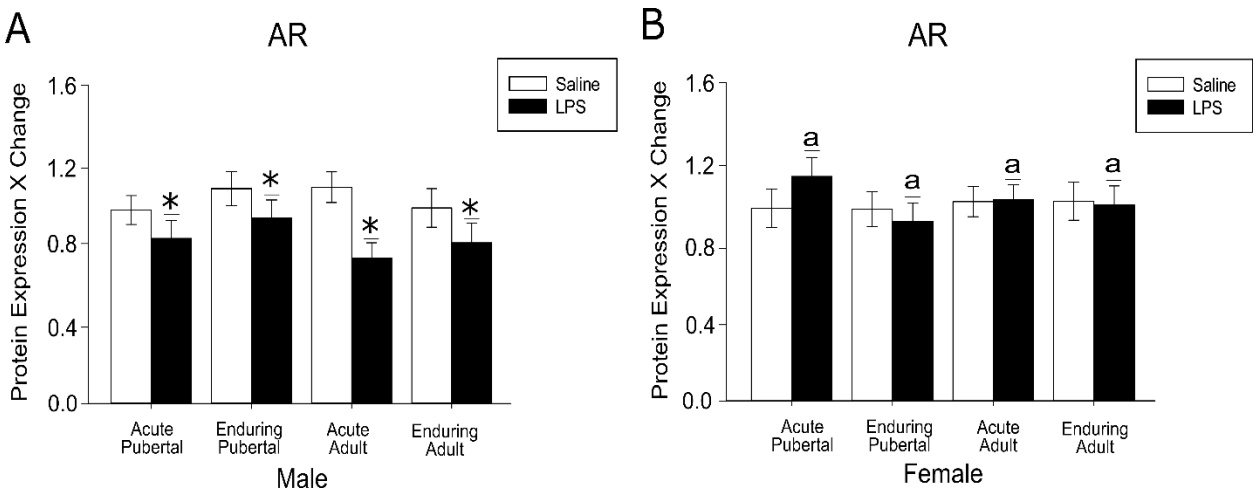

Fig. 7. Hypothalamic AR protein expression. Mean (±SEM) fold change in acute and enduring hypothalamic androgen receptor (AR) protein expression in male (A) and female (B) pubertal and adult mice treated with either saline or lipopolysaccharide (LPS). (C) Relative fold change in male AR protein expression using total protein normalization. *APS* = acute pubertal saline, *APL* = acute pubertal LPS, *EPS* = enduring pubertal saline *EPL* = enduring pubertal LPS *AAS* = enduring pubertal saline, *AAL* = acute adult LPS, *EAS* = enduring adult saline, *EAL* = enduring adult LPS. (D) Relative fold change in female AR protein expression using total normalization. The asterisk (*) denotes significant differences between saline and LPS treatment ($p < 0.05$). The *a* denotes significant differences between males and females.

3.1.9 Hypothalamic estrogen receptor alpha (ERα) protein expression

The four-way ANOVA revealed significantly less ERα expression in LPS-treated mice compare to saline-treated mice ($F_{1,63} = 31.77$, $p < 0.05$, $\eta_p^2 = 0.34$). Moreover, adult mice displayed greater ERα expression compared to pubertal mice ($F_{1,63} = 6.35$, $p < 0.05$, $\eta_p^2 = 0.09$). Furthermore, mice euthanized 4 weeks post-treatment showed greater ERα expression compared

to mice euthanized 8 hours post-treatment($F_{1,63} = 5.04$, $p < 0.05$, $\eta_p^2 = 0.07$). LPS treatment reduced ERα expression in pubertal males and females, and adult females 8 hours post-treatment compared to saline-treated counterparts (*MD* = 0.32, *SE* = 0.09, $p < 0.05$; *MD* = 0.32, *SE* = 0.09, $p < 0.05$; *MD* = 0.21, *SE* = 0.09, $p < 0.05$, respectively). Additionally, LPS-treated pubertal females showed significantly less ER*α* expression 4 weeks post-treatment compared to saline-treated counterparts (*MD* = 0.36, *SE* = 0.09, $p < 0.05$). Furthermore, LPS-treated adult females displayed significantly less ERα expression compared to male counterparts 4 weeks post-treatment (*MD* = 0.26, *SE* = 0.10, $p < 0.05$). Furthermore, both LPS-treated pubertal males and pubertal females showed significantly less ERα expression 8 hours post-treatment compared to adult male and adult female counterparts (*MD* = 0.23, *SE* = 0.09, $p < 0.05$; *MD* = 0.21, *SE* = 0.09, $p < 0.05$, respectively). Additionally, LPS-treated pubertal females showed significantly less ERα expression compared to adult female counterparts 4 weeks post-treatment (*MD* = 0.36, *SE* = 1.00, $p < 0.05$) (Fig. 8).

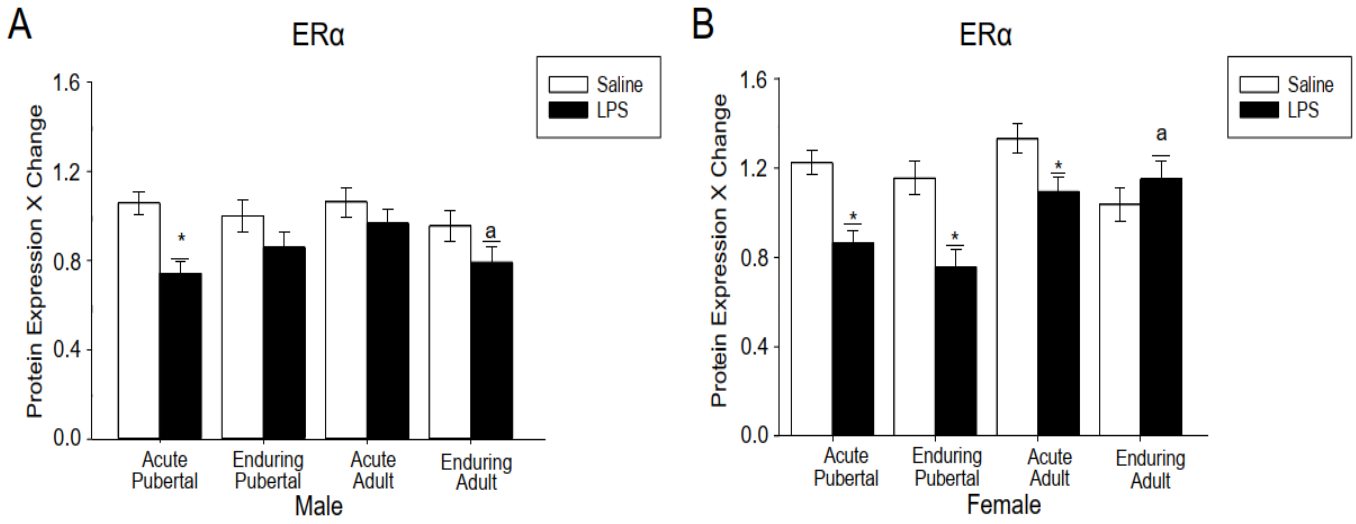

Fig. 8. Hypothalamic ERα protein expression. Mean (±SEM) fold change in acute and enduring hypothalamic estrogen receptor alpha (ERα) protein expression in male (A) and female

(B) pubertal and adult mice treated with either saline or lipopolysaccharide (LPS). (C) Relative fold change in male ERα protein expression using total protein normalization. *APS* = acute pubertal saline, *APL* = acute pubertal LPS, *EPS* = enduring pubertal saline *EPL* = enduring pubertal LPS *AAS* = enduring pubertal saline, *AAL* = acute adult LPS, *EAS* = enduring adult saline, *EAL* = enduring adult LPS. (D) Relative fold change in female ERα protein expression using total normalization. The asterisk (*) denotes significant differences between saline and LPS groups ($p < 0.05$). The *a* denotes significant differences between acute pubertal and adult groups. The *b* denotes significant differences between pubertal and adult mice. The *c* denotes significant differences between males and females.

4.1 Discussion

LPS treatment induces innate immune stress response by activating the HPA axis and consequently downregulating the HPG axis to maintain the allostatic load and redirect energy towards other systems necessary for survival (McEwen & Stellar, 1993). The aim of the current study was to examine how LPS treatment during puberty and adulthood may cause short- and long-lasting changes in HPG axis functioning in male and female mice. We found that a single dose of LPS during puberty causes sex- and age-dependent increases in sickness behavior, as well as short- or long-lasting downregulation of *Kiss1*, LH, and ERα expressions. Additionally, we observed acute downregulation of *Kiss1* and *Kiss1r* mRNA expressions, LH concentration, and ERα and AR protein expressions, as well as upregulation of IL-6 and TNFα concentrations in LPS-treated pubertal mice. However, in adult mice, LPS treatment only induced short-lasting downregulation of *Kiss1r* mRNA expression and FSH concentration, and upregulation of IL-6 and TNFα concentrations. In addition to highlighting the sensitivity of the pubertal period to immune stress, this work is the first to report that pubertal LPS treatment causes enduring

downregulation of the HPG axis and provides a possible mechanism through which pubertal LPS treatment induces an enduring sex-dependent suppression of the HPG axis.

As expected, LPS-treated mice displayed more sickness behaviors compared to saline-treated mice at least up to 8 hours following treatment. Furthermore, adult male mice showed more sickness behaviors following LPS treatment compared to adult female and pubertal male counterparts 8 hours post-treatment. Additionally, adult female mice displayed more sickness behaviors 2 hours following LPS treatment compared to their pubertal counterparts. These findings support our hypotheses and are consistent with previous findings showing age- and sex-specific effects of LPS treatment on sickness behaviors (Cai et al., 2016; Sharma et al., 2018). Such age and sex differences in response to an immune challenge may be attributed to the effect of gonadal steroid hormones on the innate immune system (Cai et al., 2016). In rodents, testosterone has been identified as an immune suppressing hormone, while estradiol is known to enhance the immune system (Wichmann et al., 1997). The immune modulating properties of estradiol likely increase resistance to infection, particularly against bacterial and parasitic agents (Klein, 2000). Although an enhanced immune response culminates in greater pathogen defense and rapid recovery, it also increases the propensity to develop autoimmune disorders in females (Rider & Abdou, 2001). In addition to sex-based immune differences, previous findings from our laboratory indicate that there are age differences in immune response, as pubertal 6-week-old mice are hyporesponsive and display less sickness behavior following LPS treatment compared to adults (Cai et al., 2016; Girard-Joyal et al., 2015; Sharma et al., 2019). Taken together, these findings show that pubertal gonadal steroid hormones contribute to the development and maturation of the immune system and give rise to age and sex differences in sickness behavior following LPS treatment.

We also found a downregulation of the HPG axis through the suppression of *Kiss1* and *Kiss1r* mRNA expressions and LH concentration in pubertal males and females, and adult male mice within 8 hours of LPS treatment. These findings replicate our previous results (Smith, Murray, Chandrasegaram, et al., 2021) and also show that in adult mice LPS treatment reduces *Kiss1* mRNA expression and LH concentration only, suggesting that adult mice are less susceptible to immune stress-induced decreases in *Kiss1r* mRNA expression. These findings are in accordance with previous work showing that LPS-treated adult female rats display a decrease in *Kiss1* mRNA expression, but no change in *Kiss1r* mRNA expression (Iwasa et al., 2008). Although the expression of the receptor remains stable after LPS exposure, it is not yet clear whether the sensitivity of the receptor is altered. One potential limiting factor of using LPS as a stressor is its effect on metabolism. LPS-induced sickness decreases food intake and induces a 10% body weight loss over the first 48 hours following treatment. Considering that previous findings have shown that food deprivation results in kisspeptin downregulation (Castellano et al., 2005), it is possible that the acute downregulation of the HPG axis may be partially attributed to the effects of LPS on metabolism. However, since the effects of LPS on metabolism subside 48 hours after treatment, we do not expect any confounding effects. Food deprivation studies typically involve 48 hours or more of complete food deprivation for kisspeptin downregulation to take effect. In contrast, our LPS dosage shows peak sickness behavior at 8 hours post treatment. By 24 hours post-treatment sickness behavior has substantially waned and mice resume eating. Nevertheless, the resiliency of *Kiss1r* in adulthood may convey that HPG axis downregulation following immune stress exposure is related to an inhibition in kisspeptin peptide production rather than a dysregulation in receptor availability.

We also found an acute downregulation of hypothalamic ERα expression following LPS treatment in pubertal males and females, as well as adult females. Moreover, we observed an acute downregulation of AR expression only in pubertal male mice following LPS treatment. In female rats, uterine LPS treatment reduced overall graafian follicle count and peripheral levels of ERα up to three days after treatment (Magata et al., 2022). Similarly, exposure to chronic mild stress reduced AR expression in adult male mice (Hung et al., 2019). These data further emphasize the importance of ERα and AR for normal HPG axis functioning and highlight their vulnerability to stressors especially during the pubertal period. ERα and AR play a critical role in regulating the HPG axis and are colocalized in kisspeptin producing neurons (Lehmann et al., 2013). Ovariectomized, adult female, kisspeptin-specific ERα knockout (KERKO) mice display robust increases in glutamatergic signaling in the arcuate nucleus (ARC) which is restored to baseline signaling with estradiol treatment (Wang et al., 2018). Similarly, non-ovariectomized KERKO mice exhibit greater LH pulse frequencies, indicating that kisspeptin-specific ERα are integral in managing HPG axis activity (Stephens et al., 2016; Wang et al., 2018). Testosterone also regulates kisspeptin in male mice. *Kiss1* knockout male rats show feminine sexual behaviors regardless of testosterone treatment (Nakamura et al., 2016). However, intracerebral kisspeptin injection increases testosterone concentration in male rats (Thompson et al., 2004), indicating a unidirectional HPG axis activation. These findings provide important implications for the regulatory action of sex hormones and their receptors on kisspeptin that warrants further examination to establish causality.

In corroboration with our hypothesis, we observed acute increases in the concentration of the pro-inflammatory cytokines IL-6 and TNFα, which is consistent with previous findings from our laboratory (Sharma et al., 2018). Moreover, our findings suggest that the LPS-induced

activation of the HPA axis and the immune system, and the resulting increase in pro-inflammatory cytokines may be directly involved in the downregulation of the HPG axis following LPS treatment. Intracerebral infusion of the proinflammatory cytokines IL-1α and IL-1β generates marked reductions in LH concentration in adult male rats that is reversed with treatment IL-1α/β antagonists (Ebisui et al., 1992). Similarly, adult female rats do not display LPS-induced *Kiss1* decreases when given the anti-inflammatory drug indomethacin, suggesting that inflammatory cytokines mediate kisspeptin signaling (Iwasa et al., 2008). Although the mechanism underlying the direct effects of cytokines on the HPG-axis are unknown, there is evidence that proinflammatory cytokines upregulate the production of nitric oxide and prostaglandins, which in turn inhibit GnRH release (Turnbull & Rivier, 1999). The cytokine-induced downregulation of the HPG axis is believed to be an evolutionary adaptation of prioritizing energy towards systems necessary for survival over reproduction to optimally balance the allostatic load (Segner et al., 2017; Turnbull & Rivier, 1999). However, when these allostatic shifts occur during critical periods of development, they may cause enduring negative changes in behavior (Blaustein & Ismail, 2013; Ismail et al., 2011; Kane & Ismail, 2017).

Our results also showed that pubertal LPS treatment causes enduring downregulation of the HPG axis in both sexes as measured by the long-lasting decrease in *Kiss1* and *Kiss1r* mRNA expressions and LH concentration. Interestingly, these enduring effects of LPS were limited to pubertal mice and did not extend to mice treated with LPS in adulthood. To our knowledge, we are the first to show enduring immune stress-induced suppression of the HPG axis during puberty in males and females. Others have reported similar findings following early postnatal stress exposure. For example, LPS treatment during the early postnatal period causes an enduring suppression of *Kiss1r* mRNA expression in adult female rats (Knox et al., 2009) and results in

elevated levels of IL-6 concentrations along with decreased LH levels in adult male rats (Thompson et al., 2004).

We also found important sex differences in the enduring effects of LPS treatment on the HPG axis. More specifically, pubertal females showed enduring downregulation of hypothalamic ERα expression compared to males. These findings are consistent with previous reports showing that pubertal shipping stress causes enduring reductions in ERα expression in the medial preoptic area, ARC, and ventral medial area of the hypothalamus in female mice, as well as a decrease in sexual receptivity, a behavioral indicator of HPG axis activity (Ismail et al., 2011). Taken together, these results suggest that while kisspeptin modulates the HPG axis activity in both males and females, ERα and AR may modulate HPG axis activity in a sex-specific manner and play an important role in immune stress-induced acute and enduring effects on HPG axis activity. However, it is unclear if this relationship is bidirectional or whether kisspeptin release is dependent on sex hormone receptors.

This study is the first to show that pubertal shifts in allostatic load can cause enduring sex-dependent downregulation of the HPG axis. We observed LPS-induced HPG axis dysfunction in both male and female, and pubertal and adult mice that extend beyond the hypothalamus. Specifically, pubertal LPS treatment causes increased sickness behavior and IL-6 and TNFα concentrations, and acute and enduring *Kiss1,* LH, and ERα downregulation. *Kiss1r* mRNA expression and AR expression are also decreased acutely. We did not identify any enduring changes in adults, but LPS treatment downregulated *Kiss1r mRNA* expression and FSH concentration acutely, in addition to upregulating IL-6 and TNFα concentrations. The enduring pubertal HPG axis dysfunction is a pinnacle finding that accentuates the magnitude of immune stress sensitivity of the pubertal period to reproductive development. Furthermore, the absence of

enduring consequences in adult mice accents the virility of puberty as a uniquely critical and sensitive period of development. Key components of the HPG axis were downregulated variably by sex, suggesting a sex-bias in the neuroimmune response during puberty and adulthood.

Acknowledgements

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Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author upon request.

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Declarations of Interest

None.

References

Provided at end of document.

Study 2 investigated the immediate and long-lasting effects of LPS stress on the HPG axis during puberty and adulthood. A key finding from this research was the enduring downregulation of Kiss1 and Kiss1R, accompanied by the significant downregulation of LH concentrations and ERα expression. The enduring nature of the downregulation of Kiss1 and Kiss1R emerged as a significant outcome, signaling a potential area for further in-depth exploration. However, it's important to note that these enduring changes did not extend to adulthood, further highlighting the vulnerability of critical HPG axis components to pubertal LPS treatment. This persistent change in Kiss1 and Kiss1R expression is of paramount importance and lays the framework for the inquiries posed in Study 3. It shifts the research trajectory towards examining the reversibility of these negative enduring neuroendocrine changes and whether there is a treatment method available to recover sexual behaviour deficits that emerge from pubertal LPS.

Study 3 delved into the enduring behavioural impacts resulting from LPS-induced disruptions to the HPG axis during puberty. It explored the hypothesis that persistent changes in adult sexual behavior, induced by LPS treatment, could be mitigated through exogenous kisspeptin administration. Focusing on reversing enduring sexual dysfunctions, this research aimed to deepen our comprehension of the neuroendocrine interactions disrupted during puberty. Additionally, the research pays special attention to potential sex differences, aimed at discerning the effectiveness of kisspeptin treatment between males and females. Study 3 aimed to offer a nuanced understanding of kisspeptin's therapeutic potential between sexes, with the goal of reversing the long-term consequences on adult sexual behavior.

CHAPTER 4: Kisspeptin's Effect on Adult Sexual Behavior Following Pubertal LPS Treatment in Male and Female Mice

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Abstract

Kisspeptin is a neuropeptide responsible for initiating puberty and regulating the hypothalamic-pituitary-gonadal (HPG) axis activity. Previous findings show that pubertal LPS treatment causes enduring downregulation of hypothalamic kisspeptin expression and impairments in sexual behavior in male and female mice. However, it is unclear whether kisspeptin treatment in adulthood can restore sexual behavior in mice treated with lipopolysaccharide (LPS) during puberty. Thus, the current study aims to address this knowledge gap. Eighty male and female mice arrived at 3 weeks of age and were treated with either saline or LPS at 6 weeks. At 10 weeks of age, mice were gonadectomized and received testosterone (T), or estradiol (E_2) and Progesterone (P_4) treatments. Mice were then treated with either a vehicle or kisspeptin prior to sexual behavior testing for 5 consecutive weeks. The results show that LPS treatment decreases the lordosis quotient in females, and mount and intromission frequencies in males. However, kisspeptin treatment successfully restored sexual behavior in males and females. The findings indicate that kisspeptin treatment in adulthood counteracts the negative impacts of pubertal LPS treatment on sexual behavior in both male and female mice. These results not only and highlight kisspeptin's therapeutic role in restoring sexual dysfunction, but also underscore its importance in maintaining sexual health.

1. Introduction.

Puberty is a critical period of development and marks the transition from a non-reproductive to a reproductive state (Ismail et al., 2011; Schulz et al., 2009; Sisk & Zehr, 2005). Pubertal development is also an important period for brain reorganization and remodelling (Romeo, 2003; Sisk & Foster, 2004; Teicher et al., 2003), which occurs as a result of rising levels of sex hormones, predominantly testosterone (T) in males and estradiol (E_2) in females. Sex hormones organize the brain to express sex-specific phenotypes which lay dormant until activated by these same sex hormones (Schulz et al., 2009; Sisk & Foster, 2004). Puberty and adolescence, while closely related, serve different yet complementary roles in development. Adolescence is a broad term that encompasses the period from the initiation of puberty until the start of adulthood (Boxer et al., 1983; Choudhury, 2010; Sisk & Foster, 2004). Adolescence is a multidimensional stage that includes physiological, psychology, and emotional transformations (Lerner & Steinberg, 2009). Puberty is distinct from adolescence in that it involves specific physiological and neurological changes leading to sexual maturation (Schulz et al., 2009; Sisk & Foster, 2004). These changes are regulated by the hypothalamic-pituitary-gonadal (HPG) axis, and are essential in establishing sexual reproductive behaviors in adulthood (Romeo et al., 2002).

The HPG axis is responsible for initiating puberty and regulating sex hormone production (Han et al., 2005; Irwig et al., 2004). Although gonadotropin releasing hormone (GnRH) was believed to exert primary control, recent findings revealed that the neuropeptide kisspeptin (Kiss1), and its receptor (Kiss1R) are at the forefront (Iwasa et al., 2008; Moore, Abbott, et al., 2018). Kisspeptin stimulation activates GnRH neurons to secrete GnRH into the hypophyseal portal vein of the pituitary. The anterior pituitary gland then releases luteinizing (LH) and follicle stimulating hormone (FSH) into the bloodstream where they bind with the gonads to begin androgen and estrogen production (Ojeda et al., 1976; Silverman et al., 1987). Androgens like T

and estrogens like E_2 provide negative feedback to hypothalamic kisspeptin, dynorphin, and neurokinin B (KNDy) neurons (Kauffman et al., 2007; Ruka et al., 2016). Sufficient feedback triggers dynorphin to downregulate kisspeptin release, whereas neurokinin B stimulates kisspeptin release when feedback is reduced (Moore et al., 2019). This system works optimally during non-stressful periods, but it is significantly downregulated by stress exposure (Oyola & Handa, 2017).

Exposure to stress and activation of the hypothalamic-pituitary-adrenal (HPA) axis, can inhibit HPG axis activity resulting in marked permanent negative consequences affecting mood disorders and sexual behavior (Sisk & Foster, 2004). For example, male and female mice displayed greater anxiety- and depression-like behaviors, respectively, in adulthood following lipopolysaccharide (LPS) treatment, a bacterial endotoxin, during puberty (Murray et al., 2019). Similarly, mice exposed to shipping stress during puberty showed enduring sexual behavior dysfunction in adulthood (Laroche et al., 2009b). Additionally, pubertal LPS treatment induced sexual dysfunction in adult C57Bl/6 female mice. Moreover, pubertal LPS-induced sexual dysfunction is not limited to inbred mice, but also extends to outbred CD1 mice (Ismail et al., 2011; Laroche et al., 2009a). Pubertal LPS treatment also downregulates glucocorticoid receptor expression in the paraventricular nucleus of the hypothalamus in adulthood (Smith et al., 2021). Glucocorticoid receptor reduction is generally associated with impaired negative feedback of glucocorticoids to the HPA axis and it has also been linked with depression (Ridder, 2005). The stress-induced inhibition of the HPG axis is likely due to a stress-induced downregulation of kisspeptin expression.

Kisspeptin is a neuropeptide that is central to the HPG axis activity (Messager et al., 2005). More specifically, it is responsible for activating the HPG axis, initiating puberty and

regulating sex hormone production (Smith et al., 2005). We have previously shown that pubertal LPS treatment causes both acute and enduring downregulation of Kiss1 and Kiss1R in both males and females (Smith et al., 2021, 2023). Moreover, LH is also reduced in pubertal LPS-treated male and female mice (Smith et al., 2021). Reductions in LH concentration were found to be enduring in LPS-treated pubertal, but not adult, male and females (Smith et al., 2023). Although we have shown that pubertal LPS treatment decreases hypothalamic kisspeptin (Smith et al., 2021, 2023) and impairs sexual behavior (Ismail et al., 2011; Laroche et al., 2009a) it is unclear whether restoring kisspeptin via exogenous injection could re-establish normal sexual behavior in both pubertally stressed male and female mice.

Therefore, we aimed to identify whether kisspeptin treatment can restore sexual behavior in adult male and female mice treated with LPS during puberty. We hypothesized that in adulthood, LPS-treated pubertal male mice will show reduced mount, intromission, and ejaculation frequencies, whereas female mice will show reduced lordosis quotient. Additionally, we hypothesize that mice treated with kisspeptin will exhibit a typical pattern of sexual behavior, as identified in our saline-treated group.

2. Methods

2.1 Animals

Male (n=40) and female (n=40) mice arrived at three weeks of age from Charles River Laboratories. Upon arrival the mice were segregated into all male or all female rooms in groups of two or three in polycarbonate cages (17 cm x 28 cm x 12 cm [height x width x length]) with *ad libitum* access to food (Teklad Global Diets, Envigo) and water. Cages were bedded with corn cob (Cat# 7097, Envigo, Mississauga, CA), and included one piece of nestlet (Cat# NES3600, Ancare, Bellmore, USA) and a cardboard hut (Cat# XKA2455, Ketchum, Brockville, CA) for basic enrichment. A temperature of 24 ± 2 °C, humidity of 40%, and a reversed 14:8 light-dark

cycle (lights on between 8:00 pm and 10:00 am) was maintained throughout. All procedures were approved by the Animal Care Committee of the University of Ottawa.

2.2 Procedure

Eighty CD1 mice (40 males and 40 females) arrived to the lab at three weeks of age and were allowed to acclimate for 3 weeks. Mice were treated with either saline- or LPS at six weeks of age and were monitored for sickness behaviors. At 10 weeks of age, in adulthood, mice were gonadectomized and treated with carprofen once a day for three days. Following one week of recovery, mice were treated with either saline or KP for 5 weeks, 2 hours prior to sexual behaviour testing. Mice were euthanized following the completion of sexual behavior testing at 16 weeks of age, and brains were perfused and extracted for future analysis.

2.3 Lipopolysaccharide (LPS) Solution

Lipopolysaccharide (LPS) was prepared by dissolving lyophilized LPS, obtained from Escherichia coli serotype O26:B6 (Sigma Aldrich Canada, Cat. No. L3755), into a 0.9% (w/v) sterile aqueous sodium chloride (saline) solution (Ricca Chemical Company, Cat. No. R7210000-4A7210-1), following the protocol outlined by Murray et al. (2019). This solution was administered intraperitoneally to mice at six weeks of age, aligning with the end of the light cycle. The chosen dose, 1.5 mg/kg of LPS, is documented to cause mild sickness symptoms lasting up to 48 hours (Cai et al., 2016; Ismail, Garas & Blaustein, 2011). Notably, administration during the stress-sensitive period of puberty, specifically at six weeks, is associated with enduring adverse effects on a range of behaviors, both reproductive and non-reproductive (Laroche et al., 2009; Ismail et al., 2011; Blaustein & Ismail, 2013; Kolmogorova et al., 2019). Following the administration of saline or LPS, mice were observed for 48-hours and sickness behaviours were recorded.

2.4 Sickness Behavior

Sickness behaviors were scored independently by two raters blind to treatment conditions at 2h, 4h, 6h, 8h, 24h, and 48 hours following LPS or saline treatment as previously described in (Kolmogorova, et al, 2017). Briefly, mice were given a score between 0 and 4 with 1 point for each sickness behavior observed (huddling, piloerection, ptosis, and lethargy) at each time point. Sickness behavior scores were averaged between the two raters.

2.5 Surgery

Gonadectomy was performed on both male and female mice to regulate hormone fluctuations, ensuring consistent conditions for assessing sexual behavior. In females, it eliminated the variability associated with the estrous cycle and prevented pregnancy, maintaining a stable sexual receptivity baseline. In males, the procedure facilitated precise control over hormone levels. This approach standardized hormonal backgrounds across subjects, providing a reliable platform for accurately evaluating the effects of interventions on sexual behavior. Gonadectomy took place during adulthood to ensure that the pubertal organizing effects of endogenous hormones could be complete. In females, a small incision was made to the lower left ventral skin and to the muscle layer to locate the uterine horns and ovaries in female mice. Once located, the uterine horns were tied with absorbable suture, and the ovaries were removed. The muscle layer was sutured with absorbable suture, and the skin incision was closed with wound clips. Topical transdermal bupivacaine was applied after the skin had been closed and once again 6 hrs later. Mice were injected subcutaneously with Carprofen at a dose of 5 mg/kg for 3 days.

In males, a small ventral midline incision was made to the scrotum and the skin was retracted to expose the tunica. The tunica was pierced, and the testes were removed by using forceps to pull off and cut each testicle. All deferential vessels and ducts were tied with

absorbable suture and were replaced back into the tunica. Skin incisions were closed with sutures. Topical transdermal bupivacaine was applied after the skin had been closed and once again 6hrs later. Mice were injected subcutaneously with Carprofen at a dose of 5 mg/kg. Additionally, a SILASTIC capsule implant measuring an outer diameter of 2.16mm and an inner diameter of 1.02mm, was subcutaneously placed between the shoulder blades, and the skin will be closed with sutures.

2.6 Hormone Treatment

Female mice were given subcutaneous injection of E_2 benzoate (2µg/100ul sesame oil) 48 hours before and progesterone (P_4) (100µg P_4/100ul sesame oil) 4 hours before sexual behavior testing (Ismail et al., 2011). Males received the SILASTIC implant containing 60% T diluted in cholesterol (Ismail et al., 2011). The dosage and timing of the synthetic kisspeptin-10 (KP) administration were meticulously selected based on empirical evidence indicating its efficacy in modulating sexual behaviour. Specifically, all mice received a subcutaneous injection of KP, diluted in sterile saline, at a concentration of 0.52 µg/kg, or a corresponding vehicle (sterile saline), precisely 2 hours prior to the commencement of sexual behavior testing. This dosage and administration schedule were adopted from Hellier et al. (2018), where it was demonstrated to significantly enhance sexual behaviour in female mice. The study by Hellier et al. (2018) is particularly notable as it provides robust evidence of the effectiveness of peripheral administration of kisspeptin in eliciting measurable behavioral changes, marking it as a seminal reference for the operational parameters of kisspeptin administration in experimental settings. The adoption of this protocol in the current study is grounded in the aim to replicate and extend these findings, examining the potential of kisspeptin to mitigate stress-induced alterations in sexual behaviour observed following pubertal LPS treatment.

2.7 Sexual Behavior

To assess sexual behavior in males, mice were placed in a plexiglass chamber where sexual behavior was recorded with a video camera. Males were placed in the chamber for a 5-minute habituation period. Then, a female stimulus mouse was introduced into the chamber for 20 minutes where mount, intromission and ejaculation frequencies and latencies were scored. A mount was scored when the male places both forepaws on the female's hind region in a copulatory position with or without penile insertion. Mounting that included penile insertion was scored as intromission, and ejaculation is defined by the release of semen.

To assess sexual receptivity in females, a sexually experienced stimulus male was placed within a plexiglass arena where sexual behavior can be recorded with a video camera for scoring. Females were introduced to the chamber 5 minutes later. Sexual behaviors were score and lordosis quotient (LQ) was calculated. LQ is defined as an elevation of the hindquarters and an arching of the back. LQ was computed via number of intromissive bouts (defined by successful penetrations)/total number of mounts x 100). Lordosis behavior was scored until the female receives 20 mounts or the male ejaculated to prevent unnecessary stress on the female mouse.

2.8 Euthanasia

All mice received an intraperitoneal injection of Euthanyl the day after sexual behavior testing concluded (Euthansol; Merck Animal Intervet Canada Corp; Kirckland, Quebec). Mice were intracardially perfused with 20 mL of 0.9% saline, followed by 20 mL of 4% paraformaldehyde (PFA). Brains were extracted and post-fixed in 10 mL of 4% PFA for 2 hours at 4o C. Then, brains were transferred to 10 mL of 30% sucrose solution. Twenty-four hours later, the brains were again transferred to a 30% sucrose solution and stored for further analyses.

2.9 Statistical Analysis

All data were initially screened for outliers using box plots. The dataset contained 5 outliers which were winsorized to the next highest or lowest value in the group dataset to mitigate their impact on the analysis. Within this total, outliers were distributed across different variables and groups. For one variable (Week 1 – Mount Latency) two outliers were found. The other three were distributed across three groups (Week 1 – Mount; Week 1 – Intromission; Week 2 – Mount Latency). A two-way mixed methods repeated measures ANOVA was used to examine the effects of saline or LPS treatment on sickness behaviour, as well as vehicle or kisspeptin treatment on sexual behavior in males and females. Partial eta squared (η_p^2) was used to determine the effect size of significant main effects and interactions. When appropriate, pairwise comparisons were analyzed using a Bonferroni correction factor on SPSS V21 software. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Sickness Behavior

The 2 x 2 mixed methods ANOVA violated Mauchly's test of sphericity ($p < 0.05$) so all within subject effects were analyzed with Greenhouse-Geiser corrections. The within-subjects ANOVA found a significant main effect of time ($F_{(5,290.81)} = 7.76$, $p < 0.05$, $\eta_p^2 = 0.10$). The 2 x 2 between-subjects ANOVA revealed a significant main effect of treatment ($F_{(1,47)} = 3949.66$, $p < 0.05$, $\eta_p^2 = 0.98$). The pairwise comparisons showed that LPS-treated females display more sickness behaviors at 6 hours (mean difference [MD] = 0.55, standard error of the mean [SEM] = 0.12, $p = 0.00$) and 8 hours compared to male counterparts (MD = 0.23, SEM = 0.06, $p = 0.01$), whereas LPS-treated males showed more sickness behavior at 24 hours compared to female counterparts (MD = 0.66, SEM = 0.12, $p = 0.00$). As expected, all LPS-treated males and females showed significantly more sickness behaviors compared to their saline counterparts at each time point.

3.2 Sexual Behavior

Mount frequency

The 2 x 2 mixed methods ANOVA violated Mauchly's test of sphericity ($p < 0.05$) so all within subject effects were analyzed with Greenhouse-Geiser corrections. The within-subjects ANOVA found a significant main effect of time ($F_{(3.36,124.98)} = 4.56$, $p < 0.05$, $\eta p^2 = 0.12$). The between-subjects ANOVA found a significant interaction between LPS and KP treatment ($F_{(1,36)} = 24.33$, $p < 0.05$, $\eta p^2 = 0.41$) for mount frequency. The pairwise comparisons indicated that saline- and vehicle-treated males showed greater mount frequency than LPS-treated counterparts at week 1 (*MD* = 0.75, *SEM* = 0.26, $p = 0.00$), week 3 (*MD* = 2.55, *SEM* = 0.49, $p = 0.00$), week 4 (*MD* = 2.12, *SEM* = 0.50, $p = 0.00$), and week 5 (*MD* = 2.46, *SEM* = 0.54, $p = 0.00$). Saline- and KP-treated males showed greater mount frequency than their LPS-treated counterparts at week 3 (*MD* = 1.00, *SEM* = 0.47, $p = 0.04$), and week 5 (*MD* = 1.85, *SEM* = 0.52, $p = 0.01$). LPS- and KP-treated males showed greater mount frequency than saline-treated counterparts at week 1 (*MD* = 0.55, *SEM* = 0.26, $p = 0.04$), week 3 (*MD* = 2.91, *SEM* = 0.49, $p = 0.00$), week 4 (*MD* = 1.27, *SEM* = 0.50, $p = 0.01$), and week 5 (*MD* = 1.36, *SEM* = 0.54, $p = 0.02$). In saline- and vehicle-treated mice mount frequency increased from week 1 to week 2 (*MD* = 1.50, *SEM* = 0.29, $p = 0.00$). In saline- and KP-treated mice mount frequency increased from week 1 to week 2 (*MD* = 1.20, *SEM* = 0.29, $p = 0.00$) and week 3 to 4 (*MD* = 1.70, *SEM* = 0.52, $p = 0.02$). In LPS- and vehicle-treated mice, mount frequency increased from week 1 to week 2 (*MD* = 1.61, *SEM* = 0.31, $p = 0.00$), and from week 3 to 4 (*MD* = 1.83, *SEM* = 0.55, $p = 0.02$). In LPS- and KP-treated mice, mount frequency increased from week 1 to week 2 (*MD* = 1.00, *SEM* = 0.30, $p = 0.03$) and from week 2 to week 3 (*MD* = 1.85, *SEM* = 0.42, $p = 0.00$; see Fig 1.).

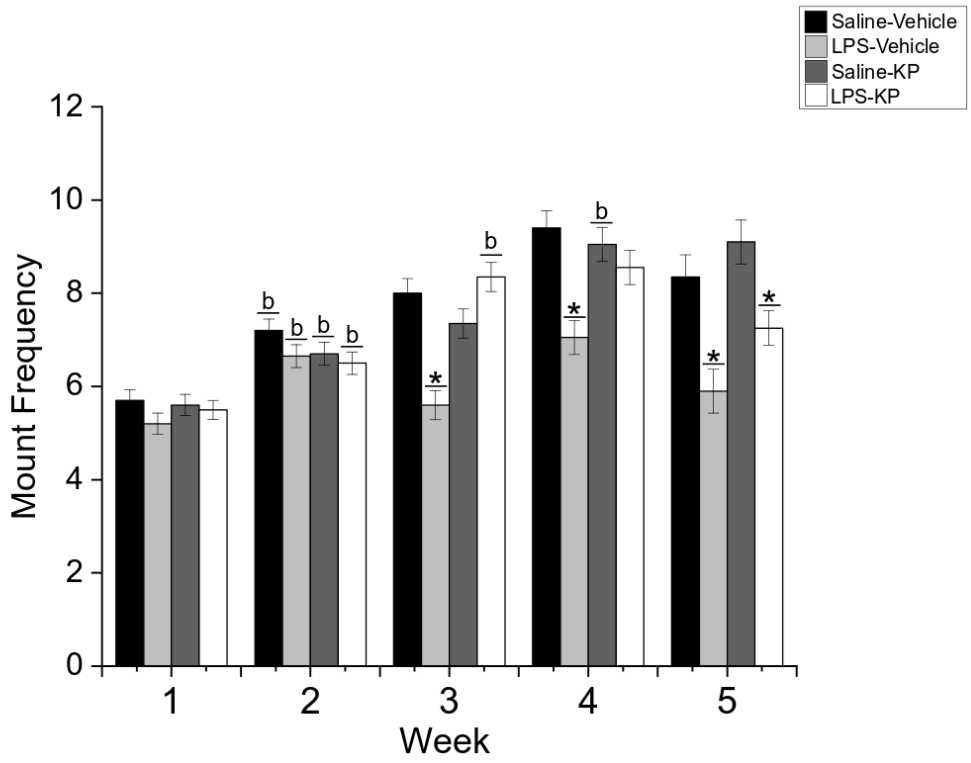

Fig 1. Mount Frequency. Lordosis Quotient in adult male mice from 11 to 16 weeks of age treated with saline or LPS and Vehicle or Kisspeptin. The asterisk (*) denotes significant differences ($p < 0.05$) between saline and LPS-treated mice. The *a* denotes significant differences between LPS-vehicle and LPS-KP-treated mice. The *b* denotes significant week-over-week differences.

Mount Latency

The 2 x 2 mixed methods ANOVA did not violate Mauchly's test of sphericity ($p > 0.05$) so all within subject effects were analyzed without any corrections for sphericity. The within-subjects ANOVA found a significant main effect of time ($F_{(2.98,104.45)} = 5.12$, $p < 0.05$, $\eta p^2 = 0.13$). The between-subjects ANOVA did not find any significant main effects or interactions.

Male mice displayed shorter mount latency from week 4 to 5 (*MD* = 51.71, *SEM* = 11.53, *p* = 0.00).

Intromission frequency

The 2 x 2 mixed methods ANOVA violated Mauchly's test of sphericity ($p < 0.05$) so all within subject effects were analyzed with Greenhouse-Geiser corrections. The within-subjects ANOVA found a significant main effect of time ($F_{(2.91,101.92)} = 3.36$, $p < 0.05$, $\eta p^2 = 0.09$). The between-subjects ANOVA revealed a significant main effect of LPS treatment ($F_{(1,36)} = 13.49$, $p < 0.05$, $\eta p^2 = 0.28$), where saline-treated males displayed significantly more intromissions than LPS-treated counterparts (*MD* = 0.51, *SEM* = 0.14, *p* = 0.00). Saline-treated male mice showed significantly more intromission in week 4 (*MD* = 0.61, *SEM* = 0.26, *p* = 0.03), and week 5 (*MD* = 1.42, *SEM* = 0.45, *p* = 0.00) compared to LPS-treated counterparts. Saline-treated males intromitted significantly more from week 1 to 2 (*MD* = 2.10, *SEM* = 0.20, *p* = 0.00), week 2 to 3 (*MD* = 1.73, *SEM* = 0.26, *p* = 0.00), and week 3 to 4 (*MD* = 1.20, *SEM* = 0.28, *p* = 0.00). LPS-treated males intromitted more from week 1 to 2 (*MD* = 2.33, *SEM* = 0.20, *p* = 0.00), and week 2 to 3 (*MD* = 1.63, *SEM* = 0.27, *p* = 0.00; see Fig 2.).

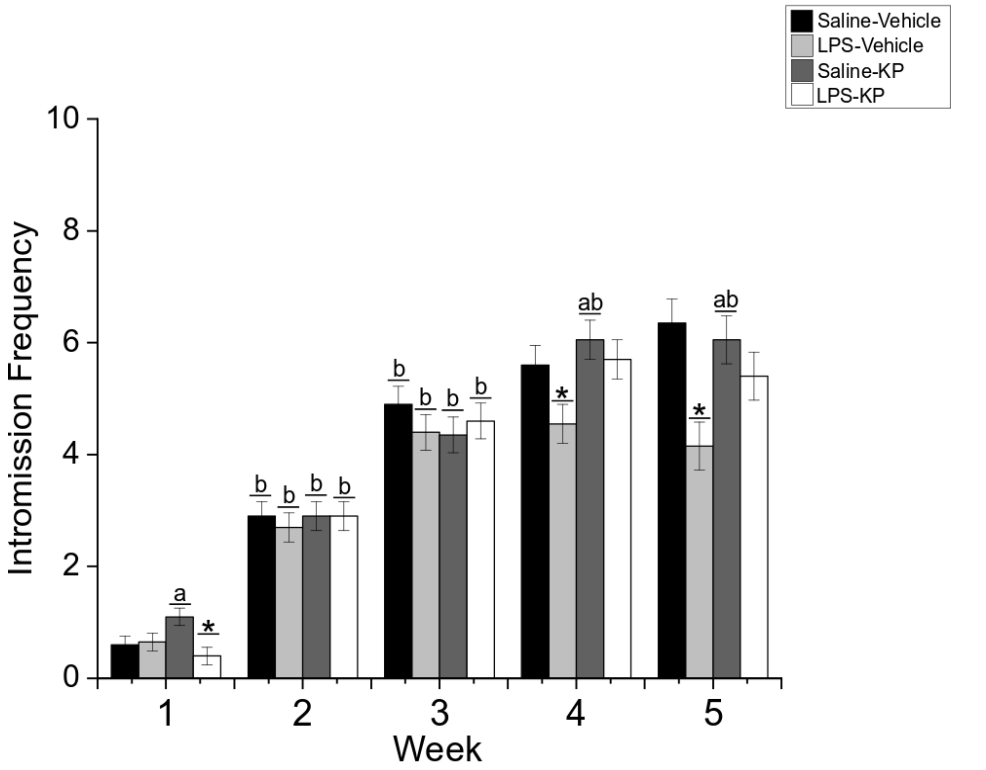

Fig 2. Intromission Frequency. Lordosis Quotient in adult male mice from 11 to 16 weeks of age treated with saline or LPS and Vehicle or Kisspeptin. The asterisk (*) denotes significant differences ($p < 0.05$) between saline and LPS-treated mice. The *a* denotes significant differences between LPS-vehicle and LPS-KP-treated mice. The *b* denotes significant week-over-week differences.

Intromission Latency

The 2 x 2 mixed methods ANOVA violated Mauchly's test of sphericity ($p < 0.05$) so all within subject effects were analyzed with Greenhouse-Geiser corrections. The within-subjects ANOVA found a significant main effect of time ($F_{(1.69,35.49)} = 16.49$, $p < 0.05$, $\eta p^2 = 0.44$). The between-subjects ANOVA did not reveal any significant effects of treatment ($F_{(1,36)} = 0.01$, $p > 0.05$, $\eta p^2 = 0.00$) or KP ($F_{(1,36)} = 1.45$, $p > 0.05$, $\eta p^2 = 0.07$). The pairwise comparisons revealed

significantly shorter intromission latency between week 2 and 3 (*MD* = 89.71, *SEM* = 28.31, *p* = 0.04), and week 4 and 5 (*MD* = 39.46, *SEM* = 12.26, *p* = 0.04).

Ejaculation frequency

The 2 x 2 mixed methods ANOVA violated Mauchly's test of sphericity ($p < 0.05$) so all within subject effects were analyzed with Greenhouse-Geiser corrections. The within-subjects ANOVA found a significant effect of time ($F_{(2.60,91.00)} = 4.67$, $p < 0.05$, $\eta p^2 = 0.12$). However, the between-subjects ANOVA did not identify any significant main effects or interactions. Saline- and vehicle-treated males ejaculated significantly more from week 3 to 4 (*MD* = 0.60, *SEM* = 0.17, *p* = 0.01). Saline- and KP-treated males ejaculated significantly from week 2 to 3 (*MD* = 0.50, *SEM* = 0.11, *p* = 0.00).

Lordosis Quotient

The 2 x 2 mixed methods ANOVA violated Mauchly's test of sphericity ($p > 0.05$) so all within subject effects were analyzed without any corrections for sphericity. The within-subjects ANOVA revealed a significant main effect of time ($F_{(4,144)} = 307.60$, $p < 0.05$, $\eta p^2 = 0.90$)The between-subjects ANOVA revealed a significant LPS treatment x KP interaction ($F_{(1,36)} = 21.88$, $p < 0.05$, $\eta p^2 = 0.38$). Vehicle- and saline-treated females displayed significantly greater LQ in week 2 (*MD* = 0.12, *SEM* = 0.04, *p* = 0.00), week 3 (*MD* = 0.17, *SEM* = 0.04, *p* = 0.00), week 4 (*MD* = 0.23, *SEM* = 0.03, *p* = 0.00), week 5 (*MD* = 0.20, *SEM* = 0.03, *p* = 0.00) compared to LPS-treated counterparts. KP- and saline-treated females displayed significantly greater LQ in week 5 (*MD* = 0.11, *SEM* = 0.02, *p* = 0.01) compared to LPS-treated counterparts. LPS- and KP-treated females displayed significantly greater LQ in week 2 (*MD* = 0.08, *SEM* = 0.03, *p* = 0.01), week 3 (*MD* = 0.14, *SEM* = 0.04, *p* = 0.02), week 4 (*MD* = 0.14, *SEM* = 0.04, *p* = 0.01), and week 5 (*MD* = 0.15, *SEM* = 0.03, *p* = 0.00) compared to vehicle-treated counterparts. Saline- and

vehicle-treated females displayed greater LQ from week 1 to 2 (*MD* = 0.28, *SEM* = 0.04, *p* = 0.00), week 2 to 3 (*MD* = 0.15, *SEM* = 0.04, *p* = 0.02), from week 3 to 4 (*MD* = 0.18, *SEM* = 0.04, *p* = 0.00). Saline- and KP-treated females displayed greater LQ from week 1 to 2 (*MD* = 0.28, *SEM* = 0.04, *p* = 0.01), and week 2 to 3 (*MD* = 0.22, *SEM* = 0.04, *p* = 0.01). LPS- and vehicle-treated females displayed significantly greater LQ from week 1 to week 2 (*MD* = 0.21, *SEM* = 0.04, *p* = 0.01), while LPS- and KP-treated females displayed significantly greater LQ from week 1 to 2 (*MD* = 0.26, *SEM* = 0.04, *p* = 0.00), and week 2 to 3 (*MD* = 0.15, *SEM* = 0.04, *p* = 0.00; see Fig 3.).

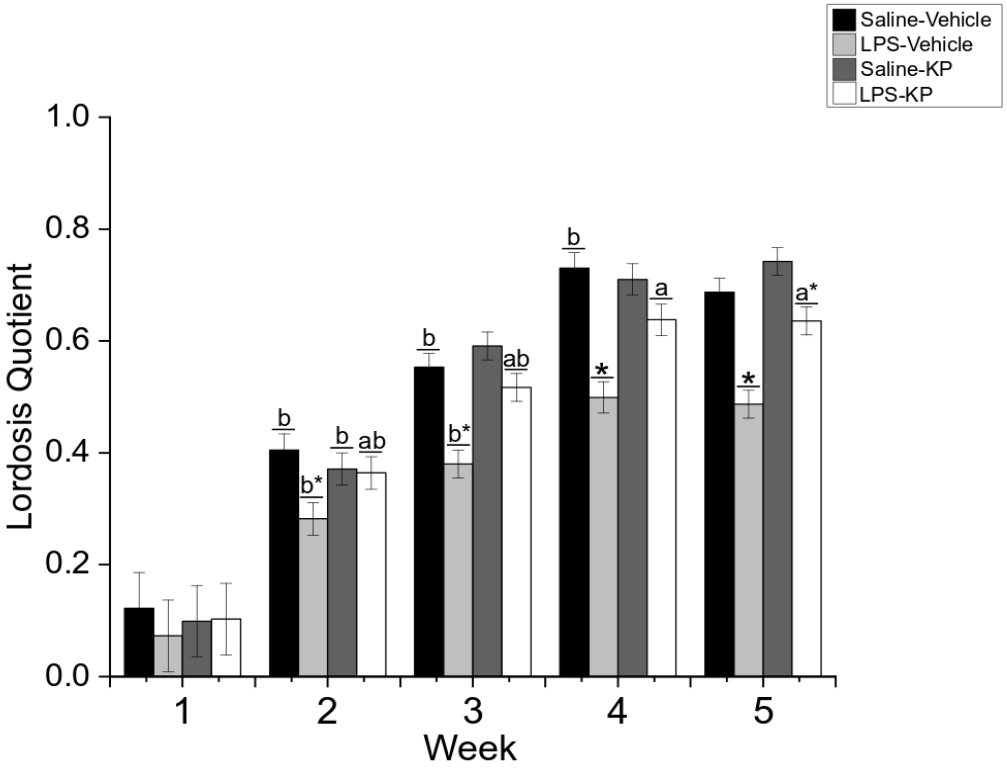

Fig 3. Lordosis Quotient. Lordosis Quotient in adult female mice from 11 to 16 weeks of age treated with saline or LPS and Vehicle or Kisspeptin. The asterisk (*) denotes significant differences ($p < 0.05$) between saline and LPS-treated mice. The *a* denotes significant

differences between LPS-vehicle and LPS-KP-treated mice. The *b* denotes significant week-over-week differences.

4. Discussion

It is well known that the HPA and HPG axes interact in an allostatic manner to maintain physiological homeostasis, with the HPA axis often inhibiting the HPG axis under stress to prioritize immediate survival over reproductive functions. A single dose of the immune stressor, LPS, during the pubertal stress-sensitive period (6 weeks of age) is sufficient to cause enduring changes in sexual behavior and kisspeptin expression in both male and female mice (Laroche et al., 2009a; Smith et al., 2023). However, it is unclear whether it is possible to restore sexual behavior functionality via kisspeptin treatment. Therefore, we investigated whether kisspeptin treatment in adulthood could improve sexual function in mice treated with LPS during puberty. As expected, pubertal LPS treatment caused enduring sexual behavior dysfunction in both male and female mice, as measured by reduced mount and intromission frequency and latency, and LQ, respectively. As anticipated, pubertal KP treatment led to amelioration of sexual behavior dysfunction in female mice, as evidenced by increases to LQ. Interestingly, this effect was not observed in male mice, but the reason for this sex-specific response is currently unknown. The findings from this research implicate kisspeptin as a mechanism responsible for healthy sexual behavior in female.

As expected, LPS treatment induced sickness behaviors in all mice for less than 48 hours. In agreement with our hypothesis, female mice experienced sooner than did male mice, however, male mice continued showing more sickness behavior for a longer period than did females, suggesting that male mice recovered slower than female counterparts. These sex differences in immune response are consistent with previous findings from our lab (Cai et al., 2016; Esposito et

al., 2022; Murack et al., 2023; Sharma et al., 2019; Smith et al., 2023) and is likely due to sex differences in sex hormone concentrations. T and E_2 are the prevailing sex hormones in males and females, respectively. Unlike T, which quells the immune system, E_2 has an immune enhancing effect (Palaszynski et al., 2005). For example, both intact adult females and castrated adult males showed greater levels of peripheral TNFα and IFNγ compared to intact adult males after being treated with autoantigens (Palaszynski et al., 2005). Additionally, intact male mice show lower expression of toll-like receptor 4 (TLR4), the receptor responsible for binding LPS, compared to castrated counterparts (Rettew et al., 2008). These findings indicate that sex differences in sex hormone concentrations play an important role in the sex difference in immune response and sickness behavior.

In line with our hypothesis, we observed that pubertal LPS treatment resulted in long-lasting deficits in sexual behavior for both male and female mice. Specifically, male mice treated with LPS displayed a significant reduction in both mounting and intromission frequency from week two through week five when compared to their saline-treated counterparts. Similarly, female mice treated with LPS showed a reduction in lordosis quotient (LQ) compared to saline-treated mice, reaching a plateau after the initial weeks of the study. These results are consistent with previous findings showing that six-week-old mice showed considerably diminished sexual behavior in adulthood when treated with LPS during puberty (Laroche et al., 2009a). Although this effect was found in inbred mice initially, a later investigation showed that pubertal LPS-induced sexual behavior dysfunction extends to outbred mice, as well (Ismail et al., 2011). In conjunction with these earlier findings, our lab detected acute and enduring HPG axis dysfunction (Smith, 2021, 2023). For example, LPS-treated pubertal mice showed acute and enduring decreases in hypothalamic *Kiss1* and *Kiss1R* mRNA. However, adult LPS-treated mice

did not show the same enduring outcomes, suggesting that stress-induced HPG axis dysfunction is limited to the critical pubertal developmental period. These findings emphasize the significant impact of LPS treatment during puberty on long-term sexual behavior, providing valuable insights into the role of stressors during critical developmental periods.

Expanding upon our initial observations of the detrimental effects of LPS treatment on long-term sexual behavior dysfunction, we further investigated whether we could improve sexual behavior by restoring kisspeptin. In females, exogenous KP treatments alleviated sexual behavior dysfunction, as measured by increased LQ. Aligning with our hypotheses, LPS- and KP-treated female mice showed significantly greater increases in sexual behavior, compared to vehicle-treated counterparts. The restorative effects of KP in our data is consistent with previous findings indicating that Kiss1 knockout female mice treated with KP displayed a restoration in sexual behavior compared to vehicle-treated counterparts (Hellier et al., 2018). Furthermore, KP treatment directly into the ventromedial hypothalamus of adult female mice increased LQ in females by roughly 20% compared to their saline-treated counterparts (Bentefour & Bakker, 2021). However, further research is necessary to comprehensively understand the underlying mechanisms driving this observation. Ultimately, our data substantiates the notion that KP treatment reverses the negative enduring changes to female sexual behavior from pubertal LPS treatment and implicates kisspeptin as the mechanism responsible for female sexual behavior dysfunction.

The sex-specific outcomes observed in response to KP treatment may be linked to differences in the development and function of Kiss1R expression between males and females post pubertal LPS treatment. Notably, our previous study observed enduring downregulation of Kiss1R expression in males post-LPS treatment, a pattern not identified in females (Smith et al.,

2023). This suggests that the success of kisspeptin treatment in rectifying sexual behaviour might be contingent on the presence of functional Kiss1R; if Kiss1R is significantly diminished, as previously seen in males, but not females, the efficacy of kisspeptin treatment could be compromised, given the reduced number of receptors for the kisspeptin peptide to bind. However, the precise mechanisms underlying these sex-specific responses warrant exploration. Further research is needed to elucidate the pathways of Kiss1R and their implications for treatment strategies. Until then, addressing sexual dysfunction in males with kisspeptin treatment may present a challenge, reflecting the nuanced interplay of neuroendocrine factors that govern sexual behaviour.

While our findings underscore the pivotal role of kisspeptin ameliorating sexual dysfunction due to pubertal LPS treatment, they also hint at the possibility of a maximal threshold efficacy. The significant improvement to sexual behavior in both LPS-treated female mice, did not reach sexual behavior performance observed in saline-treated animals. For example, we observed no significant sexual behavior differences between saline- and kisspeptin-treated mice and saline- and vehicle-treated counterparts. Our data suggests that the effects of kisspeptin on sexual behavior may have an inherent limit, and this threshold appears to be reached at physiological kisspeptin levels. Earlier studies in mice involving intracerebral infusion of kisspeptin demonstrated an increase in LH at a dose of 1 femtomole, with a peak response at 10 femtomoles, followed by a plateau effect with subsequently higher doses (Gottsch et al., 2004). When considering these findings, it is possible that exceeding physiological kisspeptin levels does not result in further enhancements in sexual behavior. However, it is essential to underscore the necessity for a comprehensive pharmacological analysis of kisspeptin treatment to conclusively establish its dosage-dependent effects on sexual behavior. Further investigations

are warranted to unravel the complex relationship between kisspeptin dosage and its effects on reproductive physiology.

Conclusion

Our study confirms that a single dose of LPS during puberty leads to long-term sexual dysfunction in both male and female mice. Importantly, we identified that reduced kisspeptin levels are a likely mechanism behind these effects. However, while restoring kisspeptin levels successfully reinstated female sexual behavior functionality, these effects did not extend to males. Our findings offer valuable insights for potential targeted treatments for female sexual dysfunction, especially if similar mechanisms exist in humans.

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Declaration of Competing Interesting

The authors declare no conflict of interest.

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References

References provided at end of document.

CHAPTER 5: General Discussion

Puberty is a critical period of development characterized by rapid neuronal growth and pruning. Puberty is also a period of sexual maturation. However, the precarious nature of rapid changes to the brain increases its vulnerability to stress. Exposure to stress triggers the activation of the HPA axis which can potentially disrupt healthy development and lead to adverse enduring changes. Within this context, it is important to note that both the HPA and HPG axes undergo maturation during puberty. Current research supports the notion that HPA axis activation can result in the suppression of the HPG axis. While this trade-off is typically transient and serves to maintain allostasis, stress during puberty may ultimately lead to permanent dysfunction of the HPG axis. However, the mechanism responsible for enduring HPG axis dysfunction is currently unknown. Although, kisspeptin, a neuropeptide responsible for initiating puberty, shows promise as a key player contributing to HPA axis-induced downregulation of the HPG axis. Kisspeptin's function within the HPG axis is well-known, however, it remains unclear how the HPA axis influences kisspeptin and downstream HPG axis activity.

The objective of this thesis was to determine kisspeptin's involvement as a mechanism for these negative enduring changes. Three studies were conducted to address the following research questions: 1) Does LPS treatment acutely reduce kisspeptin and its receptor in the hypothalamus of male and female mice? 2) Is LPS capable of inducing enduring changes to kisspeptin and its receptor, and does it permanently diminish downstream HPG axis factors such as ERα, AR, LH and/or FSH? 3) Can kisspeptin restore sexual behavior, implicating kisspeptin as the mechanism responsible for HPG axis downregulation? This thesis effectively addresses each of these questions, providing significant contributions to the advancement of knowledge in the field. Importantly, it sheds light on the intricate relationship between stress-induced

kisspeptin downregulation and the resulting impairment in HPG axis function. The initial investigations aimed to establish the effects of pubertal stress on the kisspeptin system, specifically we aimed to discern stress-induced changes to hypothalamic *Kiss1* and *Kiss1R* mRNA levels.

1. Stress-induced kisspeptin and kisspeptin receptor reduction

The neuropeptide kisspeptin and its receptor Kiss1R play a pivotal role within the HPG axis by initiating the release of GnRH, subsequently triggering LH and FSH secretion. LH and FSH, in turn, stimulate the production of sex hormones such as E_2 and T. Importantly, the negative feedback of both sex hormones is mediated by kisspeptin, situating it as a critical component of HPG axis regulation. However, stress potently downregulates kisspeptin and its receptor which may threaten the negative feedback process regulating hormone release. To illustrate this point, intracardiac LPS treatment, restraint stress, and central injection of CRH all potently downregulate mPOA and ARC expression of kisspeptin and its receptor in gonadectomized adult female rats (Kinsey-Jones et al., 2009). In comparison, intravenous LPS treatment only reduced whole hypothalamus *Kiss1* mRNA in adult female rats (Iwasa et al., 2008). Furthermore in adult male rats treated with 0.5mg/kg, LPS treatment induced decreases to hypothalamic *Kiss1* mRNA, but not Kiss1R (Iwasa et al., 2008). Collectively these studies provide compelling evidence of kisspeptin's sensitivity to stress treatment. However, limitations to these designs create curiosity as to whether LPS affects kisspeptin expression during puberty, how this effect is contrasted between males and females, and whether it is enduring.

While stress-induced changes in kisspeptin and its receptor have been outlined in previous reports, this work adds to the current literature by addressing two important factors missing from previous work: how stress impacts kisspeptin expression both over time and by

based on sex. Study 1 and 2 replicated previous findings by showing that acute, intraperitoneal LPS treatment reduces hypothalamic *Kiss1* mRNA. More importantly, Study 1 also showed for the first time that LPS treatment reduces hypothalamic *Kiss1* mRNA expression in both male and female pubertal mice. Although we observed some reductions to adult hypothalamic *Kiss1* mRNA in Study 2, it's important to note that it was not significant, and therefore restricts this observation to pubertal mice. In addition to *Kiss1* LPS treatment acutely reduced hypothalamic *Kiss1R* mRNA in pubertal and adult male mice in the second study, but not the first. In females, however, the data found that hypothalamic *Kiss1R* mRNA expression paradoxically increased in response to LPS treatment in pubertal and adult females. As we expected, hypothalamic *Kiss1* mRNA remained downregulated in LPS-treated pubertal males and females through to adulthood, whereas LPS-treated adult males and females showed increased hypothalamic *Kiss1* mRNA levels four weeks post-treatment. Enduring decreases in hypothalamic *Kiss1R* mRNA in pubertal and adult LPS-treated males, but not females perhaps suggests that receptor expression changes are sexually dimorphic. Given the dynamic changes in *Kiss1* and *Kiss1R* expression that we observed, further investigation is warranted to understand the underlying mechanisms that drive these sexually dimorphic changes.

2. HPG axis receptors in the brain are affected by LPS.

Kisspeptin is one of many intricate components that make up the expansive HPG axis network. But it is important to broaden the scope of our analysis to include various other HPG axis components, such as hypothalamic and pituitary *GAD67* mRNA, whole brain ERα protein, hypothalamic AR protein, hypothalamic, and mPFC were examined to capture a complete picture of the central HPG axis components. GABA transmission has long been suspected to function as a potential link contributing to the downregulation of the HPG axis by the HPA axis.

To illustrate, intracerebral LPS treatment in adult hormone-replaced OVX female rats inhibits pulsatile LH secretion, but this effect is reversed when GABA receptor antagonist, bicuculine is administered (Li et al., 2015). Unexpectedly, our RT-qPCR analyses did not reveal significant differences for pituitary nor hypothalamic *GAD67* mRNA across any treatment or age condition. However, more recent data suggests that LH pulsatile frequency is dependent on the rate of hypothalamic GABAergic firing frequency (McIntyre et al., 2023). It is therefore plausible to insinuate that examining the electrophysiological properties of GABAergic neurons than relative mRNA levels is more meaningful to GABA's influence on the HPG axis than simply the absolute levels of *GAD67* mRNA (McIntyre et al., 2023). Nonetheless, these data point us away from examining *GAD67* in our attempt to elucidate the mechanism responsible for stress induced HPG axis downregulation. While our findings raise questions about the precise role of GABA within the HPG axis, the complexity of this system warrants examination from multiple angles. To that end, this thesis examined the effects of pubertal stress on two HPG axis components responsible for binding to sex hormones, ERα and AR.

Collectively, ERα and AR contribute critically to HPG activation and regulation (Moore, et al., 2018). These receptors are highly colocalized in hypothalamic kisspeptin neurons and receive feedback from the respective sex hormones to modulate kisspeptin firing (Smith et al., 2005). Additionally, these receptors contribute substantially to the workings of the HPG axis. For example, AR-suppressed castrated adult male mice supplemented display substantially lower ARC *Kiss1* mRNA compared to wildtypes. However, this relationship is reversed after AR-supressed mice are treated with T (Smith et al., 2005). On the other hand, female ERα knockout mice show accelerated pubertal onset, undergoing puberty at roughly PND 15, nearly 3 weeks prior to their wild-type counterparts (Mayer et al., 2010). Moreover, female ERα knockout mice

showed a decreased LH surge and blunted activity of AVPV kisspeptin neurons compared to wildtype females (Wang et al., 2019). The interconnected roles of AR and ERα in both male and female physiology underscore the pivotal role that these receptors play in the HPG axis. Recognizing the influential nature of these receptors on the HPG axis, we extended our approach to investigate LPS-induced acute, and enduring changes to AR and ERα in male and female, pubertal and adult mice.

This thesis explored the changes to both the AR and ERα to understand the varying impacts of LPS treatments across sexes and age groups. Unlike in the case of hypothalamic AR protein where only modest decreases in protein expression was observed in males, LPS treatment produced sex and age differences in whole brain ERα protein expression. Acute reductions in ERα protein were seen in pubertal males, as well as in pubertal and adult females; however, enduring changes were specifically noted in LPS-treated pubertal females. Robust reductions in ERα and not AR are unsurprising considering that few kisspeptin studies have supported a substantial role of AR in HPG axis functionality. For example, adult female wild-type and androgen receptor knockout (ARKO) mice showed comparable estrous cycling and fertility despite ARKO mice completely lacking AR (Walters et al., 2018). Conversely, ERα downregulation tended to cooccur with reductions to *Kiss1* mRNA and decreases to peripheral LH changes, indicating a that ERα affects the HPG axis more substantially than AR. Although both receptors are important to HPG axis functionality, these findings indicate that AR may play a lower supportive role in the negative feedback processes to the more influential ERα. Future research should aim to further delineate the relative contributions and mechanisms of action of AR and ERα in regulating the HPG axis, especially under conditions of stress or hormonal

imbalances. Building on this, it is essential to consider the downstream components of the HPG axis, such as the gonadotropins LH and FSH.

3. Acute and Enduring LH and FSH response to LPS

Gonadotropins like LH and FSH are produced and released from the pituitary gland where they stimulate sex hormone, sperm, and ovarian production (Döhler & Wuttke, 1974; Saez, 1994). LH and FSH are the first HPG axis byproducts to enter the circulatory system and interact with the rest of the body. Sex hormones, such as T and E_2, activate cellular receptors throughout the body and the brain where they ultimately provide negative feedback to hypothalamic KNDy cells (Helena et al., 2015). Consequently, gauging levels of gonadotropins like LH and FSH provides a viable method for assessing the health of the HPG axis. Stress treatments, such as chronic unpredictable stress, has been shown to effectively lower LH and E2 levels in peripubertal female mice (Zhao et al., 2021). Moreover, LPS treatment decreases LH pulse frequency in adult female rats compared to saline-treated counterparts (Lee et al., 2019). Interestingly, inconsistent FSH concentration changes are found following stress treatments. LPS treatment diminishes LH, but not FSH serum concentrations in both prenatal male and female mice (Solati et al., 2012). Yet, the effects of FSH appear to be more nuanced; for instance, LPS treatment diminishes LH, but not FSH serum concentration in prenatal male and female mice, whereas, LPS increases FSH concentrations in adult female mice, despite reducing E_2 (Lv et al., 2021). Notably, previous studies have largely ignored the enduring nature of these changes, although they are critical to acknowledge to fully understand the HPG axis. In light of this, this thesis examined the peripheral concentrations of LH and FSH to achieve a comprehensive understanding of the HPG axis response to stress, in addition to appreciating the contributions of sex- and age.

The investigations into the effects of LPS treatment during the pubertal sensitive period revealed a complex and sex-dependent impact on gonadotropin levels, challenging both our initial hypothesis and existing understanding of HPG axis regulation. As predicted, LPS treatment during the pubertal sensitive period acutely and induced sex-dependent changes in gonadotropins concentration. In both Studies 1 and 2, LPS treatment decreased serum LH in pubertal male mice, and in adult male and female mice compared to their saline-treated counterparts. Surprisingly, serum levels remained consistent between LPS- and saline-treated pubertal female mice in Study 1, but unexpectedly decreased from LPS treatment in Study 2. Contrary to our hypothesis, pubertal LPS treatment failed to induce enduring changes in pubertal or adult males and females. While it's established that LH is directly regulated by the HPG axis, these findings suggest the possibility of a compensatory mechanism that maintains LH levels even after significant suppression of hypothalamic Kiss1. Although more research in the long-term effects of LPS treatment on LH is required to identify whether a compensatory mechanism exists. Nonetheless, having elucidated the enduring resilience of LH to LPS treatment, across age groups, it's essential to explore the nuanced sex-dependent variations in LH serum levels.

Intriguingly, we identified sex-dependent LH changes where pubertal male mice showed greater baseline serum concentrations than females in Study 1, but not Study 2. It is feasible to suggest that baseline differences in LH serum are due to female estrous cycling. However, it is important to note that baseline LH concentrations are susceptible to estrous cycling, where LH is roughly 33% greater in female rats during proestrus than diestrus (Faccio et al., 2013). To further explore the influences of female estrous cycling on LH levels, future experiments should consider the estrous cycle to validate these findings. By considering the hormonal variations

across estrous cycle stages, we can gain deeper insights into the sex-specific regulation of LH and its impact on reproductive processes.

Similarly, to LH, this thesis identified sex-dependent effects on FSH concentration where females, regardless of treatment condition, show diminished serum levels in both Study 1 and Study 2 compared to males. To support this finding, FSH recordings from birth to PND 36 report that male serum levels are substantially greater than females (Michael et al., 1980). Furthermore, elevated FSH in prepubertal male rats was also reported by Moguilevsky and colleagues (1977), as well as Döhler & Wuttke (1974), however, the latter report demonstrated FSH equalization between the sexes after PND30. The disparity in FSH levels between males and females during early development may contribute to sex-specific differences in reproductive maturation and hormonal regulation and vice versa. There may exist critical stages of FSH production and release between males and females, but this postulation has yet to be explored. These findings emphasize the importance of examining both sexes in biological research, particularly to those pertinent to endocrinology. Further investigations exploring the underlying mechanism responsible for sex specific FSH variations could provide valuable insights into the regulation of reproductive processes between the sexes. Having illuminated the complex interplay of hormones such as LH and FSH in both sexes, it's crucial to expand our lens to include another key player in stress and reproductive regulation: the immune response.

4. Immune response affects males and females separately.

Building on our understanding of the complex hormonal landscape, it is essential to consider the immune system, as it is the first line of defense against LPS. LPS treatment potently activates the HPA axis and triggers the release of proinflammatory cytokines, such as IL-6 and TNFα, through the TLR4 receptors. This cascade triggers an acute immune response, leading to

behavioral symptoms like ptosis, huddling, lethargy, and piloerection for up to 48 hours. Like kisspeptin, the TLR4 is abundant in the hypothalamus, particularly in close proximity to, or colocalized with GnRH releasing neurons within the median eminence (Haziak et al., 2014; Magata et al., 2022). Central administration of proinflammatory cytokine IL1β and TNFα leads to inhibited GnRH release, as well as LH and FSH in adult female rats (Ebisui et al., 1992; Rivier & Vale, 1990). As well, treatment with the anti-inflammatory drug, indomethacin, prevents hypothalamic kisspeptin mRNA downregulation in female rats (Iwasa et al., 2008). This intricate milieu offers potential to the regulatory capacity of cytokines on the kisspeptin system. Therefore, a nuanced understanding of the immune system is crucial for advancing our knowledge of LPS on the HPA axis.

Sickness behavior data were convergent across all three studies reporting that pubertal female mice who received LPS showed greater sickness intensity compared to males. Consistent with predictions, LPS-treated male mice showed sickness symptoms for a lengthier time frame than females. These findings not only corroborate earlier data, but also contribute to the growing consensus that females experience intensified sickness behaviors, whereas symptoms in males persist for a longer time. Not surprisingly, Study 2 demonstrated that LPS evokes considerably greater serum IL-6 concentration in adult mice over pubertal mice of both sexes. However, we did not detect the same trend in serum levels of TNFα. It is currently unclear why adult mice are showing greater cytokine expression, however, it is possible that puberty is a period of stress hyporesponsivity. For example, LPS-treated pubertal female rats (McCormick et al., 2020) and pubertal male mice displayed reduced IFNγ and IL1β serum concentrations (Cai et al., 2016) compared to their adult counterparts. More recently, adult LPS-treated male mice showed increased serum concentrations of proinflammatory IFNγ, IL-1β, IL-6 compared to pubertal

counterparts (Murray et al., 2023). Stress hyporesponsivity is well-documented during neonatal development, but it may extend to the pubertal period, as well. Alternatively, pubertal animals may show lower levels of IL-6 concentration eight hours after LPS treatment due to quicker recovery time from a hyporesponsive reaction, in comparison to adults. To support this, body temperature recordings between pubertal and adult mice following LPS treatment indicates a quicker return to a healthy body temperature in pubertal males (Cai et al., 2016). It should be emphasized that with the exception of the report by Cai and colleagues (2016), there is limited research contrasting hour-by-hour immune system responses between pubertal and adult animals (Cai et al., 2016). The substantial contribution of the immune system to stress regulation warrants further investigation to fully understand how the immune system is impacting HPA axis activation and HPG axis downregulation in pubertal and adult animals. In addition to understanding the complexities of the immune system following LPS treatment, it's important to address how immune stress influences over behaviors. Sexual behavior serves as a critical non-molecular gauge of HPG axis functionality.

5. Sexual behavior is decreased by pubertal LPS.

Assessing sexual behavior serves as a benchmark practice for evaluating HPG axis health and offers a lens through which HPG axis dysfunction can be observed. We and others have shown that LPS treatment on mice at different developmental stages. Specifically, six-week-old mice subjected to LPS treatment show a marked diminishment in sexual behavior in adulthood relative to mice treated with LPS at either four, five, seven, eight, nine, or ten weeks of age (Laroche et al., 2009a). Similarly, shipping stress at six weeks of age induces greater sexual behavior deficits compared to other mice between four, five, seven, eight, nine or ten weeks of age (Laroche et al., 2009b). Although, outside of this small collection of findings, little is known

about pubertal LPS-induced sexual behavior dysfunction. Contributing to this existing literature has been a valuable component of this thesis, enabling further exploration into age- and sex-related behavioral variations that have yet to be studied in detail.

This thesis brings greater clarity to the effects of LPS treatment on sexual behavior by not only supporting prior work but also offering novel insights to the interplay between stressors and sexual behavior in rodents. As was expected, pubertal LPS treatment induced sexual behavior deficits in both male and female mice. The data revealed a significant decrease in both mounting and intromission activities from the third week to the fifth week compared to saline-treated males. Comparing week-over-week findings, LPS-treated males demonstrated a stagnant level of mount activity from week two to week five. This suggests that despite gaining more sexual experience over time, there was little to no improvement in their sexual activity, unlike mice in the other treatment conditions. In contrast, saline- and vehicle-treated counterparts continued to improve their mount activity from week three to week five. Alongside the mount data, intromission frequency remained fairly consistent from week one to week three. Whereas weeks four and five saw notably higher intromissions among saline- and vehicle-treated mice compared to those treated with LPS. With the exception of a few details, LQ in female mice largely saw the same pattern as males. Saline-treated females exhibited noticeable increases in LQ from week one-two, two-three, and three-four finally maintaining at the fifth week. Contrary to male sexual behavior, LQ increased between week one-to-two and two-to-three in LPS-treated females where it hit a ceiling for the remaining weeks. Still, saline-treated mice outperformed LPS-treated mice in every week besides the first. An interesting distinction emerged when we examined sex-based sexual behavior responses. Notably, LPS-treated female mice began showing significantly reduced LQ compared to their saline-treated counterparts at week two, one week earlier than

when males began showing LPS treatment differences in mount frequency, and two weeks earlier than intromission frequency. This suggests the possibility that females are more vulnerable to enduring stress-induced sexual behavioral changes. Although the increased vulnerability to female mice is concerning, they also benefitted substantially greater than males when undergoing kisspeptin treatment.

7. Kisspeptin restores sexual behaviour functionality.

Our investigation reveals that synthetic kisspeptin (KP) treatment ameliorates the pubertal LPS-induced sexual dysfunction in females, but not males. Our findings partially corroborate our initial hypotheses, as the KP-treated female mice exhibited notable enhancements in sexual behaviors in comparison to those treated with a vehicle solution. In line with our initial observations that long-term sexual behavior dysfunction was adversely impacted by LPS treatment, we explored the possibility of restoring normal sexual behavior through exogenous KP treatment. When administered KP treatment, the LPS-treated female mice demonstrated marked improvement in LQ from week two to week five compared to vehicle- and LPS-treated counterparts. The restorative properties of KP treatment in females observed in this thesis falls in line with previous findings, where adult female kisspeptin knockout mice showed little interest in male stimulus mice until they were treated with subcutaneous KP (Hellier et al., 2018).

Unexpectedly, KP- and LPS-treated males did not exhibit improvements in sexual behavior. This could be linked to the findings from Study 2, which indicated that LPS treatment led to a lasting downregulation of Kiss1R expression specifically in male mice, but not in females (Smith et al., 2023). This downregulation of Kiss1R in males suggests that the exogenous KP may lack sufficient receptor sites to bind to, thereby diminishing its potential

therapeutic efficacy. The presumed mechanism behind KP treatment was to supplement endogenously reduced levels of kisspeptin. However, for this supplementation to be effective, an adequate number of Kiss1R receptors must be available for the exogenous KP to interact with. The absence of a similar downregulation of Kiss1R in females might explain why they did not exhibit the same lack of response to KP treatment as males. This discrepancy underscores the importance of Kiss1R availability in mediating the effectiveness of KP-based therapies. Further research is imperative to delve deeper into this mechanism, particularly to understand the conditions under which KP treatment can be optimally effective, and how the interplay between kisspeptin and its receptor is modulated following stressors like LPS in both sexes. Our data advocate for KP's role in counteracting the long-lasting adverse modifications in female sexual behavior due to LPS exposure during the formative pubertal stage. Nevertheless, a more comprehensive investigation is essential for fully understanding the mechanisms that contribute to this observed phenomenon. Based on our data, we conclude that KP treatment is effective in mitigating the enduring negative effects that pubertal LPS treatment has on the sexual behavior of female mice. As a result, our research identifies kisspeptin as a pivotal factor in the mediation of female sexual dysfunction. Our findings contribute to the existing body of knowledge and open up the possibility of new, targeted hormonal treatments in this area.

Our data somewhat aligns with preclinical research highlighting kisspeptin's central role in the hypothalamic-pituitary-gonadal (HPG) axis and its profound influence on sexual motivation and behaviour (Thurston et al., 2022). For example, kisspeptin administration increased activity in the reward-associated regions (nucleus accumbens), and emotional salience areas (insula) in men and women during exposure to erotic stimuli, as measured by fMRI (Mills et al., 2023). This effect extended to the hypothalamus, a key hub for sexual attraction. These

findings suggest a central mechanism of action whereby kisspeptin potentiates neural circuits underlying sexual desire and motivation in humans, ultimately translating into functional improvements. Notably, these changes in brain activation directly correlated with significant increases in self-reported sexual desire and satisfaction, underscoring the functional translation of the neuroendocrine effects. Specifically in men, kisspeptin further manifested its ameliorative effects through increased penile tumescence (Mills et al., 2023). This finding not only corroborates the hypothesis that kisspeptin enhances sexual behaviour but also provides tangible evidence for its ability to facilitate physical arousal, a crucial component of the human sexual response. Importantly, these improvements occurred without affecting other vital parameters like heart rate or blood pressure, suggesting a selective modulation of the sexual response system (Mills et al., 2023).

In conclusion, this thesis, demonstrates the profound potential of kisspeptin as a novel therapeutic approach for sexual behaviour dysfunction. Together these reports signify that kisspeptin may indeed be the mechanism behind dysfunctional sexual behavior, and ultimately stressed-induced HPG axis downregulation. Future research should focus on optimizing dosing regimens, exploring treatment duration, and examining potential long-term effects to fully realize the clinical potential of this exciting new therapeutic candidate. Therefore, targeted kisspeptin treatments could offer a promising route for therapeutic interventions. As such, future inquiries are essential to elucidate the intricate dynamics between varying dosages of kisspeptin and their corresponding effects on reproductive physiology.

8. Limitations

There are important limitations within the studies that make up this thesis. Acknowledging these constraints will lead to a more nuanced and accurate interpretation of the

findings. First, specificity is lacking in the RT-qPCR component of the first two studies. The size of the mouse brain and the relatively large brain tissue requirement for RT-qPCR resulted in a larger than intended brain tissue excision. The hypothalamus is a large brain region that contains several small and highly specialized nuclei. Capturing tissue from only the nuclei of interest may lead to more refined and pronounced observations. Second, administering peripheral kisspeptin treatment greatly enhances the ecological validity of our findings, although central administration of kisspeptin may have increased effect size potential and decreased variability. In line with the preceding comment, enhancing the specificity of kisspeptin treatment holds significant value for scientific analysis. Third, KP treatment in the absence of a KR antagonist (or including KR knockout mice) limits the scope of our mechanistic understanding. Despite procuring a wealth of data regarding the therapeutic effects of KP, the degree to which the receptor influences sexual behavior, and overall HPG axis functionality, is elusive. Lastly, sex differences in hormonal milieu should be given greater consideration by capturing androgen and estrogen data, as well as limiting female brain tissue collection to a specific estrous stage. In addition to the analyses already performed, correlational analysis between HPG axis biomarkers and sex hormones may provide greater insights into the specific roles of sex hormones.

9. Future Directions

This thesis widens the limited body of research pertaining to using kisspeptin therapeutically to restore sexual dysfunction in both male and female mice. Though much is known about kisspeptin, there is a distinct lack of knowledge surrounding its practical implications. Future studies should continue expanding on the findings of this thesis by identifying the mechanism through which infertility, precocious puberty, and diseases like polycystic ovarian syndrome and hypogonadism occur. Furthermore, the impact of sex

differences on kisspeptin expression and the KNDy neurons requires greater elucidation. The KNDy body of research presents a high-resolution general mechanism for HPG axis functionality yet fails to impactfully describe contributions from sex hormones and their corresponding receptors. Likewise, proinflammatory cytokines and the TLR4 commonly instigate hypothalamic kisspeptin attenuation and represent a potential model for HPA and HPG axis crosstalk. Accounting for the immune systems contribution is requisite for gaining a deeper comprehension of how these two neuroendocrine systems manage allostasis. Moreover, investigations can expand upon the cellular pathways responsible for the synthesis and release of kisspeptin as potential targets for HPG axis viability. Certain cases of HPG axis dysfunction may not stem from inadequate kisspeptin or kisspeptin receptor levels. Instead, they could result from an impaired ability to synthesize or transport the peptide and/or protein.

10. Conclusions

This thesis expands existing knowledge concerning kisspeptin, providing concrete evidence that both complements and advances existing knowledge. The culmination of these studies ultimately found that exogenous kisspeptin administration successfully restores female sexual behavior following pubertal LPS treatment. First, the HPG axis' vulnerability to HPA axis activation is confirmed by the fact that LPS treatment acutely diminishes kisspeptin, its receptor, and LH in a sex-specific manner. Second, hypothalamic Kiss1 mRNA is subject to enduring downregulation in pubertal, but not adult, LPS-treated male and female mice. However, hypothalamic Kiss1R and AR are acutely and enduringly diminished by LPS in a manner that is uniquely observed in pubertal and adult males. Pubertal males and females as well as adult females experience acute reductions in ERα, whereas only pubertal females experience enduring reductions. Third, pubertal LPS treatment and the subsequent HPG axis dysfunction translates to

detrimental adulthood sexual behaviors for both sexes. However, sexual behavior is restored through subcutaneous kisspeptin treatments in female LPS-treated pubertal mice.

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