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Mechanisms of Obesity Induced Impairment of Reproduction

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

by

Nancy M. Lainez

June 2019

Dissertation Committee:

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The Dissertation of Nancy M. Lainez is approved:

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To Dr. Djurdjica Coss: You saw past my insecurities and never let me quit, and for that I am forever grateful.

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DEDICATION

This dissertation is dedicated to the many strong and independent women in my life. To my mom, who will always inspire me to be strong during times of adversity and to prove to myself that I can do anything. To my Tia Sara, I want to thank you for always being proud of me and believing in me when I didn't believe in myself. A mama pita, le quiero decir gracias por sus oraciones y por amar me como su propia hija, and to Tia Nini, I will always be grateful for your endless enthusiasm and excitement for my accomplishments. I also would like to thank my cousin Mimi for being my role model, I look up to you and I appreciate your encouragement and support. Finally, to my niece Kennedy, you showed me true strength and courage, watching you flourish and overcome so much at such a young age showed me to persevere and motivated me to keep going.

ABSTRACT OF THE DISSERTATION

Mechanism of Obesity Induced Impairment of Reproduction

by

Nancy M. Lainez

Doctor of Philosophy, Graduate Program in Biomedical Sciences

University of California, Riverside, June 2019

Dr. Djurdjica Coss, Chairperson

The increase of obesity among US men and women of age 20-39 has resulted in social and economic consequences. Obese individuals are at increased risk of developing reproductive issues. Obese women present with menstrual irregularities, pregnancy complications, and infertility due to anovulation, while obese men present with low testosterone and sperm count. Mammalian reproduction is regulated by the hypothalamic-pituitary-gonadal axis. Gonadotropin releasing hormone (GnRH) neurons, scattered in the preoptic area of the hypothalamus, synthesize and secrete GnRH to act on the anterior pituitary to stimulate the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from gonadotrope cells. LH and FSH then act on the gonads to promote steroidogenesis and gametogenesis.

Nutritional status exerts their effects on reproduction by modulating GnRH levels. Previous studies have reported that metabolic pathways do not directly act on GnRH neurons. Pro-inflammatory cytokines, tumor necrosis factor (TNF) α , interleukin (IL)-6 and IL-1 β , which are elevated in circulation and in the central nervous system (CNS), including the hypothalamus, of obese individuals, have been implicated in altering GnRH levels in rodents challenged with lipopolysaccharide, but how these cytokines mediate their effects is unknown and controversial. Thus, we investigated mechanisms by which inflammatory signals influence GnRH neurons and GnRH gene expression to provide insight into the etiology of obesity-induced infertility.

Our studies conducted in C57BL/6J mice demonstrated sex differences in the response to diet induced obesity with respect to inflammation and hypothalamic function. We proposed neuro-immune mechanisms of obesity induced impairment of reproduction whereby neuroinflammation and subsequent decrease in GnRH neuron spine density was specific for male mice, while protection in females was independent of ovarian estrogens. We also investigated direct effects of neuroinflammation-induced cytokines on GnRH gene expression by examining signaling pathways and mechanisms in male mice and in GnRH expressing cell line, GT1-7 cells. We identified an additional mechanism of obesity induced impairment of reproduction where LIF directly repressed GnRH mRNA via cFOS, that is induced in GnRH and other neurons that are proximal to fenestrated capillaries of diet-induced obese male mice.

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Chapter 1: Introduction

1.1 Hypothalamic Pituitary Gonadal Axis

The hypothalamus is a forebrain region located below the thalamus. It is delineated by the lamina terminalis and optic chiasm rostrally, mammillary bodies caudally, the walls of the third ventricle medially and by the internal capsule laterally. The hypothalamus is comprised of several nuclei containing neurons that express a number of neuropeptides and neurohormones implicated in the regulation of a wide range of homeostatic processes, including food intake, thirst, energy expenditure, body weight, circadian rhythms, thermoregulation and reproduction [1]. These neurons can project to other brain regions or other hypothalamic nuclei, allowing communication between them. The preoptic area (POA), arcuate nucleus (ARC), anteroventral paraventricular (AVPV), are hypothalamic nuclei heavily involved in the regulation of reproduction, while the ARC, lateral hypothalamus area (LHA) and ventromedial hypothalamic nucleus (VMH) are involved in regulating satiety and food intake [1]. The hypothalamus contains several circumventricular organs that lack a blood-brain barrier, most notably organum vasculosum laminae terminalis (OVLT) located in the rostral region, and median eminence (ME) located at the ventral border. These areas possess specialized arrangements of blood vessels called the hypophyseal portal system, with capillary endothelial cells that allow diffusion of large molecules in the central nervous system (CNS) where the neurons involved in metabolism and reproduction may be able to sense them.

Reproduction is controlled by the hypothalamus-pituitary-gonadal axis. The final brain output that regulates reproduction is hypothalamic neuropeptide gonadotropin releasing-hormone (GnRH), secreted by a small population of hypothalamic neurons. There are about 800 GnRH neurons scattered in the forebrain of a mouse. GnRH neuron cell bodies are localized to the preoptic area (POA) and send long axon projections to the median eminence, where GnRH is released in a pulsatile manner into the hypophyseal portal blood system. GnRH is carried to the anterior pituitary where it binds to its receptor on gonadotrope cells, stimulating the synthesis and secretion of gonadotropin hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [2]. LH and FSH act through their receptors located in the gonads to regulate gonadal development and function. LH acts on Leydig cells, in males, and theca cells, in females, to promote testosterone production in both sexes, and to stimulate ovulation in females. FSH works on Sertoli cells to stimulate spermatogenesis via synthesis of androgen binding protein in males, while in females FSH acts on granulosa cells to stimulate folliculogenesis prior to antral stage and to stimulate estrogen synthesis from testosterone produced by theca cells. Estrogen and testosterone feedback at the level of the hypothalamus and pituitary to regulate GnRH, LH, and FSH [3]. GnRH neurons integrate afferent neurons, metabolic hormones and sex steroids in the regulation of reproduction [3].

1.2 Obesity Effects on Reproductive Fitness

Obese individuals are at increased risk of developing reproductive issues.

According to the world health organization, obesity, defined as a body mass index (BMI) of $\geq 30 \text{ kg/m}^2$, is linked to an increased risk of ischemic heart disease, type II diabetes, and reproductive disorders for both men and women [4], [5]. Over the past 30 years prevalence of obesity has increased steadily with over 30% of men and women classified as obese, including those of reproductive age (20-39). In the recent years, increase in obesity has disproportionately affected children and young individuals, which may lead to longer term consequences on a number of homeostatic processes, including reproductive function.

1.2.1 Negative Effects of Obesity in Men

Previous studies have reported that weight gain and obesity is associated with suppression of testosterone and reduced levels of sex hormone binding globulin [6], [7]. In obesity, reductions in testosterone is accompanied by reduced levels of LH [6]. Many studies are in agreement that sperm number and quality are negatively impacted by obesity [8], [4] - [9]. Increased BMIs ($>25 \text{ kg/m}^2$) in males are associated with lower sperm concentration and fewer total spermatozoa compared with subjects with normal BMIs ($20 - 25 \text{ kg/m}^2$) [10], [11]. Meta-analysis of 21 reports and a total of 13000 men, associated obesity with increased prevalence of azoospermia and oligozoospermia [5].

Studies in humans were confirmed using obese Zucker rats that demonstrated decreased sperm production and increased sperm DNA fragmentation compared to their lean littermates [10], [9]. In addition, sperm binding and fertilization rates during in vitro fertilization (IVF) are reduced when the male partner is overweight or obese [12]. Since obesity mediated reduction in testosterone is observed with reduced LH levels, and age-associated reduction in testosterone is accompanied with an increase in LH, due to the negative feedback, obesity likely impairs central, hypothalamic function, while age affects testicular function [6].

1.2.2 Negative Effects of Obesity in Women

Similar to what is observed in overweight and obese men, overweight and obese women are more likely to have reduced fertility accompanied by reduced levels of LH [13], [4]. Obesity among women include early onset of puberty, menstrual irregularities, pregnancy complications, infertility and spontaneous abortions [14], [15]. A previous report found that among women seeking to become pregnant, obese women experience longer times to conception regardless of age and menstrual cycle patterns [16]. It has been established that obese women have lower levels of gonadotropins [17], [18]. A study conducted in 25 gynecologically normal and regularly menstruating age matched obese women had significantly reduced serum levels of LH [17] consistent with other studies where the amplitude of LH was dramatically reduced in obese women compared to controls [18]. In a larger study that evaluated 833 women, heavier women had longer cycles and lower levels of LH [19].

Studies conducted in mammals are consistent with what is observed on obese women. In female mice, diet induced obesity resulted in increased estrous cycle length, suggesting obese mice ovulated less [20]. Female rhesus macaques placed on western diet (WSD) for 6 months, a high calorie and high fat diet, presented with reduced LH pulse amplitude [21]. After 14 months on the diet, body fat increased from 2% to 15-19%, and LH pulse amplitude remained low [21]. These studies suggest that the level of dysregulation may be at the hypothalamus.

1.3 Regulation of GnRH secretion

1.3.1 Kisspeptin

Kisspeptin (KISS), is a 54 amino acid neuropeptide important for the onset of puberty and support of reproductive function in the adult. KISS was first shown to be critical for reproduction in humans that carry mutation in *KISS1* gene and fail to undergo pubertal development and are infertile [22], [23]. Intracerebroventricular (ICV) injection of KISS in immature female rats caused precocious puberty [24]. ICV [25], intraperitoneal (IP) [26], and intravenous [27] injections with KISS elicited robust secretion of LH in adult male mice, sheep, and human males respectively.

KISS signals through the G protein coupled receptor 54, GPR54 expressed throughout the hypothalamus and on GnRH neurons in adult male rat, male and female juvenile and adult mice, and male rhesus monkey [28], [26], [25], [29]. While expression of GPR54 on GnRH neurons in humans, have not yet been reported, KISS and GPR54 were immunolocalized and co-expressed with GnRH in the fetal olfactory mucosa and FNC-B4 cells, primary culture of human fetal GnRH-secreting neuroblasts [30]. The importance of KISS-GPR54 signaling was identified when studies reported that loss of function mutation of GPR54 resulted in hypogonadotropic hypogonadism and absence of puberty in humans [23]. Consistent with these studies, mice deficient for GPR54 experience hypogonadotropic hypogonadism, with pituitary gonadotropes that are capable of responding to exogenous GnRH, indicating that KISS is upstream of GnRH [22, 23, [31].

In rodents, KISS neurons are found in the AVPV and in the ARC and send projections to the POA, where GnRH neurons are abundant [32]. KISS neurons in the AVPV project to GnRH cell bodies, while those from the ARC project to GnRH terminals [33]. AVPV is a sexually dimorphic area, larger [34] with more KISS neurons in the female than in the male [35], while the ARC shows no sex differences in rodents [36]. Similar sex differences were reported in humans [37], which may indicate that these two populations serve different functions. *KISS1* mRNA is increased by estradiol in the KISS neurons in the AVPV, and this population is thought to regulate estrogen positive feedback during the preovulatory LH surge [38], [39]. In the ARC, estrogen treatments resulted in lower *KISS1* mRNA [38], [39]. Thus, KISS neurons in the AVPV has been suggested to generate the

preovulatory GnRH/LH surge in the female [40], while those in the ARC are thought to be involved in the negative feedback by estrogen.

In the adult mammalian brain, GnRH neurons secrete GnRH in a pulsatile manner. Since GnRH neurons are scattered in the basal forebrain, significant effort has been made to identify the pulse generator that would activate and synchronize dispersed GnRH neurons. Previously, rhythmic activity of KISS neurons from the ARC were found to coincide with GnRH/LH pulses [36], [41] - [42]. Others reported that mutation of GPR54 in mice had no effect on hypothalamic production of GnRH peptide GnRH [23]2, 23, [31].

KISS likely regulates GnRH secretion, but not GnRH peptide production.

These data suggest that KISS is a major regulator of GnRH secretion, so much so that recent review posited that KISS neurons in the ARC nucleus synchronize GnRH neurons and drive the pulsatile secretion of GnRH [41].

1.3.2 Neurotransmitters Glutamate and GABA

Glutamate is regarded as the main fast excitatory neurotransmitter in the central nervous system. The three types of ionotropic glutamate receptors are α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), comprised of 4 subunits GluR1-4 and GluR A-D, N-methyl-D-aspartic acid (NMDA), comprised of subunits NR1-3 with NR1 as the obligatory subunit, and kainate-type glutamate receptors comprised of GluK1-5 subunits [43]. These receptors are found at the postsynaptic dense (PSD) structure of the neuron [44]. PSDs can be found on dendritic spines of many neurons. GnRH neurons not only

express all three types of glutamate receptors [45] - [46], but also exhibit spines on the soma and primary dendrite [47].

Spontaneous excitatory postsynaptic stochastic current (sEPSC) studies, conducted in brain slices from adult male and female mice demonstrated that 50% GnRH neurons tagged with GFP exhibit sEPSCs resulting from activation of AMPA/kainate receptors while 31% of GnRH neurons displaying sEPSCs was mediated by NMDA receptors, and 19% resulted from both [48], supporting that GnRH neurons receive glutamatergic input. Currents recorded in GnRH neurons located in the AVPV produce a fast rise and decay time when evoked by AMPA, while decay time for NMDA is much slower. There are studies suggesting the involvement of glutamatergic pathways in puberty onset and in evoking preovulatory GnRH surge [49] - [50].

The γ -aminobutyric acid or GABA, is classically regarded as the inhibitory neurotransmitter in the central nervous system. There are three classes of GABA receptors, GABA_A ligand-gated ion channel, GABA_B metabotropic seven-transmembrane domain G-protein coupled receptor, and GABA_C considered a subclass of GABA_A. These receptors are located on both pre- and post-synaptic membranes. GnRH neurons express both GABA_A and GABA_B receptors with studies reporting both inhibitory and excitatory actions of GABA on GnRH/LH secretion. GABAergic neurons in the AVPV express estrogen receptors and are thought to play both inhibitory and excitatory roles to regulate the LH surge, since following GABA release during the LH surge they exhibit changes in the expression of GAD67, the enzyme that converts glutamate into GABA [51] - [52]. Studies that focused on the direct action of GABA on GnRH neurons using both GT1-7 cells [53]

- [54] and transgenic mice [55] - [56] found that the activation of GABA_A receptors excited both GT1-7 cells and GnRH neurons in mouse hypothalamic slices. The GABA_A receptor gates an intrinsic ion channel that mainly passes chloride, thus GABA_A receptor activation can either inhibit or excite a cell depending on chloride levels. A study that recorded post synaptic currents of GnRH neurons in adult fed and fasted transgenic female mice, in diestrus, in which green fluorescent protein (GFP) is genetically targeted to GnRH neurons, determined that the principal ligand-activated chloride channel in GnRH neurons is GABA_A receptor [57].

This study showed that direct GABA application elicited robust action potentials of GnRH neurons, in the presence of high or low chloride levels, from both fed and fasted mice. GnRH neurons from fasted female mice had significantly lower post synaptic current from fed female mice. This may suggest that in fasted mice, GABAergic drive on GnRH neurons is reduced.

Although studies have shown that activation of GABA_A receptors on the soma and proximal dendrite GnRH neurons caused action potential discharges and activation of L-type calcium channels [56], [58] other studies have reported that GABA acts on GnRH axon terminals in the ME. When GABA release near GnRH nerve terminals was activated in rats, the estrous cycle was disrupted and fertility was reduced [59]. This suggests that the effect GABA on GnRH neurons may depend on where on the neuron GABA receptors are activated and on intracellular ion concentration.

1.4 Regulation of GnRH gene expression

GnRH is a peptide hormone composed of 10 amino acids that is elevated in the onset of puberty and essential for the reproductive process [60]. The control of GnRH synthesis and, as previously discussed, release have been studied heavily. The development of GnRH secreting cell lines, GN11 (immature, migratory cells) and GT1-7 (mature, non-migratory cells) and transgenic mice enabled the investigation of GnRH gene expression and the regulation *in vitro* and *in vivo* [61], [62]. GnRH promoter contains different putative transcription factor-binding sites that are important for basal transcription activities.

The 5' flanking region of the GnRH gene is homologous between human, rats, and mice [63]. Specific binding sites for a number of different transcription factors have been found within the promoter and enhancer have been identified by studying the rat promoter.

Neuron-specific activation of the rat GnRH gene is conferred, in culture and *in vivo*, by two upstream evolutionarily conserved regulatory elements: the 300 bp enhancer (-1863 to -1571, rodent numbering) and a promoter (-173 to +1). The 173 bp promoter upstream of the transcription start site has about 80% nucleotide homology among human, rat and mouse [64] - [65]. The 300 bp enhancer provides 50–100-fold activation of GnRH gene transcription as compared to the activity of the promoter alone [65].

Several transcription factors have been identified to act on the enhancer or promoter as regulators of GnRH gene transcription, including the homeodomain proteins Oct-1, Six6, SCIP/Oct-6, Dlx2 and Msx1, and Otx2; the zinc finger protein GATA-4; nuclear factor-1; and CCAAT/enhancer-binding protein- β [64 - 66].

Octamer transcription factor Oct-1 is a critical regulator of GnRH transcription and confers a 20-fold induction of the *GnRH* gene at the proximal promoter region at -47/-40 and within the enhancer at -1785 to -1771 bp [67]. Mutation of the Oct-1 binding site within -1785 to -1771 bp resulted in 95% reduction in transcriptional activity [67]. Thus, Oct-1 is important for basal transcriptional activity of the GnRH enhancer [67]. Within the enhancer region, two GATA sites, -1710 to -1715 bp and -1743 to -1748 bp, bind two GATA factors, GBP-A1/A2 and GBF-B1, which were found to be necessary for full enhancer activity [68]. The function of homeodomain proteins may be determined by the interaction of cofactors, which can enhance or inhibit the interactions between the homeodomain proteins Msx1, a repressor, and Dlx2, an activator, compete for homeodomain ATTA sites [69]. Two of these sites are located on the proximal promoter (-41 to -38 bp and -54 to -51 bp) and two sites on the enhancer (-1620 to -1617 bp, and -1634 to -1631 bp) [69, 70]. Specific interactions of transcription regulators with specific cofactors affect the activity of the promoter and result in specific targeting of expression of GnRH in GnRH neurons. Cofactor, groucho-related gene (GRG) 4 that interacts with Msx/Dlx homeodomain factors also interacts with Oct-1 to repress Oct-1-mediated activation [70]. Cofactors that interact with Oct-1 resulting in activation of GnRH transcription include, GRG5, Pbx1b and Prep1 [71, 72]. These antagonistic functions may be important during the onset of puberty, when GnRH increases. Knock-out (KO) of Six6 leads to loss of GnRH neurons during late development.

The orthodenticle homeobox (Otx) site in the promoter, found to bind Otx2 protein, was shown to be important for basal and enhancer driven transcription of the GnRH gene. In addition, two Otx2 binding sites, TTATC, -319 to -315 bp and TAATCC, -257 to -252 bp, were shown to be important for appropriate neuronal expression of *GnRH* [66, 73]. Other homeodomain protein required for normal GnRH neuron development includes Six6.

A previous study demonstrated that treatment of GT1-7 neurons with human chorionic gonadotropin (hCG), a GnRH inhibitor, resulted in an increase in phosphorylated Fos, Jun and cAMP response element binding protein (CREB) [74]. Overexpression of Fos and CREB inhibited promoter activity in the -3026 to +116 bp construct [74]. The specific binding site for FOS and CREB were not identified. We and others have shown that repression of GnRH, in GT1-7 cells is mediated through cFOS [75] - [76]. 12-*O*-tetradecanoyl phorbol-12-acetate (TPA) resulted in a dose and time dependent reduction in GnRH mRNA expression [77]. Repression of GnRH by TPA was found to be dependent on the proximal promoter, which previous studies identified activator protein-1 (AP-1) site in this region [76, 78]. It was found that the increase in *c-fos* and *c-jun* mRNA levels mediated this repression, but did not bind the AP-1 site, and required Oct-1, Prep/Pbx1a [77], [76]. In our studies we determined that LIF activated the MAPK pathway specifically p38 to repress GnRH, mediated by cFOS. We furthermore, identified the regulatory element that mediates this repression.

Though the development of GnRH neurons and control of GnRH expression has been studied heavily, further investigation is needed to understand the combination of transcription factors and the effect of surrounding environment required to fine-tune GnRH expression.

1.5 Feeding Circuitry

Normal function of the hypothalamic-pituitary-gonadal (HPG) axis, is sensitive to nutritional status [79]. States of over- and under- nutrition have resulted in disruption of the HPG axis in both humans and rodents. In the CNS, metabolic cues are coordinated by anorexigenic proopiomelanocortin (POMC) neurons and orexigenic neuropeptide Y (NPY) from the ARC nucleus and orexin from the lateral hypothalamus (LHA) [80]. These neurons regulate food intake by sensing levels of leptin and insulin, and it is proposed that they convey metabolic status to HPG axis. Though studies have examined the involvement of neuropeptides produced by POMC, NPY, and orexin neurons in communicating metabolic status to the HPG axis, results remain controversial.

1.5.1 POMC

Pro-opiomelanocortin (POMC) is an anorexigenic precursor found in neurons in the ARC that co-express cocaine and amphetamine-regulated transcript (CART). POMC neuronal activation reduces food intake and decreases energy expenditure. Melanocortins, such as adrenocorticotrophic hormone (ACTH) and anorexigenic α -melanocyte-stimulating hormone (α -MSH), and orexigenic β -endorphin (β -END) an opioid peptides are products cleaved from POMC precursor. The receptors for melanocortins have been classified into five subtypes, MC1R, MC2R, MC3R, MC4R, and MC5R. The classical α -MSH receptors is MC1R while MC2R is the classical ACTH receptor. Orexigenic β -END act on μ - and κ -receptors.

While higher levels of leptin and insulin inhibits NPY and orexin neurons, POMC neurons are activated by leptin and insulin. POMC neurons express leptin receptors and insulin receptors. Mice lacking leptin receptor specifically on POMC neurons are mildly obese and hyperleptemic [81]. When insulin receptors are deleted from POMC neurons, there is no observed change in body weight, but when both leptin receptor and insulin receptors are deleted from POMC neurons, severe insulin resistance and diabetes results [82]. Leptin activates POMC neurons to stimulate the secretion of α -MSH, which in turn binds to neurons that express MC4R to suppress appetite. On the other hand, POMC neurons release β -END which stimulates feeding. This suggests a more complex role of POMC neurons in the regulation of feeding.

POMC products, α -MSH and β -END have differential effects on reproduction. While α -MSH stimulates lordosis behavior in female rats, β -END inhibits GnRH/LH secretion. POMC neurons make direct contact with GnRH neurons in the rat brain ([83]). Brain slices from adult female mice where GnRH neurons are genetically linked to green fluorescent protein GFP were treated with neuropeptides made by POMC neurons, α -MSH, β -END, and CART assessed using loose-patch recordings. Firing rate of 69% of GnRH neurons were increased with α -MSH. When GnRH neurons were exposed to CART, firing rate increased for 25% GnRH neurons α -MSH. β -END application resulted in decreased rate of firing in 30% of GnRH neurons [79]. Thus, POMC products have opposing effects on a common pathway.

1.5.2 NPY

Neuropeptide Y (NPY) is an orexigenic neuropeptide synthesized by neurons in the ARC and the dorsomedial hypothalamic nucleus (DMH) that are activated during states of low energy, such as lactation or starvation. Ghrelin, secreted by the stomach and intestine, activate NPY neurons to promote food intake. NPY signals through G-protein coupled receptors, several of these receptor subtypes have been identified, Y1, Y2, Y3, Y4, Y5 and Y6. Although NPY can act directly on POMC neurons through its receptor, Y1 [84], studies have shown female and male mice that were genetically modified to overexpress NPY, *Npy^{tet/tet}*, maintained a similar body weight as WT mice [99]. This same study showed that when NPY expression was reduced in these same mice, induced by Dox treatment, no difference in food consumption or refeeding responses were observed when compared to

control ([85]). This suggests that neuropeptides released by NPY may be playing a compensatory role. Previous studies have explored neuropeptides, agouti-related protein (AgRP) and γ -aminobutyric acid (GABA), released by NPY neurons that originate in ARC as potential candidates in the regulation [86], [87]. AgRP was reported to not be required for the regulation of energy homeostasis [88] while GABA was shown to inhibit anorexigenic POMC neurons [89]. This suggests that NPY is not necessary for maintaining normal feeding behavior and that GABA might compensate for the absence for NPY.

In addition to regulating food intake, NPY neurons and the neuropeptides they release have been reported to have both stimulatory and inhibitory roles on the GnRH/LH system. In a study conducted in ovariectomized rhesus macaques, intracerebroventricular administration of NPY inhibited LH pulses, while local administration of NPY into the median eminence stimulated the release of GnRH [90]. In C57BL6 mice and rats, chronic central administration of NPY resulted in hypogonadism, while acute ICV administration of NPY stimulated the release of LH from ovarian steroid-primed ovariectomized rats [91], [92]. These opposing effects of NPY on GnRH, are thought to be due to the complex interplay of neuropeptides released by NPY neurons, AgRP and GABA, and the NPY receptors activated.

Previous studies have shown that NPY neurons, including those that express AGRP and GABA, from the ARC make contacts with the majority GnRH neurons [93, [86], [93], [94]. Brain slices from adult female GnRH green fluorescent protein (GFP) mice were prepared for loose patch recording experiments treated with AgRP resulted in reduced activity of 10% of GnRH neurons and stimulation of 25% of GnRH neurons [79]. In ovariectomized rhesus monkeys central infusion of AgRP into the third ventricle resulted in decreased LH pulse frequency [95].

Studies conducted in ovariectomized female Sprague-Dawley rats that were primed with estradiol and progesterone and received GABA^A receptor agonist, muscimol, intracerebroventricularly, had no effect on LH release [92]. When NPY was given to these rats alone, LH release was stimulated [92]. When GABA^A receptor agonist was added with NPY, NPY stimulated LH release was reduced [92]. This suggests that the co-release of NPY and GABA by NPY neurons may inhibit the release of LH. On the other hand, others have reported that in sections prepared for postsynaptic current recordings of GnRH neurons from fasted adult female mice showed GnRH currents were reduced compared to fed mice, while slices from fed mice treated with NPY decreased postsynaptic current frequency. This suggests, since NPY is increased with food deprivation, and GABA induced action potentials of GnRH neurons from fed mice, and NPY reduced postsynaptic current frequency of GnRH neurons from these mice, that NPY is involved in the decrease of excitatory GABA secretion on GnRH neurons [96].

Other studies investigated the effect of NPY receptors activation on GnRH neuron activity. Y1/Y2/Y5 receptor agonist, pNPY, resulted in reduced firing of 46% of GnRH neurons, but a highly selective Y5 receptor agonist, [cPP1-7, NPY19-23, AL³¹, Aib³², Gln³⁴]-hPP, had no effect on GnRH neuron excitability [79]. To further examine NPY receptor activation on GnRH neurons, an agonist for receptors Y1/Y4/Y5, with high affinity for Y4 and Y5, was shown to inhibit 30% and stimulate 50% of GnRH neurons. A Y4 agonist, rPP, increased firing rate in 50% of GnRH neurons. This suggests that NPY may suppress GnRH neurons through Y1 and stimulate through Y4 receptors [79]. To date, only receptor Y1 and Y5 have been reported to be expressed on GnRH terminals and cell bodies, respectively [93], [94]. While this study showed that Y5 agonist had no effect on GnRH neurons from female mice, others have reported that infusions of Y5 agonists, PYY₃₋₃₆, in the lateral ventricle of male Sprague-Dawley rats and male C57BL/6J mice result in reduced levels of LH, testosterone, and reduced testicular weight [97]05, [91]. Together these studies describe a complex regulatory role of NPY neurons on GnRH neuron activity.

1.5.3 Orexin

The hypothalamic neuropeptide, orexin, is mainly known for its role in sleep and wakefulness and is also involved in the control of feeding and drinking. Neurons that express orexin are found in the lateral hypothalamus (LHA) and send projections throughout the brain, including the POA where GnRH cell bodies are found and to the mediobasal hypothalamus and ME where GnRH axon terminals project.

There are two forms of orexin, orexin A and orexin B, with orexin A being more physiologically potent due to its resistance to degradation. Similar to NPY, orexin has both stimulating and inhibiting effects on GnRH/LH secretion. A previous study demonstrated that GnRH neurons express orexin 1 receptors, and that orexin neurons contact GnRH neurons from both male and female rats [98]. Tissue explants from male and female rats in proestrus show that orexin A stimulates GnRH release from the hypothalamus, while in diestrus or low estrogen states orexin A is inhibitory. Expression of orexin receptor 1 was also observed in GT1-7 cells and treatment with orexin resulted in increased GnRH mRNA expression and stimulated the release of GnRH [99].

In addition, studies using both rat and primate brains have shown that orexin neurons not only express leptin receptors, but also express receptors for NPY, such as Y1 and Y4 [100], [98]. Orexin neurons were also shown to make synaptic contact with NPY neurons in the ARC and vice versa [100]. These studies suggest that orexin may be stimulating NPY release and enhancing feeding behavior. On the other hand, since orexin neurons express NPY receptors, this suggest NPY could exert some of its effects via orexin neurons in the LHA.

In summary these studies describe a complex neuronal network linking metabolism and reproductive fitness, whereby POMC, NPY, and Orexin neurons mediate effects of metabolic cues on GnRH neurons. However, none of these studies explain negative effects of obesity.

1.6 Adipose Tissue as an Endocrine Organ

Adipose tissue (AT) has been established as a regulator of systemic nutrient and energy homeostasis. Adipose tissue (AT) is composed of adipocytes, connective tissue, and non-adipocyte cells including macrophages, immune cells, endothelial cells, preadipocytes and fibroblasts. AT is involved in the regulation of appetite, energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure, and lipid metabolism.

The adipocyte is the chief storage of triglycerides and communicates with different organ systems through the secretion of polypeptides, called adipokines, including leptin and adiponectin.

1.6.1 Leptin

Leptin is a protein product of the obese (*ob*) gene, which is secreted by adipocytes and functions as a satiety factor to regulate food intake by signaling to the brain. Leptin provides a link between metabolism and reproduction, and studies suggest that these two systems are integrated in the hypothalamus. Leptin receptor KO mice experience delayed onset of puberty in males and females and infertility [101]. Both male and female mice that lacked leptin (*ob/ob* mice), resulted in sterility, and leptin treatment restored reproduction [102], [103]. Leptin communicates nutritional status to the central nervous system via orexigenic neuropeptide Y (NPY) and anorexigenic proopiomelanocortin (POMC) neurons in the ARC of the hypothalamus where KISS neurons are found [80]. Studies have shown that lean ovariectomized ewes with reduced levels of *KISS1* gene expression, ICV

treatment with leptin resulted in partial restoration of *KISS1* mRNA, while ICV treatment with kisspeptin peptide reduced POMC and increased NPY gene expression [104].

Both POMC and NPY neurons have shown to express leptin receptor mRNA [101], [105], [106] and send projections to KISS neurons [107]. Studies have shown leptin to exert its effects on GnRH release by acting through KISS neurons [108], [104]. Studies conducted in castrated adult male C57BL6 mice, leptin deficient ob/ob mice, and leptin treated ob/ob mice, showed *KISS1* mRNA expression levels in the ARC decreased in leptin deficient mice and increased in leptin treated mice. This study also showed that *KISS1* mRNA expressing cells expressed leptin receptor [108]. In ovariectomized, fasted and fed, Wistar female rats using pull push perfusion technique leptin induced GnRH, LH, α -MSH, and PRL secretion in a dose dependent manner [109]. This study proposed that leptin acts directly on both GnRH cell bodies and axons to stimulate the secretion of GnRH and suggested that α -MSH may play an intermediary role in stimulation of GnRH secretion by leptin. Others have shown that specific leptin receptor KO in GnRH neurons of female and male mice did not affect fertility compared to control [101]. In addition, deletion of leptin receptor from hypothalamic KISS neurons in mice did not affect puberty onset or fertility [110]. Thus, suggesting that leptin signaling on GnRH or KISS neurons is not required for onset of puberty and fertility. Though there may be conflicting data implicating the direct or indirect action of leptin on GnRH neurons to regulate fertility, it is likely that intermediary peptides carry out these actions [79], [111].

In obesity, leptin levels are elevated, and even though studies in mice show that leptin treatment in ob/ob mice, that lack leptin, resulted in reversal of obesity and reduced calorie intake, obese humans are resistant to leptin. Thus, the biological efficacy of leptin in obesity may be limited.

1.6.2 Adiponectin

Adiponectin is secreted by AT and acts to increase insulin sensitivity, fatty acid oxidation, energy expenditure, and reduction of liver gluconeogenesis. Unlike leptin, adiponectin levels are negatively correlated with body mass index, specifically abdominal fat accumulation [112]. Thus, adiponectin is thought to contribute to insulin insensitivity. Fat deposits in two main depots, visceral and subcutaneous. Subcutaneous fat accounts for 80% of all body fat. Visceral fats accounts for 10-20% of total body fat in men and 5-8% in women. Visceral deposits produce more adipokines than subcutaneous deposits. Adiponectin is produced in visceral fat, but its levels are decreased with abdominal obesity [113], [114]. A study analyzing levels of adiponectin in serum, showed that non-obese and obese women have higher levels of adiponectin than men [115], [116], which may be explained by the fact that men have higher percent visceral fat than females.

Previous studies suggest that adiponectin is implicated in reproduction. Adiponectin suppressed GnRH secretion in immortalized GnRH neuron cell line, GT1-7 [117]. In the female mouse brain adiponectin decreased GnRH neuron activity [118]. In male rats, adiponectin inhibited testosterone secretion [119]. Although male patients with metabolic syndrome have lower levels of adiponectin, they still present with reduced levels

of testosterone suggesting another level of regulation is responsible. In primary rat granulosa cells, adiponectin, had no effect on the secretion of progesterone and estradiol production, but in the presence of insulin like growth factor (IGF), secretion of progesterone and estradiol were increased [120]. Thus, in females, adiponectin may be important during pregnancy, when levels of IGF, progesterone and estrogen increase.

1.7 Obesity as a chronic inflammatory state

Obese adipose tissue is characterized by inflammation and progressive infiltration by macrophages as obesity develops [121]. Visceral deposits contain a higher density of macrophages than subcutaneous deposits. The number of macrophages present white adipose tissue correlated with BMI (in humans), adiposity and adipocyte size in both humans and mice, and their functional activity is proportional to the degree of obesity [122]. In obesity, adipocytes begin to secrete low levels of tumor necrosis factor alpha (TNF) α , which can stimulate preadipocytes to produce monocyte chemoattractant protein-1 (MCP-1, or CCL2 chemokine) [121]. Endothelial cells also secrete MCP-1 in response to cytokines. CCL2 chemokines is a ligand for CCR2 that recruits monocytes and macrophage activation [121]. Increased secretion of leptin may also contribute to macrophage accumulation by stimulating transport of macrophages to adipose tissue and promoting adhesion of macrophages to endothelial cells, respectively.

1.7.1 Macrophages in adipose tissue

In lean individuals, macrophages resemble an M2 phenotype that secrete anti-inflammatory cytokines such as arginase which blocks inducible nitric oxide synthase (iNOS) activity [123]. With the development of obesity there is a change in macrophage phenotype to M1. M1 macrophages secrete pro-inflammatory cytokines TNF α , IL-6, and IL-12, and generate reactive oxygen species such as nitric oxide via activation of iNOS [124]. Adipose macrophage density increases with obesity, which is likely due to invasion of macrophages from the bloodstream. Invasion and activation of macrophages into adipocytes initiates production of inflammatory cytokines.

1.8 Neuroinflammation

Hypothalamic inflammation is becoming increasingly recognized as a pathological characteristic of obesity in animals and humans. Increased inflammation in the central nervous system (CNS) of obese individuals may contribute to neuropathologies associated with diseases of the CNS, such as cerebral ischemia and dementia [125], [126]. Characterizing cellular interactions underlying these inflammatory responses may have important consequences for systems regulated by the hypothalamus, such as reproduction. The central nervous system (CNS) has long been regarded as an immune-privileged site due to the blood brain barrier (BBB), yet evidence supporting a role for classical pro-inflammatory cytokines interleukin (IL)-1 β , tumor necrosis factor (TNF) α , IL-6, involvement in both the normal physiology of the nervous system and pathology is accumulating [127]. Inflammatory cytokines may play a role in neuronal migration, cell

proliferation, and synaptic activity. Glial cells of the brain, microglia and astrocytes, are involved in maintaining homeostasis in the CNS [128], [129]. Microglia are resident immune cells that survey the parenchyma and act as scavengers of debris, while astrocytes maintain the BBB and support synapse function [128], [129]. Upon stimulation, microglia mediate neuronal and glial injury and death through production of cytokines that can trigger astrogliosis and recruit cells of the immune system, such as peripheral macrophages [130]. Obese individuals have increased levels of pro-inflammatory cytokines, IL-1 β , IL-6, and TNF α in circulation, which can be attributed to the increase in adipose tissue (AT) and accompanied macrophage infiltration [20, 131].

1.8.1 TNF α and IL-1 β

In the CNS TNF α and IL-1 β have both homeostatic and pathophysiological roles. In the healthy CNS, TNF α regulates synaptic plasticity, learning and memory, sleep, food and water intake and astrocyte-mediated synaptic strength [132]. In several CNS diseases, microglia upregulate TNF α expression contributing to excitotoxicity by inhibiting glutamate transport on astrocytes and by increasing localization of ionotropic glutamate receptors to synapses [133].

In the hypothalamus, in the OVLT, during infection, TNF α and IL-1 β mediate the physiological and behavioral responses in sickness such as, inducing fever, inhibiting food intake, causing nausea and fatigue [133-135]. TNF α and IL-1 β mediate their effects through activation of downstream signaling molecules: nuclear factor-kappa B (NF κ -B), Janus kinase and signal transducers and activators of transcription pathway (JAK-STAT) and mitogen activated protein kinases (MAPK) [136-138]. TNF α and IL-1 β also activate transcription factors to produce IL-6.

1.8.2 IL-6

IL-6 was originally identified as a factor that induced maturation of B-cells into antibody producing cells. IL-6 plays a role in the regulation of immune reactions and inflammation, but is also involved in regenerative activities [139], [140]. IL-6 bound to membrane-bound IL-6 receptor or the soluble form of the IL-6 receptor interact with gp130 to signal. In naïve states, IL-6 contributes to normal function of the brain regulating neuron function and neurogenesis. IL-6 deficiency leads to reduced sensitivity of sensory neurons, resulting in decrease in temperature sensitivity in IL-6 KO mice. In the CNS, neurons, astrocytes, microglia and endothelial cells are potential sources of IL-6. In models of brain injury and infection, induced by LPS, or diet-induced obesity, IL-6 is upregulated where it is involved in modulating inflammation, apoptosis, and oxidative stress to promote recovery and healing. Previous studies report more rapid healing and recovery after traumatic brain injury in mice that overexpressed IL-6 in the CNS [141]. IL-6 KO mice showed increase oxidative stress and impaired neuroglial activation [142], suggesting a

neuroprotective role of IL-6 in brain injury. Although LPS induces IL-6 in both astrocytes and microglia, TNF α and IL-1 β have been shown to induce IL-6 production from astrocytes and not microglia, in *in vitro* studies [143]. Consistent with these studies, preliminary studies in our lab has shown that in diet-induced obesity, astrocytes produce IL-6.

1.8.3 Leukemia inhibitory factor (LIF)

LIF is a member of IL-6 family that is induced during inflammatory response [144]. However, its functions are not limited to inflammation: LIF has been demonstrated to play a crucial, non-redundant role in embryo implantation in both mice and humans [145-147]. LIF also maintains stem cells and regulates differentiation of germ cells [148, 149]. In the brain, LIF regulates neuronal function and neuronal response to injury [150-152]. LIF binds its specific receptor, which, similarly to the other members of the IL-6 family, recruits and signals through the GP130 signals transducer, activating JAK-STAT pathway [146]. However, LIF was not previously analyzed in obesity. Our data are first show increased levels of LIF mRNA in the hypothalamus of diet induced obese mice [20].

Neuroendocrine cells express various cytokine receptors and TNF α , IL-1 β and IL-6 are likely to play a central role in the inhibition of the reproductive axis observed during severe illness. Very little is known about the precise mechanisms involved and available data regarding the effects of individual cytokines on the neuroendocrine reproductive axis remain controversial. LPS challenged rodents exhibited reduced levels of LH and GnRH mRNA, diminished release of LH and GnRH, and increased levels of pro-inflammatory

cytokines, such as $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 in the circulation [153] - [154]. Centrally administered cytokines also provoked reduced LH and GnRH levels, but the mechanism whereby these cytokines mediate their effects is unknown [155] - [156]. Our previous study postulated that impairment of GnRH neurons stems from reduction in spine density and consequently the connectivity of the GnRH network [20]. However, GnRH neurons express several cytokine receptors [157] and inflammation-induced cytokines may directly regulate intracellular signaling pathways in the GnRH neurons.

Chapter 2: Diet-Induced Obesity Elicits Reduction in Spines on GnRH neurons from Male but not Female Mice

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Abstract

Increasing prevalence in obesity has become a significant public concern. C57BL/6J mice are prone to diet-induced obesity (DIO) when fed high-fat diet (HFD), and develop metabolic syndrome, making them a good model to analyze mechanisms whereby obesity elicits pathologies. DIO mice demonstrated profound sex differences in response to HFD with respect to reproductive hormone levels and hypothalamic function. We determined that males are prone to DIO, while females are resistant. Ovariectomized females, on the other hand, are susceptible to DIO, implying protection by ovarian hormones. Males, but not females, exhibit changes in hypothalamic neuropeptide expression. Surprisingly, ovariectomized females remain resistant to neuroendocrine changes, showing that ovarian hormones are not necessary for protection. This study elucidates a potential mechanism of obesity-induced impairment of hypothalamic function whereby obese males exhibit reduced levels of excitatory post-synaptic protein, PSD, 95. And exhibit reduced synapses in GnRH neurons. Changes that are potentially responsible for diminished levels of gonadotropin hormones, testosterone, and sperm numbers, which corresponds to the observations in obese humans. Taken together, our data implicate sex-specific differences in obesity-induced impairment of the hypothalamic function with potential consequences for reproduction and fertility.

Introduction

Over half of the US population is classified as overweight and a full third is classified as obese [158]. The number of obese people has increased steadily over the last 30 years [159]. This increase in obesity has coincided with an increase in co-morbidities, such as type 2 diabetes, cardiovascular disease, stroke, and hypothalamic disorders, including reproductive disorders that cause infertility [160-163]. Deleterious effects of obesity on fertility include irregularities in menstrual cycles, abnormalities in the oocyte development, anovulation, and increased risk of miscarriages in women [163]; and inferior sperm quality, reduced sperm quantity, and lower testosterone levels in men [164]. Currently, 18% of couples require assisted reproductive technologies to become pregnant [165], a portion of which may stem from widespread obesity [166, 167]. Although several hypotheses have been put forth [168-172], mechanisms whereby obesity negatively affects reproductive function are unknown.

The hypothalamus in the basal forebrain controls feeding and satiety, thermoregulation, thirst, circadian rhythms, metabolism, and mammalian reproduction. In the control of reproduction, the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) is the final brain output that regulates expression and secretion of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gonadotropes [173], which in turn stimulate steroidogenesis and gametogenesis in the gonads [3, 174]. In the rodent hypothalamus, the majority of GnRH cell bodies are scattered in the preoptic area surrounding organum vasculosum laminae terminalis (OVLT), while secretion occurs at the terminals in the median eminence (ME).

Both OVLT and ME are circumventricular areas with a leaky blood-brain barrier, whereby hypothalamic neurons perceive changes in the circulation and neuropeptides reach the portal circulation [175]. GnRH secretion is synchronized by upstream regulatory neuronal populations [176]. These regulatory neurons integrate sex steroid feedback from the gonads [177], and external or environmental factors, such as stress [178, 179], and exposure to endocrine disruptors [180, 181]. Metabolic stimuli and energy status, such as anorexia nervosa, excessive exercise, malnutrition, and obesity are also integrated with reproductive function at the level of the hypothalamus [169-172, 182, 183]. However, since metabolic signals do not influence GnRH neurons directly [101, 184, 185], we investigated other potential mechanisms.

To analyze the effects of obesity-induced inflammation on reproductive function we used diet-induced obese (DIO) mice. Significant strain differences were observed in response to high-fat diet (HFD) and A/J, FVB/NJ and BALB/cJ strains are resistant to DIO, while DBA/2J and C57BL/6J gain weight [186-188]. The C57BL/6J mouse is a particularly faithful model of the human metabolic syndrome because it develops obesity, hyperinsulinemia, hyperglycemia, and hypertension, when allowed ad libitum access to a HFD [189, 190]. Herein, we demonstrate profound sex differences in response to HFD. Specifically, C57BL/6J male mice exhibit resultant decrease in the number of synaptic spines on GnRH neurons and reduction in GnRH mRNA levels. On the other hand, female mice are resistant to neuroendocrine changes, and this protection is independent of ovarian hormones.

Materials and Methods

Animals

C57BL/6J mice were obtained from Jackson Laboratory at 3 weeks of age. After a week acclimatization, they were randomly assigned to the high-fat diet fed group (HFD, D12492, 60% kcal from fat; Research Diet, New Brunswick, NJ) and control group (Ctr, D12450J, 10% kcal; Research Diet, New Brunswick, NJ) for an indicated number of weeks. Animals were maintained under a 12-hour light, 12-hour dark cycle and received food and water *ad libitum*. All experiments were performed with approval from the University of California Animal Care and Use Committee and in accordance with the National Institutes of Health Animal Care and Use Guidelines using 16-week-old animals (3 weeks before weaning, 1 week normal chow, and 12 weeks high fat or control diet) unless indicated otherwise. During the week between weaning and experimental diet, while fed normal chow, all animals were handled daily by experienced personnel to assure habituation and minimize stress [191].

Estrous cyclicity-Starting at 12 weeks of the Ctr or HFD, female mice were assessed for estrous cycle stage with daily vaginal smears for 6 weeks. Vaginal lavage was performed daily (between 9-10 am) by flushing the vagina with distilled H₂O. Collected smears were mounted on glass slides and examined microscopically for cell types. Both groups of animals were handled daily, at the same time of day, to account for any potential stress that handling may cause. Estrous cycle stages determined during the first week of vaginal smears were not included in the analysis to allow for acclimatization. Female mice on

control diet exhibited normal 4-5 day-long estrous cycles, indicating habituation to handling. Estrous cycle length was calculated as the length of time between two successive occurrences of estrus. For subsequent studies, females were estrous cycle staged and tissue samples collected in diestrus between 9-11 am.

Sperm count-After 12 weeks on the respective diet, males were sacrificed between 9-11 am. The epididymides were dissected, macerated, and then incubated in 1 ml DMEM at room temperature for 30 minutes with shaking. Sperm was cleared with a 70 μ m cell strainer, diluted with sterile water and counted with a hemocytometer.

GnRH-GFP mice were kindly provided by Dr. Suzanne Moenter [192]. Males and females were analyzed separately to determine sex differences. At least 10 animals per sex per genotype were analyzed, unless indicated otherwise for a specific analysis in the materials and methods section. Statistical differences ($p < 0.05$) between control (Ctr) and high fat diet (HFD) fed mice were determined by Student's T-test or 2-way ANOVA where appropriate, and Tukey's test for multiple comparison.

Hormone Analysis

For serum collection, mice were sacrificed between 9-11 am by isoflurane inhalation and blood was obtained from the inferior *vena cava*. The blood was left to coagulate for 15 minutes at room temperature, and then centrifuged at 2000 RCF for 15 minutes for serum separation. Hormone assays were performed by the University of Virginia, Ligand Core. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is a fee-for-service core facility and is supported in part by the Eunice Kennedy Shriver NICHD/NIH U54-HD28934 Grant. LH was measured by the ultra-sensitive mouse LH ELISA, an in-house method. The capture monoclonal antibody (anti-bovine LH beta subunit, 518B7) is provided by Janet Roser, University of California. The detection polyclonal antibody (rabbit LH antiserum, AFP240580Rb) is provided by the National Hormone and Peptide Program (NHPP). HRP-conjugated polyclonal antibody (goat anti-rabbit) is purchased from DakoCytomation (Glostrup, Denmark; D048701-2). Mouse LH reference prep (AFP5306A; NHPP) is used as the assay standard. Intra-assay coefficient of variation is 2.2% and inter-assay coefficient of variation is 7.3% at the low end of the curve. Functional sensitivity is 0.016 ng/ml. FSH was assayed by RIA using reagents provided by Dr. A.F. Parlow and the National Hormone and Peptide Program, as previously described [193]. Mouse FSH reference prep AFP5308D was used for assay standards. Steroid hormone levels were analyzed using validated commercially available assays, information for which can be found on the core's website: <http://www.medicine.virginia.edu/research/institutes-and-programs/crr/lab-facilities/assay-methods-page> and reported in [194]. Limits of detection were 2.4 ng/ml for

FSH, 3 pg/ml for estradiol, and 10 ng/dL for testosterone. Intra- and inter-assay coefficients of variation were 6.9%/7.5%, 6.0%/11.4% and 4.4%/6.4% for the FSH, estrogen (E2) and testosterone (T), respectively. Ten animals per group were used for each hormone analysis. Statistical differences in hormone levels between Ctr and HFD groups were determined by Student's T-test, and Tukey-Kramer HSD for multiple comparisons using JMP software (SAS Institute; Cary, North Carolina).

Histological analyses and immunohistochemistry

Following Ctr or HFD mice were anesthetized, perfused with 20 ml PBS and 20 ml 4% paraformaldehyde; and tissues collected. Brains were fixed in 4% paraformaldehyde, embedded in paraffin, and cut to 20 μ m. Slides were deparaffinized in xylene and rehydrated. Antigen unmasking was performed by heating for 10 minutes in a Tris-EDTA-0.3% Triton X and endogenous peroxidase was quenched by incubating for 10 minutes in 0.3% hydrogen peroxide. Slides were then blocked with 20% goat serum and incubated with primary antiserum against GnRH (1:1000 PA1-121, Thermo Sci.) or Iba-1 for microglia (1:300 cat # 019-19741, Wako) overnight at 4°C. After PBS washes, slides were incubated with biotinylated goat anti-rabbit IgG (1:300, BA-1000, Vector Laboratories, Burlingame, CA) for 30 minutes. The Vectastain ABC elite kit (Vector Laboratories) was used per manufacturer's instructions, after which the DAB peroxidase kit was used for colorimetric staining. Slides were dehydrated in ethanol and xylene, and cover-slipped with Vectamount (Vector Laboratories).

Hypothalami from GnRH-GFP mice were sectioned to 100 μm sections and sections containing organum vasculosum laminae terminalis (OVLT) where GnRH neurons are located, stained for GFP to visualize GnRH neurons. Slides were blocked with 20% goat serum and incubated with primary antibodies against GFP (1:5000 raised in chicken), at 4°C for 48 h. After PBS washes, slides were incubated with FITC/Alexa 488 goat anti-chicken IgG (1:300, Molecular Probes, Eugene, OR) for 30 minutes. Secondary antibody-only controls were performed to determine antibody specificity. Images were obtained using confocal Leica SP2 microscope. To determine spine density, we followed our established protocol as published before [195-199], with modifications that correspond to previously described procedures for analysis of spines in GnRH neurons [47, 200]. Spines, that were identified as protrusions from the soma or axon greater than 1 μm , were counted in the individual neurons where at least 75 μm of the axon proximal to soma can be observed using z-stack. The full length of every GFP-labeled GnRH neuron, including soma and the visible length of the main process, was imaged and analyzed using confocal Leica SP2 microscope. Images were encoded for blind analysis.

Spine numbers were quantified by scrolling through the series of captured images in the z-stack using LAS X software and counted for each GFP-labeled GnRH soma and along the 75 μm length of axon, at 15- μm intervals. We counted at least 3 individual neurons from 4 different sets of mice marked by hand and using NeuroLucida program (MBF Bioscience, Vermont).

Western blot

Whole cell lysates were obtained from the dissected hypothalami from Ctr and HFD fed mice and after protein determination, the same amount of protein was run on SDS-PAGE, transferred on nitrocellulose membrane and probed for: Postsynaptic density protein 95 (PSD-95; 1:1000, Cat #3409, Cell Signaling), Synaptophysin (SYPH; 1:1000, cat. #4329, Cell Signaling), neuronal marker, Microtubule-associated protein 2 (MAP2; 1:5000, cat #ab5392, Abcam) or β -tubulin (1:1000, cat #sc-9104, Santa Cruz Biotechnology). Bands were quantified using ChemiDoc imaging system (Bio-Rad, Hercules, CA).

qPCR analyses

Hypothalami were dissected, total RNA extracted and reverse transcribed using Superscript III (Invitrogen, CA). qPCR was performed using an iQ SYBR Green supermix and an IQ5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA) with primers listed in Table 1 under the following conditions: 95°C for 15 min, followed by 40 cycles at 95°C for 20 sec, 56°C for 30 sec, and 72°C for 30 sec. The amount of the gene of interest was calculated by comparing the threshold cycle obtained for each sample with the standard curve generated in the same run. Replicates were averaged and divided by the mean value of the beta-2-microglobulin (B2M) housekeeping gene in the same sample using $\Delta\Delta$ Ct method. Preliminary studies analyzing GAPDH, Ywaz, TBP and B2M housekeeping genes determined that B2M doesn't change with diet and was subsequently used for normalization. After each run, a melting curve analysis was performed to confirm

that a single amplicon was generated in each reaction. Statistical differences in expression between genotypes were determined by Student's T-test, and Tukey's HSD for multiple comparisons using JMP software (SAS Institute; Cary, North Carolina).

Table 2.1 Antibodies used for western and immunohistochemistry

Antibody	Species	Dilution	Provider, cat # and RRID
GFP	chicken	1:5000	Abcam, ab13970; AB_300798
PSD-95	rabbit	1:2000	Cell Signaling, 3409; AB_1264242
Synaptophysin (SYPH)	rabbit	1:1000	Cell Signaling, 4329; AB_1904154
MAP2	chicken	1:5000	Abcam, ab5392; AB_2138153
GnRH	rabbit	1:1000	ThermoFisher, PA1-121; AB_325077
β -tubulin	rabbit	1:1000	Santa Cruz Biotechnology, sc-9104; AB_2241191

Table 2.2 Primers used for qPCR

Primers	Forward	Reverse
<i>Gnrh</i> (GnRH)	CTACTGCTGACTGTGTGTTG	CATCTTCTTCTGCCTGGCTT C
<i>Avp</i> (AVP)	ACACTACGCTCTCCGCTTGT	CGAAGCAGCGTCCTTTGC
<i>Pomc</i> (POMC)	CAGTGCCAGGACCTCACC	CAGCGAGAGGTCGAGTTTG
<i>B2m</i> (beta-2-microglobulin)	TGACCGGCCTGTATGCTATC CA	CAGTGTGAGCCAGGATATA GAAAGAC
<i>Gapdh</i>	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC

Results

Ovarian Hormones Protect Female C57BL/6J Mice from Diet-Induced Obesity

C57BL/6J male and female mice were placed on high-fat diet (HFD) or control diet (Ctr) one week after weaning and their weights measured twice a week. As demonstrated previously [182], male mice gained weight on HFD, while female mice were resistant to diet-induced obesity (DIO) and required longer exposure to HFD to exhibit the same weight difference from mice on control diet (Fig. 2.1A, male; Fig. 2.1B, female). Preliminary studies indicated that females exposed to the HFD for the same length of time as males do not exhibit adverse effects of obesity illustrated bellow, while much longer exposure would increase the risk of confounding our results with negative effects of ageing; thus, we opted to keep the females on the HFD until they reach similar weight gain as males. To determine if ovarian estrogens, or other ovarian hormones, play a role in this sex difference, we ovariectomized (OVX) the females at 4 weeks of age and placed them on the HFD and Ctr. OVX females became susceptible to DIO (Fig. 2.1C). These results indicate that ovarian hormones are protective of DIO.

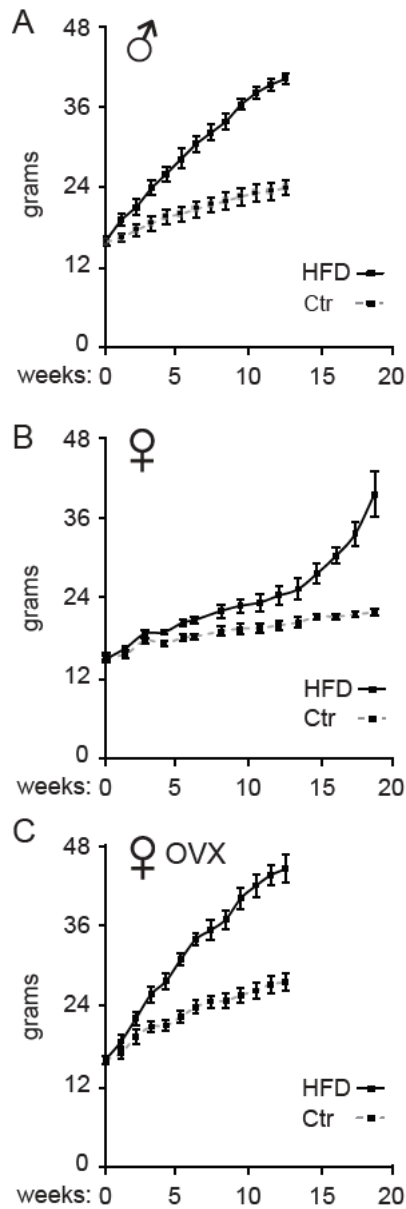


Figure 2.1: 1. Ovarian hormones provide resistance to diet-induced obesity in female C57Black/6J mice. Ten C57BL/6J mice per group were placed on control (Ctr, 10 kcal%fat, Research Diet) or high fat diet (HFD, 60 kcal%fat Research Diet) with the same sucrose levels, at 4 weeks of age. Their weights were recorded twice a week (A, males; B, females). C, ♀ OVX, female mice were ovariectomized at weaning and one week later, at 4 weeks of age placed on diets.

Ovarian Hormones are not Necessary for the Protection from Diet-Induced Obese Changes in Hormone Levels

Following 12 weeks on diets for males and OVX females, which gain weight at the same rate as males, and 19 weeks for unmodified females, we measured gonadotropin hormone and sex steroid hormone levels. Male mice on HFD exhibited 57% lower luteinizing hormone (LH), 35% lower follicle-stimulating hormone (FSH), and 40% lower intra-testicular testosterone levels (Fig. 2.2A). Consequently, HFD males had 50% fewer sperm in their epididymides (Fig. 2.2A), 22% lower seminal vesicle weight, which is dependent on the level of testosterone, while testis weight was unaffected (data not shown). Female mice exhibited longer estrous cycles (4.6 days, controls and 6.4 days, HFD), but LH, FSH or estradiol level (E_2) in diestrus were not significantly changed (Fig. 2B). Thus, female mice, although obese as a result of prolonged exposure to HFD, do not exhibit changes in gonadotropin hormone levels. LH and FSH levels in OVX females were significantly higher than LH and FSH in unmodified females due to the lack of negative feedback (compare 2.2B and 2.2C), which confirms that ovariectomy was successful. However, neither LH nor FSH in OVX females were affected by diet (Fig. 2.2C). Thus, there are profound sex differences and females are resistant to diet-induced changes in reproductive hormones. Additionally, these results demonstrate that ovarian hormones contribute to resistance to obesity, but are not necessary for the protection from hormonal changes exhibited by female mice.

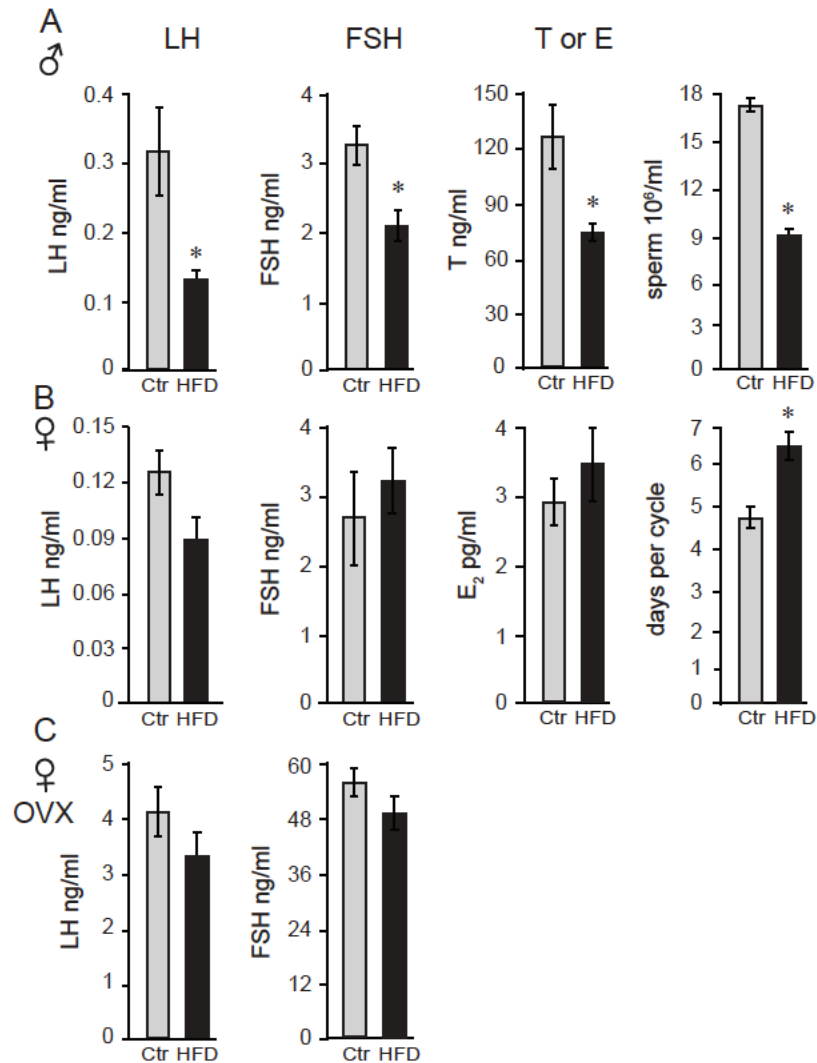


Figure 2.2: Reproductive hormones are lower in obese male mice, while both unmodified and ovariectomized females are protected from diet-induced changes. Male mice (A) on the HFD have reduced levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and intra-testicular testosterone (T), and fewer sperm. Female mice (B) lack changes in LH, FSH and estrogen (E₂), but exhibit longer estrous cycles. Ovariectomized (OVX) females (C) are protected from hormonal changes similarly to unmodified females, indicating that the ovarian hormones are dispensable for protection. Differences (*, $p < 0.05$) between control (Ctr, gray bars) and high fat diet (HFD, black bars) were determined by Student's T-test followed by Tukey's HSD test.

Ovarian Hormones are not Necessary for the Protection from Diet-Induced Obese Changes in GnRH mRNA Levels

Since LH levels are strictly regulated by GnRH neuropeptide from the hypothalamus, GnRH (*Gnrh*) expression was analyzed and revealed that male mice on HFD exhibited 46% reduced levels of *Gnrh* mRNA. To analyze specificity of repression, additional neuropeptides were assessed, and we determined that *Pomc* (POMC, proopiomelanocortin) mRNA was increased by 132%, while *Avp* (arginine vasopressin) mRNA was unchanged (Fig. 2.3A). Neither unmodified or OVX female mice, on the other hand, exhibited diet-induced changes in *Gnrh* mRNA level, although OVX females exhibited ~10-fold higher *Gnrh* mRNA levels compared to unmodified females due to the lack of negative feedback (Fig. 2.3B, C). These results again illustrate sex specific DIO-induced alterations in the neuropeptide gene expression in the hypothalamus, independent of the presence of ovarian hormones.

To determine whether a reduction in *Gnrh* mRNA levels in male mice stems from fewer GnRH neurons after exposure to HFD, we analyzed GnRH neuron number and detected no difference between diets (Fig. 2.4A). We also detected no difference in response to diet in GnRH neuron axon targeting to the median eminence (Fig. 2.4B). Thus, decrease in *Gnrh* mRNA levels correlates with the reduction in LH, testosterone and sperm count in male mice following HFD.

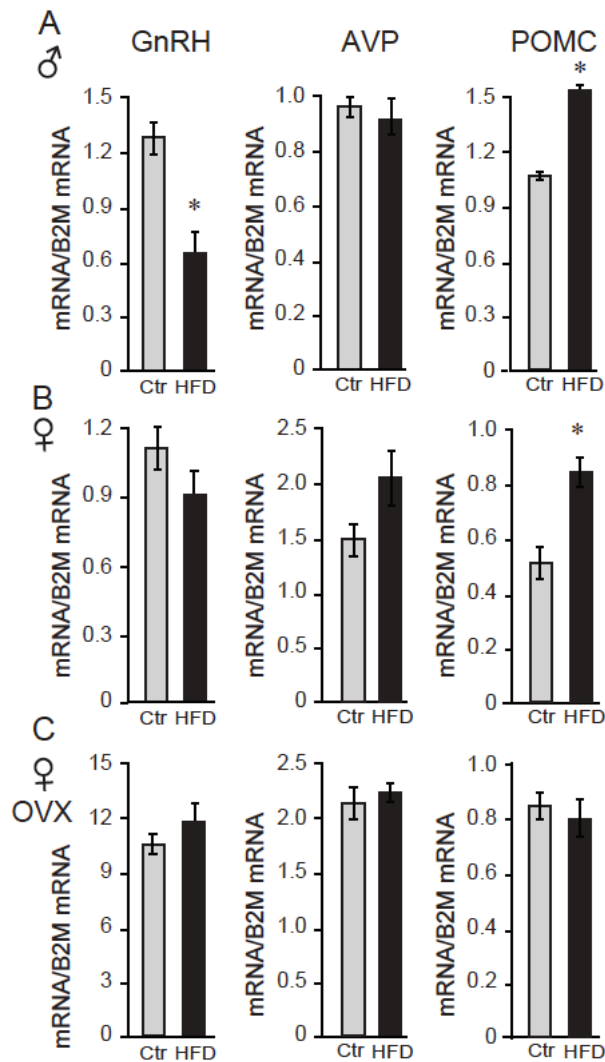


Figure 2.3: Hypothalamic peptides expression is affected with HFD. A, Male mice on HFD had 46% lower expression of *Gnrh* mRNA and 132% higher *Pomc* while *Avp* was not affected. B, Female mice did not exhibit changes in *Gnrh*, but had increased *Pomc* mRNA. C, There was no change in *Gnrh* expression in ovariectomized (OVX) females. * indicates difference between Ctr (gray bars) and HFD (black bars), determined by Student's T-test followed by Tukey's HSD test.

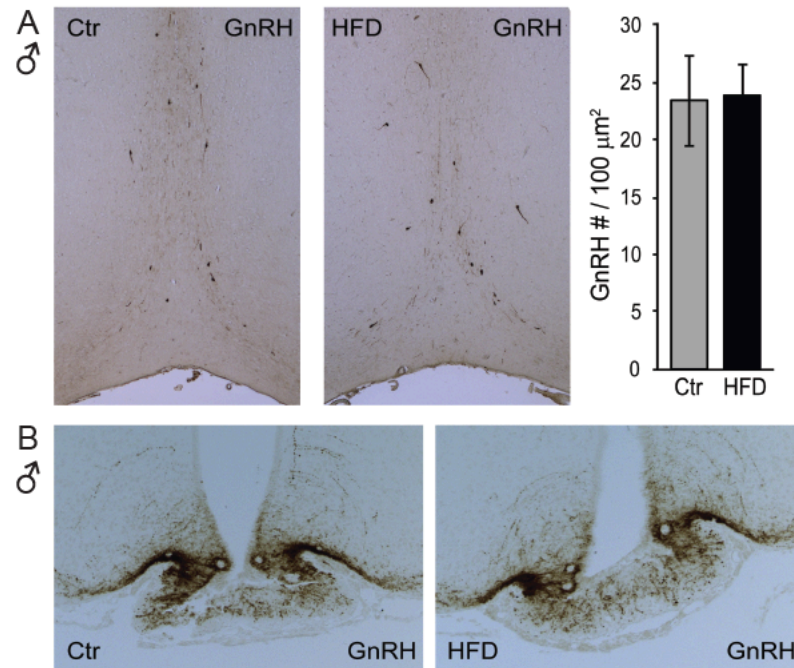


Figure 2.4: GnRH neuron number or axon targeting is unchanged in DIO mice. Coronal sections (A, top) and median eminence (B, bottom) were stained from Ctr and HFD fed male mice with anti-GnRH antibodies. No diet-mediated changes in GnRH neuron number, nor axon targeting were detected.

Decrease in PSD95 and in GnRH neuron spines density in the hypothalamus of obese male mice.

Given that we detected no changes in the number of GnRH neurons, we next determined if there are synaptic changes in the hypothalami of HFD male mice and specifically GnRH neurons. Subsequent dysregulation of GnRH neurons, which can explain reduced GnRH, LH and testosterone levels in male mice on the HFD.

We first investigated levels of synaptic molecules in the hypothalamus of mice fed control or HFD. Western blot analyses revealed lower levels of excitatory post-synaptic density (PSD) 95 protein in the hypothalami of HFD male mice, while no changes were seen in the overall levels of the pre-synaptic protein synaptophysin (SYPH), usually detected in both excitatory and inhibitory pre-synaptic sites (Fig. 2.5A). In contrast, PSD-95 and synaptophysin levels were unchanged in the hypothalami of female mice on HFD as compared to Ctr females (Fig. 2.5B). Our results show changes in synaptic protein levels in the hypothalamus of male mice that are susceptible to DIO, but not neuro-inflammation resistant female mice.

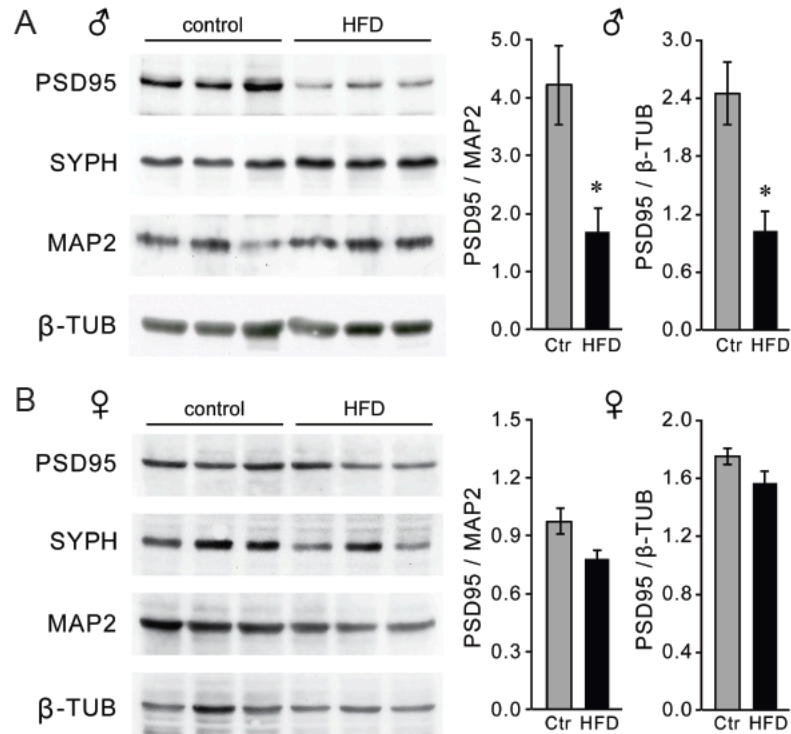


Figure 2.5: Decreased levels of PSD-95 synaptic protein in the hypothalami of male mice on the HFD. Western blot using hypothalamic lysates indicate lower levels of post-synaptic density protein 95 (PSD-95), but not of pre-synaptic marker synaptophysin (SYPH) in males (A) but not in females (B). * indicates statistical significance $p < 0.05$ determined by Student's t-test and Tukey's posthoc analysis, after quantification of PSD-95, neuronal marker MAP2 and housekeeping control β -tubulin.

We then wanted to analyze synapses of specifically GnRH neurons. To do so, we placed GnRH-GFP mice [192] on the Ctr or HFD for 12 weeks and analyzed spine density, since changes in the number of spines have been linked to alterations in neuron connectivity [195-199]. Spine density was analyzed in the GnRH-GFP neurons of the Ctr and HFD male mice, using the method described previously [47]. Spines were identified as protrusions, 1 μm – 5 μm , from the soma or from the proximal axon of these mostly unipolar neurons [201]. By scrolling through the z-stack obtained by confocal microscopy, we counted the spines on the soma and along the first 75 μm of the length of axon at 15- μm intervals. GnRH neurons from male mice on Ctr exhibited the same number of spines on the soma and in each of the 15 μm segments as determined before [200]. GnRH neurons from HFD male mice exhibit fewer spines, especially in the region of the axon that is 45 μm proximal to soma (Fig. 2.6). Therefore, obese male mice following HFD exhibit neuroinflammation and lower number of spines that may indicate reduced connectivity of the GnRH neurons, which in turn may lead to reduction in LH, testosterone and sperm numbers.

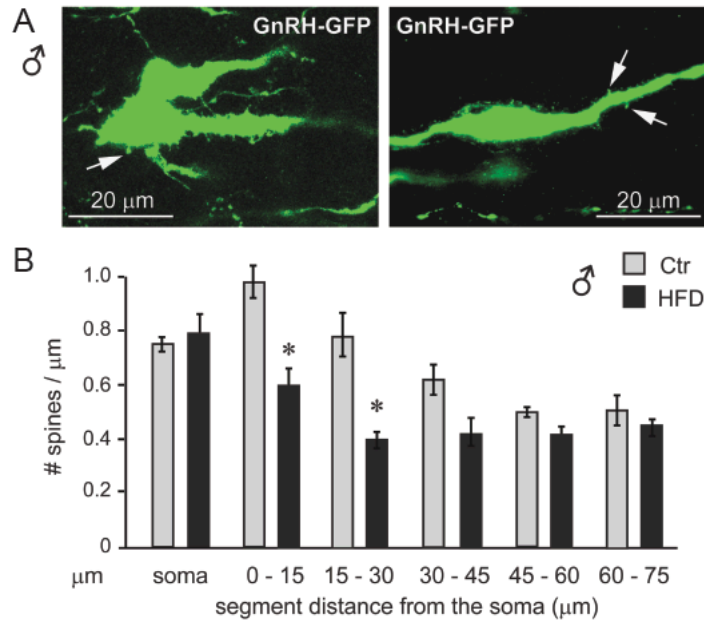


Figure 2.6: GnRH neuron spine density is lower in male mice on HFD. A, Coronal sections of the preoptic area in the hypothalamus of the GnRH-GFP mice following control and HFD were stained for GFP (green) to allow for spine count. B, Spines, identified as protrusions indicated with white arrows in A, were counted in the soma and in the visible length of the main axon in 15 μm intervals, which are indicated in the graph in comparison to the distance from the soma. * indicates statistical significance $p < 0.05$ determined by one-way ANOVA followed by Tukey's posthoc analysis.

Discussion

Herein we report several novel findings: we identify sex differences underlying the neuroendocrine response to diet-induced obesity (DIO), and that ovarian hormones protect from DIO, but are dispensable for female resistance to neuropeptide and hormonal changes; we uncover a potential mechanism of obesity-induced impairment of hypothalamic function whereby obese males exhibit lower number of synaptic proteins, which may underlie negative effects of obesity on reproductive function.

Sex differences in response to DIO were reported before in mice and in rats [202]. Based on these studies and observations in menopausal women it was hypothesized that a lack of estrogen increased adiposity, whereas estrogen replacement diminished it. Increase in adiposity following ovariectomy and removal of ovarian estrogen was observed in rodents [203, 204] and in monkeys [205]. Our results concur that ovarian estrogen is protective from DIO, but do not support the assumption that ovarian estrogen is protective from hormonal and immunological changes. Hormonal and immune changes have not been examined in response to DIO in unmodified and ovariectomized (OVX) females before. It was assumed that hormonal changes would follow weight gain in females after ovariectomy, however our data indicate that is not the case. We demonstrate that females are protected from hormonal and immune changes regardless of the gonadal status. Sex differences we observe may stem from variety of other factors, besides ovarian estrogen. In fact, extra-ovarian estrogen may play a role since the *Cyp19a1* gene, which encodes for aromatase that mediates estrogen synthesis, is expressed in other tissues, especially the brain and adipose tissue. Brain-produced estrogen may regulate GnRH neuron function

[206], while estrogen synthesis in adipose tissue may regulate deposition in various depots [207]. A few studies have analyzed the effects of the high fat diet (HFD) on the gonadotropin hormones in mice concentrating on females, since they determined that females have longer estrous cycles, findings our results support [182, 208, 209].

Due to the significant differences of various mouse strains to DIO, it is difficult to draw a direct comparison between some of these studies and ours, if other studies used a different strain or mixed strain mice [209]. Several studies identified significantly different responses to HFD and some strains, such as BALBc and FVB, were surprisingly resistant to DIO [186]. In DBA and C57BL/6J strains, that are prone to DIO, sex differences vary. Females of the DBA strain are prone to DIO, have lower pregnancy rates and lower *Gnrh* mRNA [188], while C57BL/6J females are resistant to DIO, as we have also shown herein. C57BL/6J mice, used in our study, are most often used in DIO studies since they develop insulin resistance and metabolic syndrome that matches human condition [189, 190]. Reproductive hormones analyses in DIO C57BL/6J mice determined that diestrus females do not exhibit changes in LH, which agrees with our results [208]. However, preovulatory levels of LH are lower following HFD, also aligning with the longer estrous cycles in our studies [182]. While male mice exhibited lower FSH in both of our studies, employing the new ultra-sensitive assay we also detected decreased LH levels. Therefore, male mice have lower LH, testosterone and sperm count likely due to lower *Gnrh* mRNA and reduced number of spines in the GnRH neuron. Lower *Gnrh* mRNA expression is a consistent finding in mice fed HFD. Repression of *Gnrh* mRNA in DIO mice has been reported previously [182, 188], supplemental information). Interestingly, another report analyzing

obesity-induced genome-wide changes in the brain, detected GnRH as one of the most repressed genes [210]. As stated above, acute cytokine infusion in the hypothalamus also represses *Gnrh* expression [211], suggesting that DIO repression of *Gnrh* mRNA may be mediated by increased cytokine concentration. Our studies agree that DIO diminishes *Gnrh* mRNA, and further demonstrate reduction in spine density in GnRH neurons. This may indicate that *Gnrh* expression may be regulated in an activity-dependent manner [212, 213] or that *Gnrh* gene may be repressed via activity-independent mechanisms. Activity-dependent GnRH gene regulation by afferent neurons has been implied. Hypothalamic factors involved in reproductive function, such as RFamide-related peptide 3 (RFRP-3), a mammalian gonadotropin-inhibitory hormone ortholog; senktide, a neurokinin B receptor agonist; and oxytocin; elicit changes in LH serum levels, not only via alterations of GnRH secretion but by modifications of *Gnrh* transcription [214-216]. On the other hand, GnRH neurons express cytokine receptors [157], and *Gnrh* may be repressed via activation of cytokine receptor pathways.

Reduction in GnRH spine density may indicate lower neuron excitability. A decrease in synapses following HFD was reported previously in the arcuate nucleus in both NPY and POMC neurons, which are widely studied neuronal populations in response to DIO, since they comprise feeding and satiety circuitry in the hypothalamus [217]. Specifically, elimination of inhibitory synapses on POMC neurons and excitatory synapses on NPY neurons was described. Reduction in inhibitory synapses may lead to increased POMC expression that was previously reported [217] and is consistent with our findings. Synaptic stripping, reduced levels of synaptic proteins and fewer spines following diet-

induced obesity in male C57BL/6J mice were observed in hippocampal neurons as well [218]. A decreased performance of HFD mice in cognitive tasks was attributed to the loss of synapses, fewer dendritic spines and a decrease in synaptic proteins in the prefrontal cortex [219]. Since number of spines and the levels of PSD-95 synaptic protein are linked to neuronal connectivity, fewer spines and reduced levels of synaptic proteins, may indicate changes in neuronal activity. It was postulated that either estrogen induces changes in a number of synapses on POMC neurons, since sex differences were detected in the previous study as well [220]; or that leptin is involved in synapse remodeling, since leptin is elevated in obesity and targets POMC neurons [217].

Our studies also demonstrate that GnRH neurons have fewer spines and lower *Gnrh* mRNA expression. Diminished *Gnrh* mRNA transcription and reduction in GnRH neuron activity may contribute to lower LH levels, reduced testosterone and diminished sperm numbers. Previous studies analyzing crosstalk between metabolism and reproductive function determined that GnRH neurons may integrate obesity-induced changes in glucose directly [221], but changes in insulin [184] and leptin [101, 185] are most likely relayed indirectly via neuronal afferents that synapse to GnRH neurons.

In this manuscript, we show that GnRH neurons may also mediate the effects of obesity-induced synaptic changes on reproductive function. Future studies will determine if synaptic changes result in changes in the secretion of the neuropeptide that regulates gonadotropin hormone levels and reproduction. Herein, we examine hormonal parameters to identify sex-specific effects of obesity on neuroendocrine function.

Chapter 3: Diet-Induced Obesity Elicits Macrophage Infiltration in the Hypothalami of Male but not Female Mice

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Abstract

Increasing prevalence in obesity has become a significant public concern. C57BL/6J mice are prone to diet-induced obesity (DIO) when fed high-fat diet (HFD), and develop chronic inflammation and metabolic syndrome, making them a good model to analyze mechanisms whereby obesity elicits pathologies. DIO mice demonstrated profound sex differences in response to HFD with respect to inflammation. First we demonstrated that males on HFD had heavier visceral fat pads compared to females on HFD. Microglial activation and peripheral macrophage infiltration is seen in the hypothalami of males, while females are protected from the increase in inflammatory cytokines regardless of the presence of ovarian hormones. Strikingly, the anti-inflammatory cytokine IL-10 is increased in the hypothalami of females but not males. This study elucidates a potential mechanism of obesity-induced impairment of hypothalamic function whereby obese males exhibit reduced levels of excitatory post-synaptic density protein, PSD, 95, reported in chapter 2, located in the areas exhibiting macrophage infiltration. Taken together, our data implicate neuro-immune mechanisms underlying sex-specific differences in obesity-induced impairment of the hypothalamic function.

Introduction

Over half of the US population is classified as overweight and a full third is classified as obese [158]. The number of obese people has increased steadily over the last 30 years [159]. This increase in obesity has coincided with an increase in co-morbidities, such as type 2 diabetes, cardiovascular disease, stroke, and hypothalamic disorders [160-163]. Obesity is characterized by chronic inflammation, in addition to changes in metabolic markers [222]. Increased adiposity elicits an increase in inflammatory cytokines in the circulation [223, 224], such as: tumor necrosis factor (TNF α), and interleukins, IL-1 β , IL-6 [131], primarily due to macrophage infiltration to adipose tissue and their subsequent activation. Inflammatory cytokines have been demonstrated to negatively affect hypothalamic function [225].

To analyze the effects of obesity-induced inflammation on reproductive function we used diet-induced obese (DIO) mice. Significant strain differences were observed in response to high-fat diet (HFD) and A/J, FVB/NJ and BALB/cJ strains are resistant to DIO, while DBA/2J and C57BL/6J gain weight [186-188]. The C57BL/6J mouse is a particularly faithful model of the human metabolic syndrome because it develops obesity, hyperinsulinemia, hyperglycemia, and hypertension, when allowed ad libitum access to a HFD [189, 190]. Specifically, C57BL/6J male mice exhibit neuroinflammation. On the other hand, female mice are resistant to inflammatory changes.

Materials and Methods

Animals

C57BL/6J mice were obtained from Jackson Laboratory at 3 weeks of age. After a week acclimatization, they were randomly assigned to the high-fat diet fed group (HFD, D12492, 60% kcal from fat; Research Diet, New Brunswick, NJ) and control group (Ctr, D12450J, 10% kcal; Research Diet, New Brunswick, NJ) for an indicated number of weeks. Animals were maintained under a 12-hour light, 12-hour dark cycle and received food and water *ad libitum*. All experiments were performed with approval from the University of California Animal Care and Use Committee and in accordance with the National Institutes of Health Animal Care and Use Guidelines using 16-week-old animals (3 weeks before weaning, 1 week normal chow, 12 weeks high fat or control diet) unless indicated otherwise. During the week between weaning and experimental diet, while fed normal chow, all animals were handled daily by experienced personnel to assure habituation and minimize stress [191].

For fluorescently labeled microglia, CX3CR1-GFP mice were obtained from Jackson labs (strain 005582) and randomly placed on the respective diets at 4 weeks of age to analyze change in microglia morphology. Doubly fluorescent GFP and RFP transgenic mice obtained after crossing CX3CR1-GFP (strain 005582) and CCR2-RFP (strain 017586), and heterozygous mice for both alleles were used to distinguish monocyte recruitment to the hypothalamus from resident microglia. Statistical differences ($p < 0.05$) between control (Ctr) and high fat diet (HFD) fed mice were determined by Student's T-test or 2-way ANOVA where appropriate, and Tukey's test for multiple comparison.

Cytokine Analysis

For serum collection, mice were sacrificed between 9-11 am by isoflurane inhalation and blood was obtained from the inferior *vena cava*. The blood was left to coagulate for 15 minutes at room temperature, and then centrifuged at 2000 RCF for 15 minutes for serum separation. Cytokine levels in serum and hypothalamic protein lysates were measured using Luminex MagPix instrument and mouse ProcartaPlex 7 plex (Affymetrix eBioscience, San Diego, CA). Statistical differences in hormone levels between Ctr and HFD groups were determined by Student's T-test, and Tukey-Kramer HSD for multiple comparisons using JMP software (SAS Institute; Cary, North Carolina).

Flow cytometry

Microglial activation and immune cell influx into the hypothalamus and prefrontal cortex were characterized as previously described [226]. Tissues from the hypothalamus and cortex from each mouse was processed separately as part of a 5-mouse cohort per group, with each experiment repeated 3 times. In brief, mice were perfused with ice cold PBS, brains rapidly removed and hypothalami and prefrontal cortex cell suspensions were generated by mechanical dissociation and applied to a discontinuous 1.03/1.088 percoll gradient. Cells were collected from the interface, blocked with anti-CD16/CD32 (1:300, 553141, BD Biosciences, San Jose, CA), and incubated with anti-CD45 APC-eFluor® 780 (1:300, 47-0451, eBioscience, San Diego, CA) and anti-CD11b PerCP-Cyanin5.5 (1:300, 45-0112, eBioscience, San Diego, CA) in PBS, 5% EDTA, 0.4% BSA. Sytox™ Green dead stain (30 nM, S-34860, ThermoFisher, Chino, CA) was used to exclude dead cells

and flow analysis performed using BD LSR II Flow Cytometer. Results were analyzed using FlowJo software (Tree Star, Inc.) and statistical differences were determined by Student's T-test and Tukey's posthoc test.

Histological analyses and immunohistochemistry

To visualize GFP-labeled microglia from CX3CR1-GFP mice, and activated macrophages labeled red and microglia labeled green from CCR2-RFP x CX3CR1-GFP mice, after 12 weeks of Ctr or HFD mice were anesthetized, perfused with 20 ml PBS followed by 20 ml of 4% paraformaldehyde; brains were post-fixed in 4% paraformaldehyde, frozen in OCT, and cut to 20 μ m sections using Leica cryostat. Endogenous fluorescence was visualized with Leica microscope.

qPCR analyses

Hypothalami were dissected, total RNA extracted and reverse transcribed using Superscript III (Invitrogen, CA). qPCR was performed using an iQ SYBR Green supermix and an IQ5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA) with primers listed in Table 1 under the following conditions: 95°C for 15 min, followed by 40 cycles at 95°C for 20 sec, 56°C for 30 sec, and 72°C for 30 sec. The amount of the gene of interest was calculated by comparing the threshold cycle obtained for each sample with the standard curve generated in the same run. Replicates were averaged and divided by the mean value of the beta-2-microglobulin (B2M) housekeeping gene in the same sample using $\Delta\Delta$ Ct method. Preliminary studies analyzing GAPDH, Ywaz, TBP and B2M

housekeeping genes determined that B2M doesn't change with diet and was subsequently used for normalization. After each run, a melting curve analysis was performed to confirm that a single amplicon was generated in each reaction. Statistical differences in expression between genotypes were determined by Student's T-test, and Tukey's HSD for multiple comparisons using JMP software (SAS Institute; Cary, North Carolina).

Table 3.1 Antibodies used for immunohistochemistry and FLOW cytometry

Antibody	Species	Dilution	Provider, cat # and RRID
Iba-1	rabbit	1:300	Wako, 019-19741; AB_839504
CD45 APC-eFluor 780	rat	1:300	eBioscience, 47-0451 (clone 30-F11), AB_1548781
CD11b PerCP-Cy5.5	rat	1:300	eBioscience, 45-0112 (clone M1/70), AB_953558

Table 3.2 Primers used for qPCR

Primers	Forward	Reverse
<i>Il6</i> (IL-6)	TTCTCTGGGAAATCGTGGAA AT	TCCAGTTTGGTAGCATCCA TCA
<i>Tnfa</i> (TNF α)	ATGTCTCAGCCTCTTCTCATT CC	GCTTGTCACTCGAATTTTG AGAA
<i>Lif</i> (LIF)	ATGTGCGCCTAACATGACAG	TATGCGACCATCCGATACA G
<i>Il10</i> (IL-10)	GCTGGACAACATACTGCTAA CC	ATTTCGATAAGGCTTGGC AA
<i>B2m</i> (beta-2- microglobulin)	TGACCGGCCTGTATGCTATC CA	CAGTGTGAGCCAGGATATA GAAAGAC
<i>Gapdh</i>	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC

Results

Diet-Induced Obese Male C57BL/6J Mice Have Heavier Gonadal Fat Depots than Diet-Induced Obese Females

In chapter 2, C57BL/6J female mice were resistant to diet-induced obesity (DIO) and required longer exposure to HFD to exhibit the same weight difference from mice on control diet (Fig. 2.1A, male; Fig. 2.1B, female). Thus, we wanted to analyze adipose tissue deposition by measuring fat depots. We dissected inguinal fat pad from subcutaneous depots, and gonadal fat pad from visceral fat depots, from one side of each animal. Fat pad weights were then compared between Ctr and HFD in both males and females. Weight comparison of fat pads from Ctr and HFD-fed mice indicates that both males (Fig. 3.1A) and females (Fig. 3.1B) deposit fat in both subcutaneous (inguinal) and gonadal fat depots, since fat pads from either depot were heavier in HFD than controls animals. When we compared males to females, Ctr males had significantly heavier subcutaneous fat pads and gonadal fat pads than Ctr females (compare Fig. 3.1A and 3.1B). HFD fed males had significantly heavier gonadal fat pads than HFD females. Given the individual weight difference and profound sex differences in size, we also compared fat depots after normalizing fat pad weight to total body weight. There was a significant difference in normalized weight between gonadal fat pads in Ctr males compared to gonadal fat pads in Ctr females, indicating that male mice fed low-fat control diet exhibit higher visceral adiposity than females (Fig. 3.1C, indicated with a pound sign). We did not detect sex differences between other fat depots. Comparison of fat pads between control and HFD revealed preferential fat accumulation in select depots. Significantly, there was a difference

in subcutaneous fat pad from HFD female mice compared to subcutaneous fat pad from Ctr females, even after normalizing to the body weight (Fig. 3.1C, asterisk), implying that females preferentially increase adiposity in the subcutaneous depot. Therefore, examination of fat pads may demonstrate that male mice have higher visceral adiposity in control conditions, which corresponds to observations in humans. On the other hand, female obesity may lead to preferential increase in subcutaneous fat, which exhibits less adverse health outcomes [202].

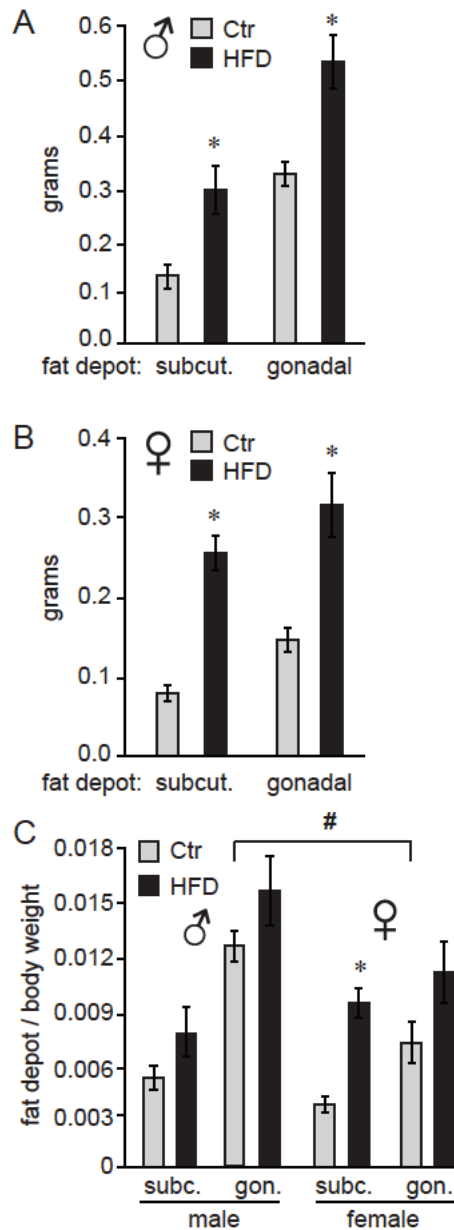


Figure 3.1: Ovarian hormones provide resistance to diet-induced obesity in female C57Black/6J mice. Ten C57BL/6J mice per group were placed on control (Ctr, 10 kcal%fat, Research Diet) or high fat diet (HFD, 60 kcal%fat Research Diet) with the same sucrose levels, at 4 weeks of age. A. Following exposure to their respective diets, mice were sacrificed and subcutaneous (subcut.) fat depots and gonadal fat depots removed from one side of each animal and weights recorded (B, males; C, females). F, fat depot weights from D and E was normalized to the whole body weight for each animal.

Sex-specific differences in inflammatory cytokines in the circulation and the hypothalami of HFD-fed mice.

Obesity is characterized by hyperleptinemia, hyperinsulinemia, hyperlipidemia, hyperglycemia, and chronic inflammation. Significant effort has been devoted to determine the effects of increased leptin, insulin, fatty acids, or glucose on GnRH neuronal function without clear outcomes [171]. We analyzed inflammatory cytokine levels following DIO in our mice and determined that inflammatory cytokines were elevated in the circulation as well as in the hypothalami of obese mice. There was no difference in serum levels of interleukin-6 (IL-6) between males, unmodified females or ovariectomized females on control diet (compare Fig. 3.2A, 3.2B and 3.2C), and all three groups exhibited significantly elevated serum IL-6 following HFD. Increased IL-6 in the serum of HFD-fed male mice (Fig. 3.2A) was previously demonstrated [227]. IL-6 levels were also increased in the hypothalamic lysates, at the protein and mRNA levels, indicating that IL-6 was induced locally in the hypothalami of the DIO mice. Additionally, hypothalamic IL-6 was increased in both male and female mice (Fig. 3.2B, C). However, increased cytokine levels were not detected in the cortex of neither male nor female mice (data not shown), indicating that neuroinflammation is specific for the hypothalamus.

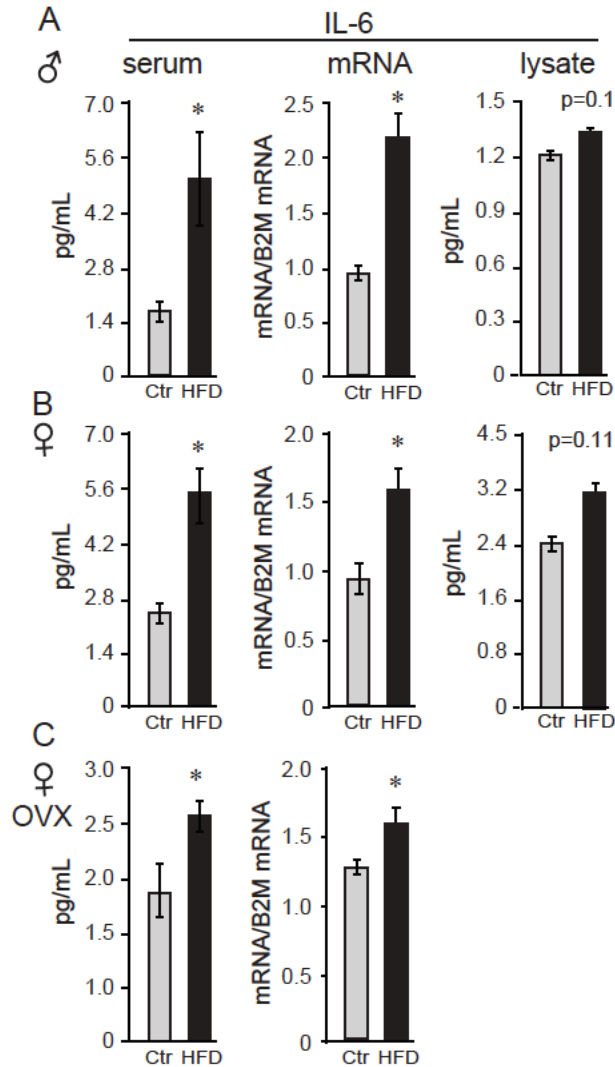


Figure 3.2: Interleukin-6 (IL-6) is increased in the serum and the hypothalamus of both male and female mice. Following Ctr (gray bars) and HFD (black bars) serum and hypothalami were collected from male (A), unmodified female (B) and ovariectomized (OVX) female mice (C). Cytokine levels in serum and hypothalamic protein lysates were measured using Luminex MagPix instrument and mouse ProcartaPlex 7 plex (Affymetrix eBioscience, San Diego, CA), while mRNA was assayed with qPCR. * indicates significant difference ($p < 0.05$) determined with a T-test and Tukey's posthoc comparison.

We sought to identify cytokines that may exhibit sex differences in response to HFD. Tumor necrosis factor (TNF) α and leukemia inhibitor factor (LIF) were examined, since shown to increase in DIO [122]. There was no sex difference in TNF α or LIF levels in serum of Ctr males and Ctr unmodified females, while removal of ovaries significantly increased TNF α concentration, but not LIF, in the serum of Ctr OVX females compared to unmodified Ctr females. TNF α and LIF increased specifically in male mice on HFD in the serum (3-fold or 300% and 4.83-fold or 483%, respectively), hypothalamic protein levels (112% and 155.6%, respectively) and mRNA expression levels in the hypothalamus (TNF α , 161% and LIF, 200%; Fig. 3.3A), but were not changed in the cortex (data not shown); nor in unmodified or OVX female mice (Fig. 3.3B, C). Therefore, IL-6 was increased in both sexes solely in the hypothalami, while TNF α and LIF are induced specifically in the hypothalami of male mice. Furthermore, while OVX females became susceptible to DIO, they remain protected from the increase in TNF α and LIF inflammatory cytokines in the hypothalamus.

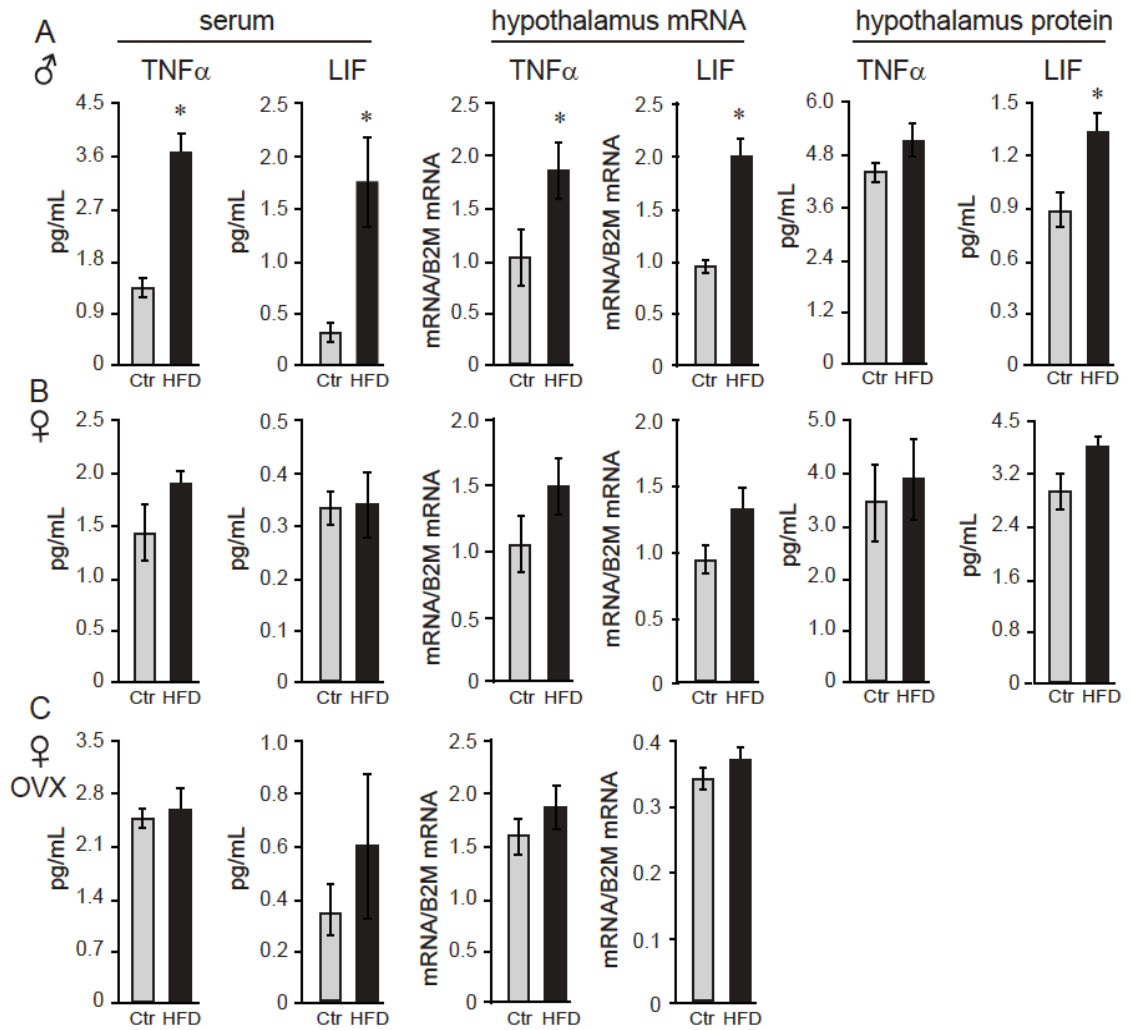


Figure 3.3: Tumor necrosis factor (TNF) α and leukemia inhibitory factor (LIF) are increased specifically in the male mice regardless of the presence of ovarian estrogens. Serum and hypothalami were collected from male (A), unmodified female (B) and ovariectomized (OVX) female mice (C) following Ctr and HFD. Cytokine levels in serum and hypothalamic protein lysates were measured using Luminex MagPix instrument and mouse ProcartaPlex 7 plex (Affymetrix eBioscience, San Diego, CA), while mRNA was assayed with qPCR. Difference (*, $p < 0.05$) between Ctr (gray bars) and HFD (black bars) were determined by Student's T-test followed by Tukey's HSD test.

To explain possible protection of female mice, we analyzed anti-inflammatory cytokines. Female mice on Ctr diet exhibit significantly higher concentration of anti-inflammatory IL-10 than Ctr male mice (3.7 pg/ml female, 1.3 pg/ml male, Fig. 3.4) in the hypothalamus, while IL-10 was below detection limit in the serum in both sexes. Exposure to HFD decreased level of IL-10 protein by 50.2% and *Il10* mRNA by 28% in male mice; however, HFD increased IL-10 protein to 188% and *Il10* mRNA to 165% in the female hypothalami (Fig. 3.4). These results indicate that following HFD, inflammatory cytokines are increased, while anti-inflammatory cytokines are decreased in male hypothalami compared to the male mice on control diet. In contrast, female mice on HFD increase anti-inflammatory IL-10 concentration in their hypothalami, while lacking heightened inflammatory response.

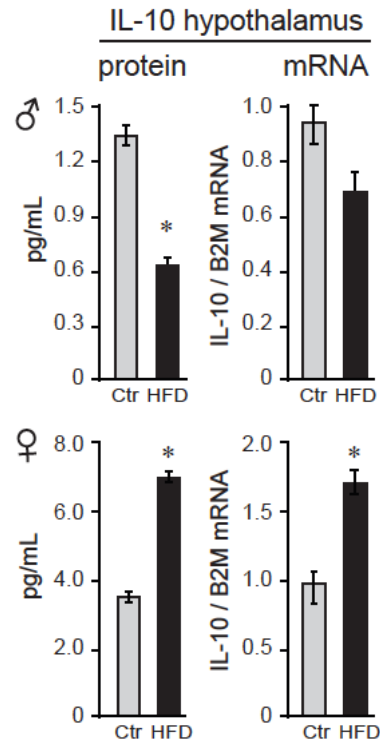


Figure 3.4: IL-10 is higher in female mice. IL-10 protein was higher in the hypothalami of female mice than male mice, and below detection in the serum in both sexes. After exposure to the HFD, IL-10, both protein and mRNA, decreased in males and increased in females. Statistical significance (*) between Ctr (gray bars) and HFD (black bars) were determined by Student's T-test followed by Tukey's test.

Regional differences in microglia activation in the hypothalamus.

Microglia activation in response to the HFD in the arcuate nucleus of the mediobasal hypothalamus has been demonstrated previously [228-231]. Using CX3CR1-GFP mice on Ctr and HFD, we also observed changes from ramified cell body with longer processes, to amoeboid, rounded cells body morphology with retracted processes, indicating activation of the microglia in the arcuate nucleus, (Fig. 3.5A, top panels). These morphological changes were not observed in the female mice (Fig. 3.5A, bottom panels). Quantification of CX3CR1-GFP positive cells demonstrated that there is no difference in the number of cells between Ctr males and Ctr females. Exposure to HFD increases the number of GFP-labeled cells in the arcuate nucleus in males, but not in females.

Identification of an increase in cell number in the arcuate nucleus in male mice on HFD prompted investigation of the regional changes in the number of Iba-1 positive cells (Iba-1, is expressed by the microglia in the brain and activated bone-derived macrophages). Increased number of Iba-1 positive cells was detected particularly around the organum vasculosum of the lamina terminalis (OVLT, Fig. 3.5B) in the rostral hypothalamus and in the median eminence (ME) and arcuate nucleus in the mediobasal hypothalamus (Fig. 3.5C), but not in the lateral preoptic area (LPO) or the paraventricular nucleus (PVN). Since PVN is located adjacent to the 3rd ventricle as is the arcuate nucleus, findings that PVN does not exhibit changes in Iba-1 positive cell number, while arcuate nucleus does, imply that the distance to the 3rd ventricle is not a contributing factor. Consistent with a lack of morphological changes, and lack of changes in the cell number in the arcuate nucleus, female mice did not exhibit any differences in Iba-1 cell number in either ME or

OVLTH hypothalamic area (data not shown). Therefore, solely in male mice, but not in female mice, HFD elicits an increase in Iba-1 positive cells in the circumventricular areas of the hypothalamus that are known to have a leaky blood-brain barrier.

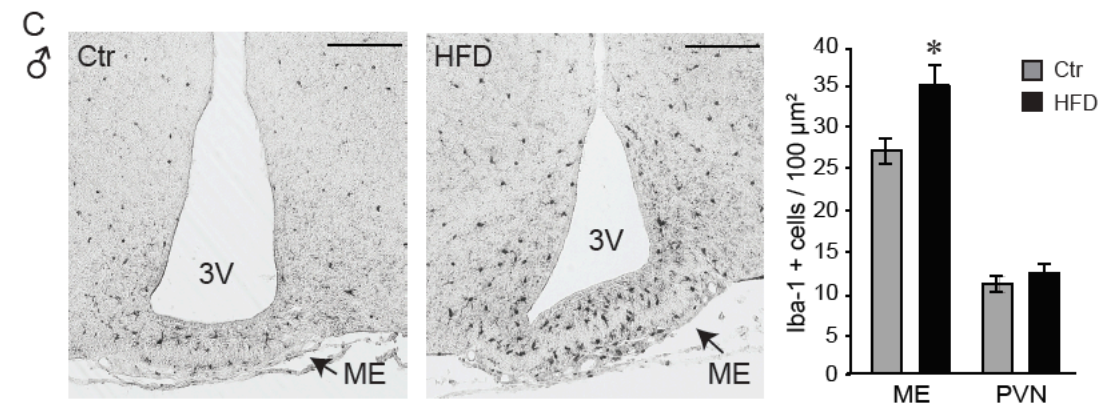
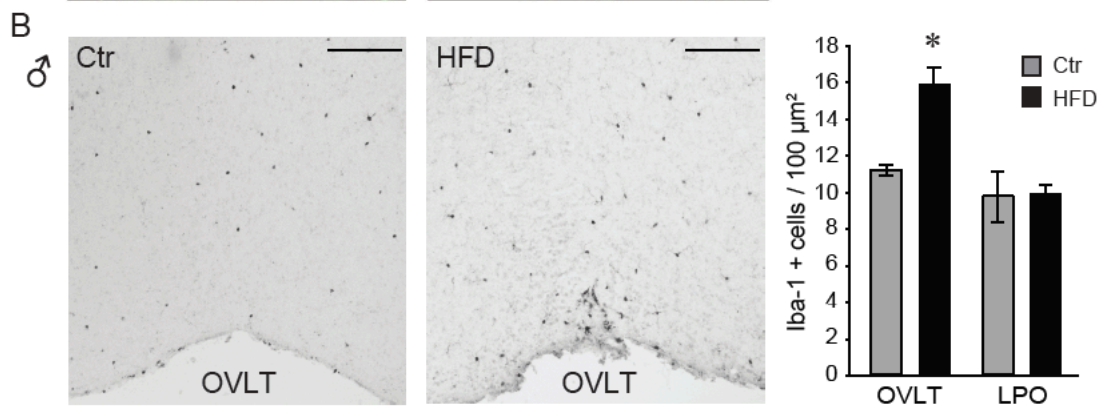
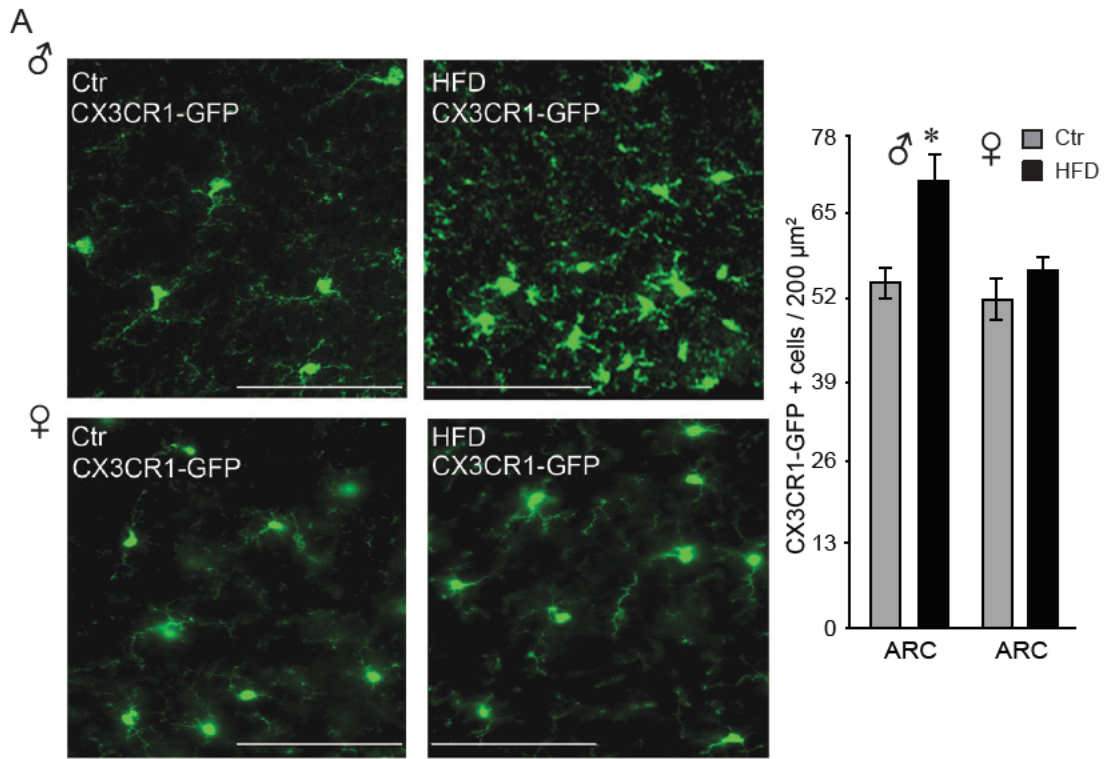


Figure 3.5: Iba-1 positive cells were more abundant in the circumventricular areas of the hypothalamus following HFD in male mice, but not in the female. A, Microglia, genetically labeled with CX3CR1-GFP, exhibit activated morphology in the arcuate nucleus after HFD specifically in male mice, but not in the female. Quantification identified that there is no difference in microglia numbers in Ctr males compared to Ctr females, or Ctr females compared to HFD females, while male mice on HFD had increased number of CX3CR1-GFP cells in the arcuate nucleus compared to the Ctr male mice, indicated with an asterisk. B, Increased number of Iba-1 positive cells around the organum vasculosum laminae terminalis (OVLT) in DIO male mice fed HFD than Ctr mice, but the same numbers in the lateral preoptic area (LPO). C, Increased Iba-1 positive cell number in the median eminence (ME) and arcuate nucleus but not in the paraventricular nucleus (PVN) in male mice on HFD. A-C, Bar indicates 100 μ m. Difference (*) between Ctr (gray bars) and HFD (black bars) were determined by Student's T-test followed by Tukey's HSD test.

Infiltration of peripheral macrophages to the hypothalamus of obese male mice.

Given that the increased Iba-1 cell numbers are observed in the areas that contain fenestrated capillaries, and that this increase is not dependent on the distance from the ventricle, we considered two possibilities, that either microglia sense the metabolic changes in the circulation that leads to their activation and proliferation, or that activated monocyte-derived macrophages enter the hypothalamus from the periphery, since increased adiposity leads to macrophage activation [224]. Microglia and macrophages can be distinguished by flow cytometry from other CNS resident cells due to the presence of CD11b, and can be distinguished from each other by their differential expression of CD45, since microglia are CD11b⁺ CD45^{low} and macrophages are CD11b⁺ CD45^{high} [226, 232]. Hypothalamus and prefrontal cortex were dissected, cells dissociated and subjected to flow analysis, which determined that macrophages are present in the hypothalami of obese male mice, but not in the cortex (Fig. 3.6A). Consistent with the increase in inflammatory cytokines, macrophage recruitment to the hypothalamus is only noted in male mice, and is not observed in the female mice on HFD (Fig. 3.6A, B).

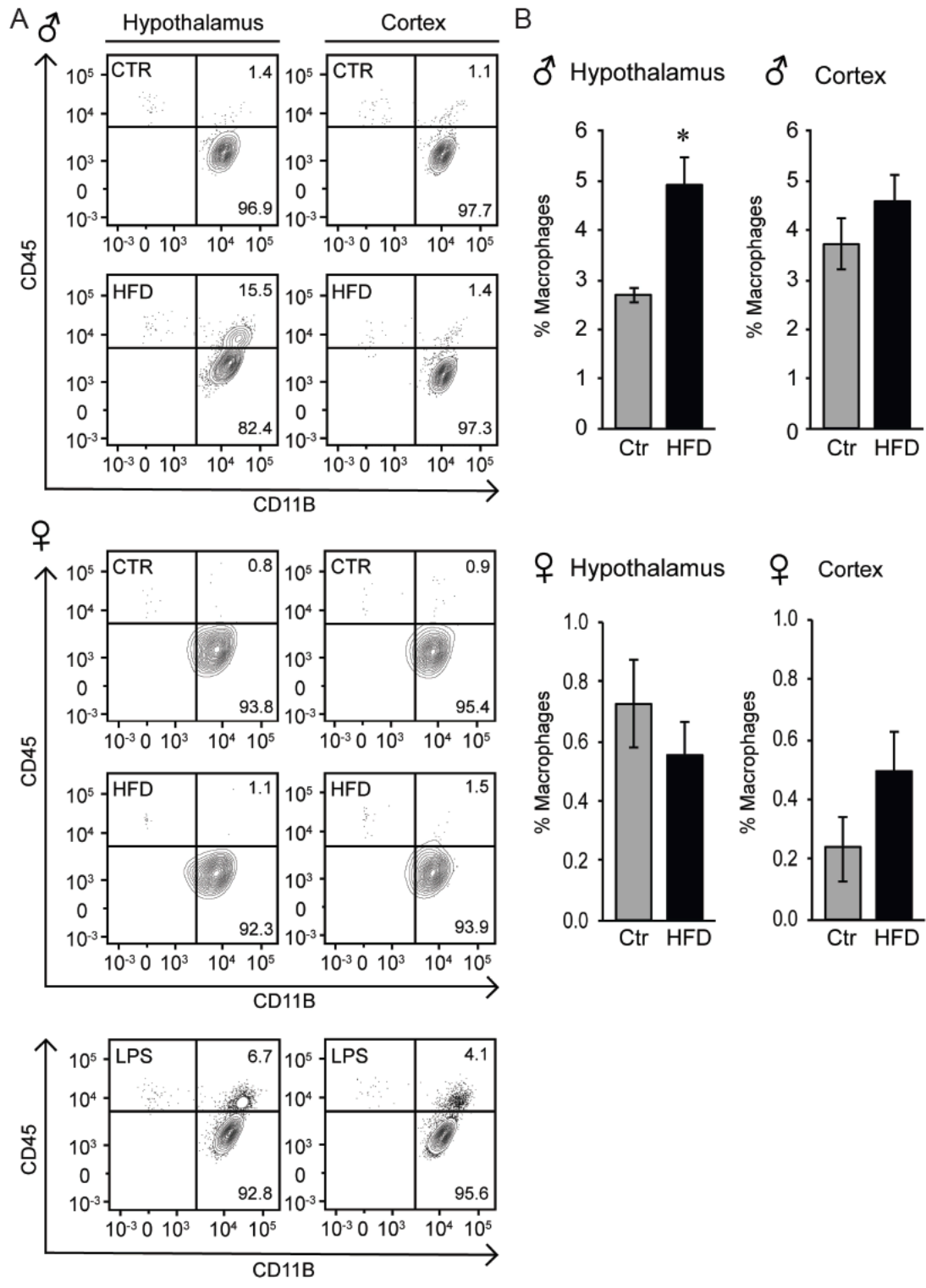


Figure 3.6: Infiltration of peripheral macrophages in the hypothalamus of the male mice on HFD but not female. A, Flow cytometry plot indicating presence of the CD45^{high} macrophage population that can be distinguished from the CD45^{low} microglia specifically in the hypothalami of HFD male mice, but not in the female. This population is not present in the cortex in either male or female mice. B, Quantification of A. Statistical significance (*) between Ctr (gray bars) and HFD (black bars) were determined by Student's T-test followed by Tukey's posthoc test.

To localize resident microglia and recruited monocyte-derived macrophages, we generated CX3CR1-GFP and CCR2-RFP double transgenic male mice. CX3CR1 is a fractalkine receptor expressed specifically by the microglia in the brain. CCR2 is a chemokine receptor involved in monocyte chemotaxis and infiltration; thus, the presence of RFP expression under CCR2-promoter control would indicate infiltrating activated macrophages. These mice were placed on a Ctr and HFD. In the Ctr, only green-labeled microglia positive for CX3CR1 are present in the arcuate nucleus of the mediobasal hypothalamus (Fig. 3.7, coronal section with 3rd ventricle on the right side of the image), while in the HFD, RFP-labeled infiltrating macrophages are localized to the brain parenchyma in addition to the GFP-labeled resident microglia. Collectively, these results indicate that peripheral macrophage recruitment to the hypothalamic parenchyma following HFD only occurs in male mice.

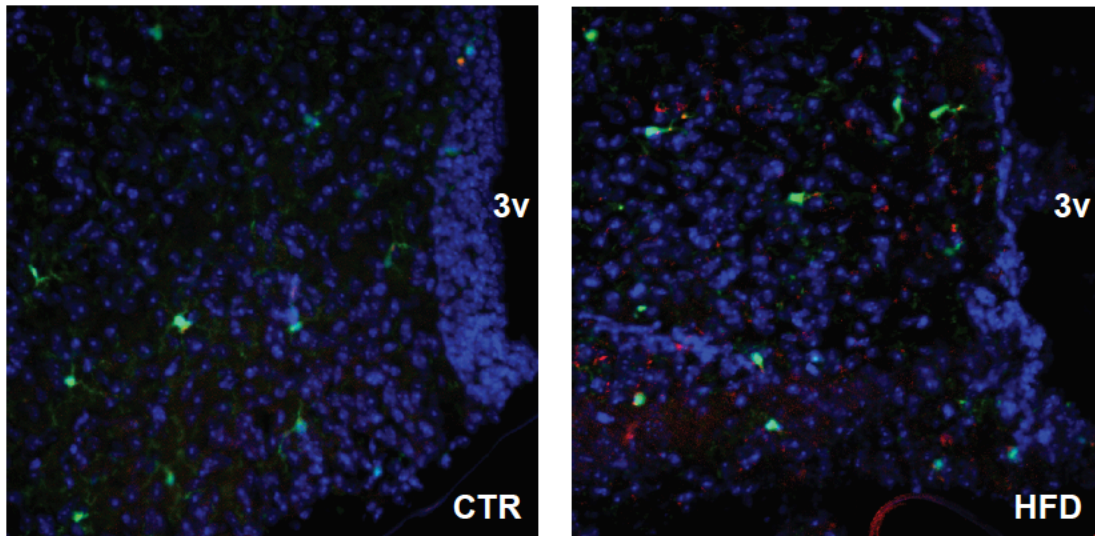


Figure 3.7: CCR2-positive macrophages localize to the parenchyma of the arcuate nucleus. Doubly fluorescent mice, where activated macrophages are genetically labeled red with CCR2-RFP and resident microglia are labeled green with CX3CR1-GFP, were placed on the control and HFD. Arcuate nuclei of mice on control diet contain only green fluorescence, while arcuate nuclei of mice on HFD contain green fluorescence of resident microglia, and red fluorescence indicating infiltration of peripheral macrophages.

Discussion

There are also profound sex differences in the immune system [233], metabolic rate and oxidative phosphorylation [234], fat deposition [204] and adipocyte number and size [235], all of which may or may not be dependent on sex steroids, gonadally or locally produced. Our results indicate that sex differences may stem from differences in inflammation or alternate fat deposition, both of which may be interdependent. Females have higher prevalence of autoimmune diseases, but exhibit lower rates of infections and fewer chronic inflammatory diseases [233]. Resistance to chronic inflammatory diseases may protect them from obesity-mediated chronic inflammation. Female mice in our study had elevated levels of anti-inflammatory IL-10 in the hypothalamus, which may provide them protection from neuroinflammation. On the other hand, we and others also determined differences in fat deposition [203, 236]. Our results agree that males have larger visceral fat depots, or gonadal fat pad, with observation in other models and humans, and that premenopausal females preferentially deposit fat into the subcutaneous depot [202]. Difference in fat deposition may also be sex steroid dependent [237, 238]; and likely provides protection for females, since subcutaneous fat is less adversely correlated with negative effects of obesity than visceral fat. Variances in fat depots, not only absolute adiposity, may impact obesity induced inflammation, since visceral fat, more abundant in males, contains more infiltrating macrophages and higher expression of inflammatory cytokines [122, 239].

Previous studies analyzing effects of obesity identified that neuroinflammation is specific for the hypothalamus and determined that microglia changes morphology specifically in the arcuate nucleus [228, 231]. In agreement, our studies also failed to detect microglia morphology changes in the cortex and detected them specifically in the hypothalami. Sex differences in microglia activation in response to obesity have also been reported previously [240]. Our studies similarly detected morphology changes specifically in the male mice. To assess the regional specificity, we further analyzed other areas besides arcuate nucleus and determined that microglia morphology changes around other circumventricular areas with fenestrated capillaries, such as OVLT. We considered two possibilities, that either microglia sense metabolic changes in the circulation via fenestrated capillaries in circumventricular areas or that increased numbers indicate that peripheral cells which label with anti-Iba-1 cross into the brain parenchyma specifically in these areas due to the lack of the blood-brain barrier. That prompted us to investigate potential peripheral cells and determine that macrophages enter the hypothalamus.

Macrophages are functionally dominant cells in obesity-induced chronic inflammation [241]. With increased adiposity, proportion of macrophages in adipose tissue increases from 10% to 50%, and they change from less inflammatory M2 phenotype to pro-inflammatory M1 phenotype [122, 224, 242, 243]. Elevated macrophage infiltration and cytokines secretion is more highly correlated with visceral adiposity [202]. Inflammatory cytokines secreted by the adipose tissue macrophages contribute to the increase in TNF α and IL-6 in the circulation and chronic inflammation in obese mice and people [131]. We determined that activated macrophages infiltrate the hypothalamus in the

DIO male mice. Macrophages are recruited to adipose tissue in part via CCR2 interaction with CCL2 that is upregulated in obesity [244]. CCR2 is involved in the macrophage recruitment to the liver in obese mice, which contributes to insulin resistance [245]. Thus, macrophages activated by increased adiposity infiltrate parenchyma of other tissues. We demonstrated that macrophages recruited to the hypothalamus of obese mice likewise express CCR2. Macrophage switch to pro-inflammatory phenotype with increasing obesity also entails a decrease in the anti-inflammatory IL-10 expression in obese adipose tissue [223, 246]. Thus, protective role of IL-10 in adipose tissue in DIO has been postulated previously but sex differences were not examined [247, 248]. We determined that female mice have higher basal levels of IL-10 in the hypothalamus and that obesity exacerbates sex differences since IL-10 is further decreased in obese males in the hypothalamus as well. In the brain, IL-10 is expressed by glial cells [249] and has a protective function [250]. We postulate that females, due to the need to handle larger weight changes during pregnancy and lactation, exhibit higher levels of protective anti-inflammatory IL-10 and that increased levels of anti-inflammatory cytokines in female may afford them protection from obesity-induced inflammation.

Given that POMC and NPY neurons are located in the arcuate nucleus, in light of our results, their synapses may be also eliminated by macrophages or activated microglia. Microglia, brain resident immune cells, are involved in synapse pruning and synapse maturation during development [251] and in the regulation synaptic transmission and activity-dependent structural remodeling in adults [252]. Peripheral, monocyte-derived macrophages, however, enter the brain in pathological conditions phagocytosing damaged

cells and engulfing synapses [253]; [254]. We demonstrated that male mice, but not females, on HFD exhibit macrophage infiltration to the hypothalamus in addition to the microglia activation, however future studies will identify which of these cells is activated first. Our studies also demonstrate that synaptic proteins are decreased in the hypothalami of obese male mice. Furthermore, we report sex specific changes in neuroinflammation and fat deposition that may explain sex differences in adverse effects of obesity. Future studies will further elucidate sex differences in adiposity and obesity-induced inflammation. Here in we examine immune parameters to identify sex-specific effects of obesity on hypothalamic function.

**Chapter 4: Leukemia Inhibitory Factor Represses GnRH Gene Expression via cFOS
During Inflammation in Male Mice**

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Abstract

The mechanisms whereby neuroinflammation negatively affects neuronal function in the hypothalamus are not clear. Our previous study determined that obesity-mediated chronic inflammation elicits sex-specific impairment in reproductive function via reduction in spine density in GnRH neurons. Neuroinflammation and subsequent decrease in GnRH neuron spine density was specific for male mice, while protection in females was independent of ovarian estrogens. To examine if neuroinflammation-induced cytokines can directly regulate GnRH gene expression, herein we examined signaling pathways and mechanisms in males *in vivo* and in GnRH-expressing cell line, GT1-7. GnRH neurons express cytokine receptors, and chronic or acute neuroinflammation represses GnRH gene expression *in vivo*. Leukemia inhibitory factor (LIF) in particular represses GnRH expression in GT1-7 cells, while other cytokines do not. STAT3 and MAPK pathways are activated following LIF treatment, but only MAPK pathway, specifically p38 α , is sufficient to repress GnRH gene. LIF induces cFOS that represses GnRH gene via the -1793 site in the enhancer region. *In vivo*, following high fat diet, cFOS is induced in GnRH neurons and neurons juxtaposed to the leaky blood brain barrier of the organum vasculosum of the lamina terminalis, but not in the neurons further away. Our results indicate that the increase in LIF due to neuroinflammation induces cFOS and represses GnRH gene. Therefore, in addition to synaptic changes in GnRH neurons, neuroinflammatory cytokines directly regulate gene expression and reproductive function, and the specificity for neuronal targets may stem from the proximity to the fenestrated capillaries.

Introduction

Gonadotropin releasing-hormone (GnRH) is the final brain output for the regulation of reproduction. GnRH neurons, which are scattered in the hypothalamus, synthesize and secrete GnRH, which acts on the anterior pituitary to stimulate the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from gonadotrope cells [2, 3]. LH and FSH act on the gonads to promote steroidogenesis and gametogenesis. GnRH neuronal processes, named “dendrons”, by Herbison group [201], form an interwoven network that receives direct synaptic and neuropeptide input from upstream regulatory neurons, most notably kisspeptin [41, 177]. This GnRH network integrates other signals that impinge on reproduction, such as stress [255, 256], endocrine disruptors [181], circadian rhythms [257, 258], metabolism [169, 171], and acute inflammation during infection [153, 259, 260].

Previous studies have implicated acute inflammation, elicited with an injection of lipopolysaccharide (LPS), in the impairment of reproductive function [153, 211, 261]. LPS challenged rodents exhibited reduced levels of LH and GnRH mRNA, diminished release of LH and GnRH, and increased levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 in the circulation [153, 154, 262, 263]. Centrally administered cytokines also provoked reduced LH and GnRH levels, but the mechanism whereby these cytokines mediate their effects is unknown [155, 156, 211, 261, 264]. More recently, our group determined that low-grade, chronic inflammation caused by high fat diet (HFD)-induced obesity may also directly affect GnRH neurons, resulting in reduced levels of LH in circulation and diminished GnRH mRNA levels in the

hypothalamus, specifically in male mice [20]. We, and others, have reported that diet-induced obese mice and people exhibited increased levels of pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6, in circulation [20, 131] and in the hypothalamus, at the mRNA level and protein level [20, 265]. Additionally, we also identified that leukemia inhibitory factor (LIF), a member of the IL-6 family, is increased in the circulation and locally produced in male mice hypothalami in obesity [20]. Interestingly, LIF is increased in a sex-specific manner only in males that exhibit reduction in GnRH mRNA and gonadotropin hormones, but not in females that lack changes in GnRH or gonadotropin hormones. IL-6, a prototypical member of the family, on the other hand, is increased in both sexes. Our previous study postulated that impairment of GnRH neurons stems from reduction in spine density and consequently the connectivity of the GnRH network [20]. However, GnRH neurons express several cytokine receptors [157] and inflammation-induced cytokines may directly regulate intracellular signaling pathways in the GnRH neurons. We are interested in delineating the mechanisms by which inflammatory cytokines influence GnRH gene expression to provide insight into the etiology of neuroinflammation-induced impairment of reproductive function. TNF α , IL-1 β , and IL-6 are key players in the regulation of immune response and inflammatory processes during infection [266]. In the central nervous system, TNF α and IL-1 β regulate synaptic plasticity, neurodegeneration, learning and memory [267-270]. During infection, both of these cytokines mediate the physiological and behavioral responses in sickness such as, inducing fever, inhibiting food intake, causing nausea and fatigue [133-135]. TNF- α and IL-1 β mediate their effects through activation of downstream signaling molecules: nuclear factor-kappa B (NF κ -B), Janus

kinase and signal transducers and activators of transcription pathway (JAK-STAT) and mitogen activated protein kinases (MAPK) [136-138]. Similarly, IL-6 is produced in response to infection and stress, and in turn stimulates various cell populations, also through the JAK-STAT and MAPK pathways [271]. In the brain, IL-6 is involved in degenerative responses. [272, 273]. However, IL-6 is also induced following TNF α or IL-1 β treatment and is involved in the negative feedback that ultimately contributes to the dampening of the immune response and activating tissue repair [274].

LIF is a member of IL-6 family that is induced during inflammatory response [144]. However, its functions are not limited to inflammation: LIF has been demonstrated to play a crucial, non-redundant role in embryo implantation in both mice and humans [145-147]. LIF also maintains stem cells and regulates differentiation of germ cells [148, 149]. In the brain, LIF regulates neuronal function and neuronal response to injury [150-152]. With respect to GnRH neurons, LIF has been shown to regulate the migration of GN11 immature GnRH neuron cell line and regulate the release of GnRH in GT1-7 cells [225, 275, 276]. LIF binds its specific receptor, which, similarly to the other members of the IL-6 family, recruits and signals through the GP130 signals transducer, activating JAK-STAT pathway [146]. Signaling pathways involved in the regulation of GnRH gene transcription by any of these cytokines have not been elucidated.

GnRH neurons in the rodent hypothalamus are located in the preoptic area surrounding organum vasculosum laminae terminalis (OVLT) and send long processes to the median eminence (ME) where secretion occurs from the terminals. Both OVLT and ME are areas that contain fenestrated capillaries and a leaky blood-brain barrier [175]. A

subpopulation of GnRH neurons extends their processes into the OVLT and across the blood-brain barrier, where they may be able to directly respond to circulating molecules, including cytokines [277]. OVLT and surrounding thermoregulatory neurons are involved in changes in body temperature and inducing fever in response to systemic inflammation. Pyrogenic, pro-inflammatory cytokines, TNF α , IL-1 β and IL-6, produced locally in the hypothalamus, or from the circulation via fenestrated capillaries in the OVLT, stimulate thermoregulatory neurons to increase the body temperature [278-281]. In addition, we postulate that these cytokines directly regulate GnRH neurons in the proximity to OVLT.

About 800-1200 GnRH neurons are scattered throughout the forebrain of a mouse [282]. This poses a challenge for molecular studies of GnRH neurons *in vivo*. GT1-7 cells are the only model of mature, terminally differentiated, GnRH-producing neurons, and have been used to identify regulatory elements and transcription factors important for GnRH transcription [283, 284]. GT1-7 cells allow for the investigation of molecular mechanisms and direct effects on GnRH gene expression without confounding variables that may be present in *in vivo* studies. Here, we combine *in vivo* mouse studies and investigate the direct effects of cytokines on GnRH gene expression in GT1-7 cells. Our results delineate the molecular mechanisms and signaling pathways that LIF activates and strongly suggest that LIF directly affects GnRH neurons to regulate GnRH gene expression in infection or obesity induced inflammation of the hypothalamus.

Materials and Methods

Animals

C57BL/6J mice were maintained under a 12-h light, 12-h dark cycle and received food and water *ad libitum*. All experiments were performed with approval from the University of California (Riverside, CA) Animal Care and Use Committee and in accordance with the National Institutes of Health Animal care and Use Guidelines. C57BL/6J male mice were placed on either a high fat diet (HFD, D12492, 60% kcal from fat; 5.21 kcal/g; protein 20% kcal; fat 60% kcal (lard 0.32 g/g diet, soybean oil 0.03 g/g); carbohydrate 20% kcal; Research Diet, New Brunswick, NJ) or control diet (CTR, D12450J, 10% kcal from fat; matching sucrose levels to HFD; 3.82 kcal/g; protein 20% kcal; fat 10% kcal (lard 0.02 g/g diet, soybean oil 0.025 g/g); carbohydrate 70% kcal; Research Diet, New Brunswick, NJ) from weaning age for 12 weeks. Mice treated with vehicle or LPS were fed standard food pellets (STD, 5053, 4.07 kcal/g; protein 24% kcal; fat 13%; carbohydrates 63%; St. Louis, MO) from weaning. Lipopolysaccharide (LPS, 2.5 mg/kg body weight) from *Escherichia coli* (catalog # L4391, Sigma, USA) was administered by intraperitoneal injection. Mice were sacrificed 24 h post LPS treatment in parallel with the corresponding control groups. At least 10 animals/diet or treatment were analyzed unless otherwise indicated, and differences from corresponding controls were compared by Student's t test. GnRH-GFP mice were kindly provided by Dr. Suzanne Moenter [192] to facilitate investigation of fluorescently labeled GnRH neurons.

Histological Analysis and Immunohistochemistry

For brain collection, animals were perfused with ice cold phosphate buffer saline (PBS) solution followed by 4% paraformaldehyde solution. Brains were post-fixed in 4% paraformaldehyde 2 h at room temperature and cryopreserved in 30% sucrose/PBS solution for 3 days at 4°C before freezing in OTC. Frozen brains from GnRH-GFP mice were sectioned to 30 µm sections and stained for GFP to visualize GnRH neurons and for GP130 or cFOS. Slides were blocked with 20% goat serum and incubated with primary antibodies against GFP (1:5000 raised in chicken, Table 1) at 4°C for 48 h. After PBS washes, slides were incubated with FITC/Alexa 488 goat anti-chicken IgG (1:300, Molecular Probes, Eugene, OR) for 1 h. Slides were then incubated with primary antibodies against GP130 (1:500, MAB4681, R&D Systems, Minneapolis, MN) or cFOS (1:300, SC-52, Santa Cruz Biotechnology, Inc. Dallas, TX) for 48 h at 4°C followed by Alexa 594 goat-anti-rat IgG (1:300, Molecular Probes, Eugene, OR) or biotinylated goat anti-rabbit IgG (1:300; Vector Laboratories, USA) and Cy5-streptavidin (1:300, 434316, Life Tech. Corp. Eugene, OR) for 1 h each at room temperature, respectively. Sections were mounted and slides covered using VectaShield mounting media with DAPI (H-1500, Vector Laboratories, USA). Secondary antibody-only controls were performed to determine antibody specificity. Images were obtained using a Leica microscope system.

To quantify the number of cFOS expressing GnRH neurons, coronal sections of the preoptic area in the hypothalamus of GnRH-GFP mice were stained for GFP (green) and cFOS (red). Three hundred GnRH-GFP neurons from each of the four male mice from control and HFD group were counted for the co-labeling with cFOS and results represented as a percent of total GFP labeled neurons. To assess expression of cFOS in other cells, two 100 μm x 100 μm areas, one area proximal to the organum vasculosum of the lamina terminalis (OVLT) and one more dorsal, in the same section were counted to quantify the number of cFOS expressing cells, where DAPI staining was used to identify cell nuclei. Statistical differences ($p < 0.05$) were determined by Student's T-test.

qPCR Analysis

Hypothalami were dissected, total RNA extracted using MicroRNA kit from Ambion and reverse transcribed using Superscript III (Invitrogen, CA). qPCR was performed using an iQ SYBR Green supermix and an IQ5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA), with primers listed in Table 2, under the following conditions: 95 °C for 15 min, followed by 40 cycles at 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 30 s. The amount of the gene of interest was calculated by comparing the threshold cycle obtained for each sample with the standard curve generated in the same run and normalized to the beta-2-microglobulin (B2M) housekeeping gene in the same sample using $\Delta\Delta\text{Ct}$ method. Replicates were averaged. After each run, a melting curve analysis was performed to confirm that a single amplicon was generated in each reaction.

Statistical differences ($p < 0.05$) in expression were determined by Student's T-test using JMP software (SAS Institute; Cary, North Carolina).

Cell Culture

GT1-7 cells, kindly provided by Pamela Mellon (University of California, La Jolla, CA), were cultured in DMEM (Cellgro, Mediatech, Inc., Herndon, VA) with 10% FBS. Leukemia Inhibitory Factor (LIF, 34-8521; eBioscience, USA) and interleukin 6 (IL-6, 216-16, PeproTech, Rocky Hill, NJ) were reconstituted in PBS containing 0.1% BSA and stored in aliquots at -80°C until use. RNA was isolated with TRIzol (Life Tech. Carlsbad, CA) and RT-PCR performed as previously described [285, 286].

Western Blot Analysis

Whole cell lysates were obtained after treatment with 10 ng/mL LIF or vehicle for times indicated, using lysis buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.5% Nonidet P-40, 0.5 mM EDTA) with 1% protease inhibitor cocktail (P8340, Sigma, USA) and 1 mM PMSF. Protein content was determined using Bradford reagent (Bio-Rad Laboratories Inc. USA). An equal amount of protein from each sample was resolved on a 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane.

Membranes were blocked in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) with 10% non-fat milk for 2 h room temperature and incubated overnight with specific antibodies for p-STAT3 (1:1000; 9134, Cell Signaling, Danvers, MA), STAT3 (1:1000, 9139, Cell Signaling, Danvers, MA), p-p38 (1:1000, 9211, Cell Signaling Danvers, MA), p38 (1:1000, 9212, Cell Signaling Danvers, MA), p-ERK1/2 (1:1000, 9101, Cell Signaling, Danvers, MA), ERK1/2 (1:1000, 9102, Cell Signaling, Danvers, MA), p-JNK (1:1000, 9255, Cell Signaling, Danvers, MA), JNK (1:1000, 9252, Cell Signaling, Danvers, MA), OCT-1 (1:500, ab66132, Abcam, Cambridge, UK), cFOS (1:1000, SC-52, Santa Cruz Biotechnology, Inc. Dallas, TX), and β -tubulin (1:1000, SC-9104, Santa Cruz Biotechnology, Inc., Dallas, TX). The bands were visualized with horseradish peroxidase (HRP)-linked secondary antibodies and enhanced chemiluminescence reagent (ECL; Amersham Bioscience), as described by manufacturer. Blots were exposed to autoradiography film (Bioexpress, USA). Each experiment was performed 3 times and representative images are presented.

Transfections

Cells were plated into 12-well plates and transfected using Fugene 6 reagent (Roche Applied Science), as described previously [286-289]. Wells were transfected with 500 ng of reporter plasmid, 100 ng of β -galactosidase reporter plasmid driven by the *Herpesvirus* thymidine kinase promoter, as an internal control for the efficiency of the transfection, and 200 ng of expression vectors or empty vector control, as indicated in the figure legends.

24 h after transfection, cells were switched to serum-free media (DMEM with 0.1% BSA) and treated with either 10 ng/mL LIF, 20 ng/mL IL-6 or vehicle for 24 h. Following treatment, cells were lysed in 0.1 M potassium phosphate buffer, pH 7.8, with 0.2% Triton X-100. Luciferase activity in the lysates was measured with a Veritas Microplate luminometer (Turner Biosystems, Sunnyvale, CA) after injection with 100 μ L of luciferase assay buffer (100mM Tris-HCl, pH 7.8, 15 mM MgSO₄, 10 mM ATP, and 65 μ M luciferin). β -galactosidase activity was measured using the Tropix Galacto-light β -galactosidase assay (Applied Biosystems, Foster City, CA). All experiments were performed three independent times and in triplicates within each experiment. Luciferase values were normalized to β -galactosidase values for each sample. Results are presented as an average of three experiments. Statistical significance, $p < 0.05$, was determined with ANOVA followed by Tukey's post hoc test using JMP software (SAS Institute; Cary, North Carolina).

Plasmids

The reporter plasmids were kindly provided by Pamela Mellon (University of California, La Jolla, CA). The -5 kb rat GnRH (-4984 to +22 relative to the transcription start site); GnRH E/P, which contains GnRH-E1 (-1863 to -1571)/GnRH-P (-173 to +112); and GnRH-P (-173 to +112) luciferase reporters have previously been described [78, 290-292]. Luciferase reporter plasmids containing mutations of AP1 binding site in GnRH enhancer and GnRH promoter, and reporter plasmids with RSV promoter fused to GnRH enhancer (GnRHe/RSVp), have also been previously described [78, 290-292]. The

expression vectors for wild type (WT) STAT3 (Stat3 Flag pRC/CMV, #8707, Addgene, Cambridge), dominant negative (DN) STAT3 (Stat3 Y705F Flag pRC/CMV, #8704, Addgene, Cambridge), constitutively active (CA) STAT3 (Stat3-C Flag pRC/CMV, #8722, Addgene, Cambridge), and luciferase reporter containing STAT3 response element (4xM67 pTATA TK-Luc, #8688, Addgene, Cambridge, MA) were purchased from Addgene and have previously been described [293]. Expression vectors for constitutively active RAS, MEK1, MEK2, MKK4, MKK7, MKK3, MKK6, and constitutively active isoforms of p38 (p38 α , p38 β , p38 λ , p38 δ) were a gift from Peiqing Sun (The Scripps Research Institute, La Jolla, CA) [294]. Expression vector for cFOS has been previously described [285, 295-297].

Table 4.1 Antibodies used for western and immunohistochemistry

Antibody	Species	Dilution	Provider, cat #
GFP	chicken	1:5000	Abcam, ab1397
GP130	rat	1:300	R&D Systems, MAB4681; clone#125623
STAT3	mouse	1:1000	Cell Signaling, 9139
p-STAT3	rabbit	1:1000	Cell Signaling, 9145
p38	rabbit	1:1000	Cell Signaling, 9212
p-p38	rabbit	1:1000	Cell Signaling, 9211
ERK 1/2	rabbit	1:1000	Cell Signaling, 9102
p-ERK 1/2	rabbit	1:1000	Cell Signaling, 9101
JNK	rabbit	1:1000	Cell Signaling, 9252
p-JNK	mouse	1:1000	Cell Signaling, 9255
OCT-1	rabbit	1:500	Abcam, ab66132
cFOS	rabbit	1:300, 1:1000	Santa Cruz Biotechnology, sc-52
β -tubulin	rabbit	1:1000	Santa Cruz Biotechnology, sc-9104

Table 4.2 Primers used for qPCR

Primers	Forward	Reverse
<i>Gnrh</i> (GnRH)	CTACTGCTGACTGTGTGTTTG	CATCTTCTTCTGCCTGGCTTC
<i>Il6</i> (IL-6)	TTCTCTGGGAAATCGTGGAAAT	TCCAGTTTGGTAGCATCCATC
<i>Tnfa</i> (TNF- α)	ATGTCTCAGCCTCTTCTCATTC	GCTTGCTCACTCGAATTTTGAG
<i>Il1β</i> (IL1 β)	GCAACTGTTTCTGAACTCAACT	CACAGCCACAATGAGTGATA
<i>Lif</i> (LIF)	ATGTGCGCCTAACATGACAG	TATGCGACCATCCGATACAG
<i>B2m</i> (beta-2-microglobulin)	TGACCGGCCTGTATGCTATCCA	CAGTGTGAGCCAGGATATAG
LIFR	TCAGTTTCAGCCAGGAGTAA	GCAATAATCAATCCCACAGA
IL6R	AAGCAGCAGGCAATGTTACC	CATAAATAGTCCCCAGTGTC
GP130	GCGTACACAGATGAAGGTGGG	GCTGACTGCAGTTCTGCTTG

Results

Inflammation in the hypothalamus induces inflammatory cytokines and represses GnRH.

Inflammation, either acute, caused by an injection of lipopolysaccharide (LPS), or chronic, elicited by high fat diet (HFD), negatively affects hypothalamic neurons and in particular, reproductive function [155, 163]. We and others postulated this impairment is due to the repression of the GnRH gene, which is the final brain signal in the control of reproduction. Inflammatory cytokines have previously been proposed to negatively regulate GnRH neurons [211, 262, 298]. We initiated our studies with an analysis of cytokine mRNA levels in the hypothalami of C57BL/6 male mice, induced by LPS injection, a bacterial endotoxin known to elicit an inflammatory response. Our studies revealed that tumor necrosis factor alpha (TNF- α , *Tnf*) mRNA was induced 22-fold, interleukin 1 beta (IL-1 β , *Il1b*) was induced 7-fold, IL-6 (*Il6*) 1.6-fold, and leukemia inhibitory factor (LIF, *Lif*) 2.2-fold after LPS injection (Fig. 4.1A). *Gnrh* mRNA level in mice treated with LPS was reduced by 72% (Fig.4.1A).

Obesity is considered a state of chronic inflammation as opposed to acute inflammation elicited by LPS injection [20, 131, 222]. Thus, we exposed male C57BL/6J mice to HFD or to control diet (CTR) for 12 weeks. Diet-induced obesity induced TNF- α 1.6-fold and IL-1 β 1.7-fold, although due to variability, it did not reach significance (Fig. 4.1B). IL-6 was significantly induced 3.1-fold and LIF 1.6-fold, compared to mice fed CTR diet (Fig. 4.1B).

Further analysis revealed significantly reduced *Gnrh* mRNA levels by 46%, compared to the controls (Fig. 4.1B). Thus, increase in locally produced cytokines, acute and chronic, correlates with repression of GnRH mRNA expression.

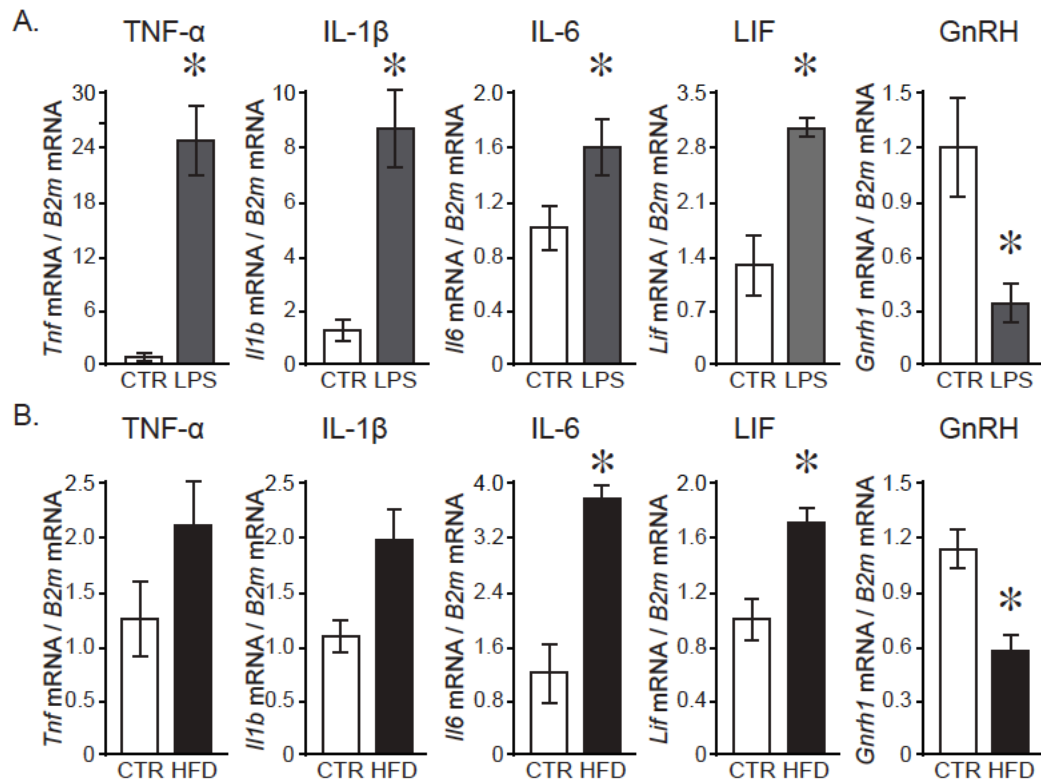


Figure 4.1: GnRH expression is repressed when cytokine levels are increased. A, Hypothalami from male mice 24 hours after injection with either vehicle control (CTR, white bars) or lipopolysaccharide (LPS, gray bars). B, Hypothalami from male mice following 12-week feeding with control (CTR, white bars) or high fat diet (HFD, black bars). Cytokine and GnRH (*Gnrh1*) expression was assayed with RT-qPCR. TNF-α, (*Tnf*) tumor necrosis factor alpha; IL-1β, (*Il1b*) interleukin 1 beta; IL-6, (*Il6*) interleukin 6; LIF, (*Lif*) leukemia inhibitory factor. * indicates significant difference ($p < 0.05$) determined with t-test.

LIF represses GnRH

We then determined whether these locally produced, inflammatory cytokines can regulate GnRH gene expression directly. Due to the scarcity and scattered location of GnRH neurons in mice, *in vivo* analysis of molecular mechanisms whereby cytokines regulate GnRH gene is not possible. To analyze direct effects of cytokines on gene expression we employed GT1-7 cells that are an established *Gnrh* expressing and GnRH secreting cell model [283]. We expressed GnRH 5 kb luciferase reporter (5 kb GnRH luc) in GT1-7 cells and treated with inflammatory cytokines. LIF repressed GnRH reporter expression by 45.7% compared to CTR (Fig. 4.2A, black bar), similar to what was observed *in vivo* with LPS injection and HFD. TNF- α , IL-1 β and IL-6 had no effect on GnRH expression. To further analyze the effect of LIF treatment on GnRH gene, RNA was isolated from GT1-7 cells treated with either vehicle or LIF, reverse transcribed and quantitative RT-PCR performed. LIF treatment reduced endogenous GnRH mRNA levels by 39.1% (Fig. 4.2B). Taken together, these results indicate that LIF represses *Gnrh* gene.

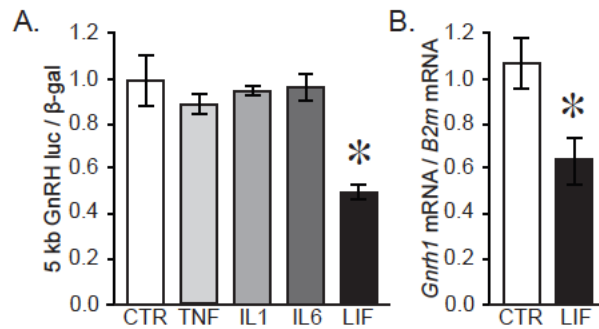


Figure 4.2: GnRH expression is suppressed by LIF. A, GT1-7 cells transiently transfected with 5 kb GnRH-reporter (5 kb GnRH Luc) treated with vehicle (CTR, white bar), or TNF- α , IL-1 β , IL-6, LIF, for 24 hours. Luciferase values were normalized to β -galactosidase values for each sample. Results are presented as an average of three experiments performed in triplicate. Statistical significance ($p < 0.05$) was determined with ANOVA followed by Tukey's post hoc test, and indicated with *. B, RT-qPCR using total RNA from GT1-7 cells treated with LIF, 10 ng/mL for 24h, demonstrates repression of endogenous GnRH mRNA (*Gnrh1*). * indicates significant difference determined with t-test ($p < 0.05$).

Since LIF and IL-6 belong to the same cytokine family, it was surprising that LIF repressed GnRH while IL-6 did not. These two cytokines share GP130 signaling receptor, but each has a specific receptor for ligand binding. We analyzed the expression of their specific receptors on GnRH neurons to explain why IL-6 had no effect on GnRH gene expression. We first demonstrated the expression of GP130 cytokine signaling receptor (red) on GnRH neurons (green) *in vivo* using immunocytochemistry of hypothalamic slices (Fig. 4.3A). This suggests that LIF and IL-6 cytokines can act on GnRH neurons in the mouse. Since antibodies for specific LIF receptor (LIFR) and IL-6 receptor (IL-6R) were not effective in immunohistochemistry, we analyzed mRNA expression of these receptors using RNA isolated from GT1-7 cells. Products of expected size for GP130 (177 bp), and LIFR (452 bp), were present in both GT1-7 cells and spleen, which served as a positive control, but absent in negative control samples lacking reverse transcriptase (Fig. 4.3B). IL-6R expression (156 bp) was absent in GT1-7 cells, but present in spleen (Fig. 4.3B). qPCR analysis reveals that the expression of IL-6R is limited in GT1-7 cells, compared to the expression of GP130 and LIFR, while expression levels of these receptors was similar in spleen (Fig. 4.3C). These results indicate that LIF can directly bind GnRH neurons and affect GnRH gene expression.

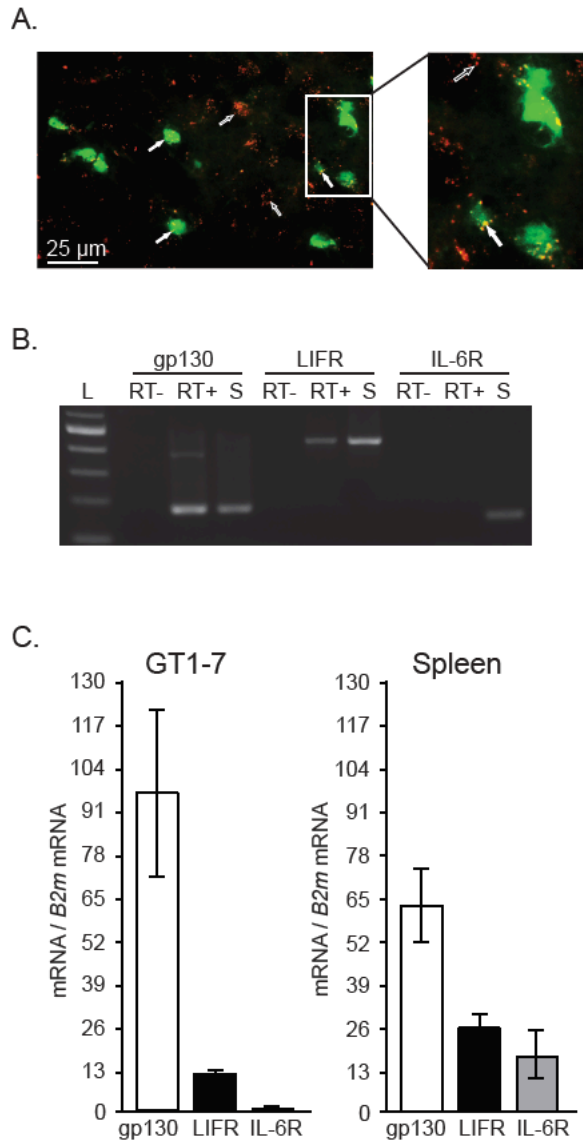


Figure 4.3: GP130 is expressed in GnRH neurons *in vivo* and in GT1-7 cells. A, Immunohistochemistry of GnRH neurons genetically labeled with GFP (green), stained for GP130 receptor (red). Bar indicates 25 μ m. White arrows indicate GnRH neurons that express GP130, while arrowheads indicate GP130 staining in non-GnRH cells. B, RT-PCR demonstrates expression of GP130 (177 bp) and LIFR (452 bp), but not IL-6R (156 bp) in GT1-7 cells (RT+), while all three receptors are expressed in the spleen (S). RT-, GT1-7 cells mRNA used without reverse transcriptase serves as negative controls; L, size ladder. C, RT-qPCR demonstrates relative expression of GP130, LIFR, IL-6R in GT1-7 cells and the spleen.

LIF functions via GnRH enhancer to repress GnRH gene

The 5 kb GnRH reporter contained two upstream regulatory elements: the 300 bp enhancer (−1863 to −1571) and the evolutionarily conserved promoter (−173 to +1), that confer neuron-specific activation of the GnRH, in culture and *in vivo* [63, 290, 299]. To map the elements necessary for LIF mediated repression of GnRH expression, GT1-7 cells were transiently transfected with a luciferase reporter containing the 5 kb regulatory region upstream of the GnRH transcription start site (5 kb GnRH), a reporter containing the enhancer and promoter without intervening sequences (GnRH E/P) or a reporter containing the promoter (GnRH P). LIF repressed luciferase activity of the 5 kb GnRH reporter and of the reporter containing the enhancer and promoter, GnRH E/P luc, by 45.7% and 42.6% respectively (Fig. 4.4A and 4.4B). Luciferase activity of the reporter containing only the promoter, GnRH P luc, did not change in response to LIF (Fig. 4.4C). Next, to examine if the enhancer is sufficient for repression, the reporter containing enhancer fused to heterologous RSV promoter was examined (GnRH E/RSVp luc). LIF treatment significantly repressed GnRH E/RSVp luc reporter by 45.2% (Fig. 4.4D). Given that GnRH E/P and GnRH E/RSVp reporters were repressed to a similar degree by LIF as the full length 5 kb GnRH reporter, these results indicate that the enhancer is sufficient and necessary for repression by LIF.

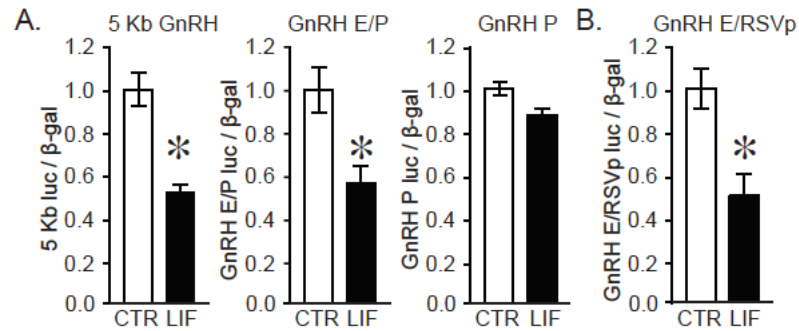


Figure 4.4: LIF represses GnRH gene expression through the enhancer region. A, 5 kb GnRH reporter containing 5 kb of the GnRH gene regulatory sequence from the transcriptional start site; B, reporter containing GnRH enhancer (E, -1863/-1571) and promoter (P, -173/+1); C, reporter containing GnRH promoter -173/+1 (P) and D, reporter containing GnRH enhancer (E, -1863/-1571) linked to the heterologous RSV (Raus Sarcoma Virus) promoter (GnRH E/RSVp luc); were transfected in GT1-7 cells and cell treated with LIF for 24h. Results demonstrate sufficiency of the enhancer for GnRH repression by LIF. Statistical significance, $p < 0.05$, indicated with *, was determined with a t-test.

STAT3 is not necessary for GnRH repression by LIF

LIF signals through GP130 to activate the signal transducer and activator of transcription (STAT) pathway. To analyze signaling pathways activated by LIF, cells were treated with LIF and western blots performed using whole cell lysate. LIF treatment resulted in increased levels of STAT3 phosphorylation (Fig. 4.5A). To determine whether STAT3 is sufficient to repress GnRH gene expression, GT1-7 cells were co-transfected with a constitutively active STAT3 mutant (CA STAT3) and STAT binding region (SBR), a reporter containing 6 copies of the STAT3 response element that serves as a positive control; or with GnRH E/P reporter. CA STAT3 overexpression induced STAT3-SBR to the similar level as induction by LIF. LIF treatment together with CA STAT3 overexpression did not further increase induction over CA STAT3 alone, indicating that CA STAT3 maximally induces SBR reporter. On the other hand, CA STAT3 overexpression did not affect the expression of the GnRH E/P reporter or its repression by LIF (Fig. 4.5B). We then analyzed necessity of STAT3 by co-transfecting dominant negative mutant STAT3 (DN STAT3) with SBR or with GnRH E/P. While transfection with DN STAT3 inhibited LIF induction of SBR positive control, DN STAT3 did not prevent repression of GnRH E/P by LIF (Fig. 4.5C). Taken together, these results demonstrate that STAT3 is not necessary for GnRH repression by LIF.

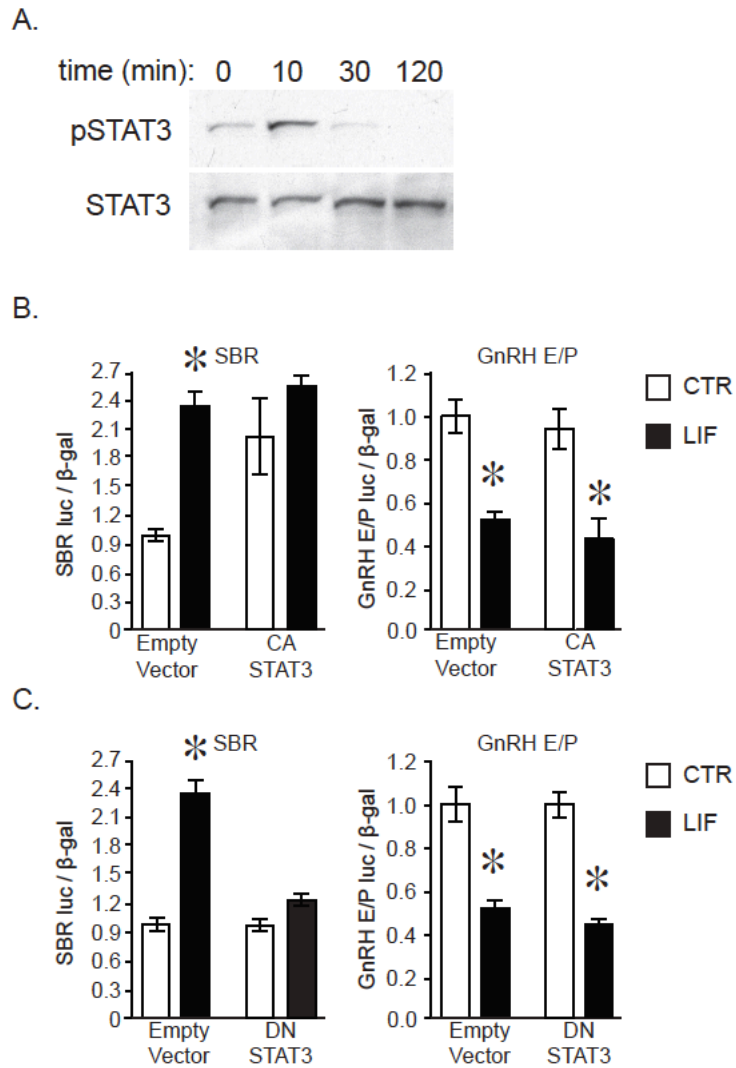


Figure 4.5: STAT3 is not sufficient or necessary for GnRH repression by LIF. A, Analysis of STAT3 phosphorylation by western blot of GT1-7 cell lysates after 10 ng/mL LIF treatment, for 10, 30 and 120 minutes. B, Co-transfection of a reporter containing 6 copies of the STAT3 response element (SBR) or GnRH E/P with a constitutively active (CA) STAT3 demonstrates that CA-STAT3 is sufficient to induce SBR, but not GnRH E/P. C, Co-transfection of a dominant negative (DN) STAT3 inhibits LIF induction of SBR-luciferase, but does not prevent LIF repression of the GnRH E/P reporter. Statistical significance (*, $p < 0.05$) was determined with ANOVA followed by Tukey's post hoc test.

LIF activates p38 to repress GnRH

LIF signaling has been shown to activate the mitogen activated protein kinase (MAPK) pathway, which include extracellular signal-regulated protein kinases (ERK1/2), p38, and the c-Jun N-terminal kinases (JNK) in a variety of other cells, including mouse embryonic stem cells, 3T3-L1, and AtT20 pituitary corticotrope cells [300, 301]. LIF treatment of GT1-7 cells resulted in phosphorylation of ERK1/2 and p38, but no changes in JNK phosphorylation were observed (Fig. 4.6A). To further delineate the necessity of MAPK signaling pathway, co-transfection assays with expression plasmid containing constitutively active RAS (CA RAS), that is upstream to the MAPK pathway, resulted in repression of GnRH expression by 51.4% (Fig. 4.6B). To identify the MAPK kinase pathway sufficient for repression, co-transfection assays were conducted using constitutively active forms of MEK1 and MEK2 that leads to ERK1/2 activation, MKK3 and MKK6 that that activate p38, or MKK4 and MKK7 which activates JNK. Constitutively active MEK1, MEK2, MKK4, MKK7 had no effect on GnRH expression. GnRH expression was repressed with constitutively active MKK3 and MKK6, which lead to activation of p38, by 40.9% and 48.2% respectively (Fig. 4.6C).

The p38 kinases have four isoforms, p38 α , p38 β , p38 γ , and p38 σ [302]. To explore the role of these isoforms on GnRH, constitutively active mutants of p38 isoforms were analyzed in co-transfection assays. Reporter activity was repressed by 31% with constitutively active p38 α , while overexpression of constitutively active p38 β , p38 γ , and p38 σ , had no effect on luciferase activity (Fig. 4.6D).

These results demonstrate that LIF represses GnRH transcriptional activity via the MAPK pathway, specifically p38, and highlights the important role that p38 α plays in this repression.

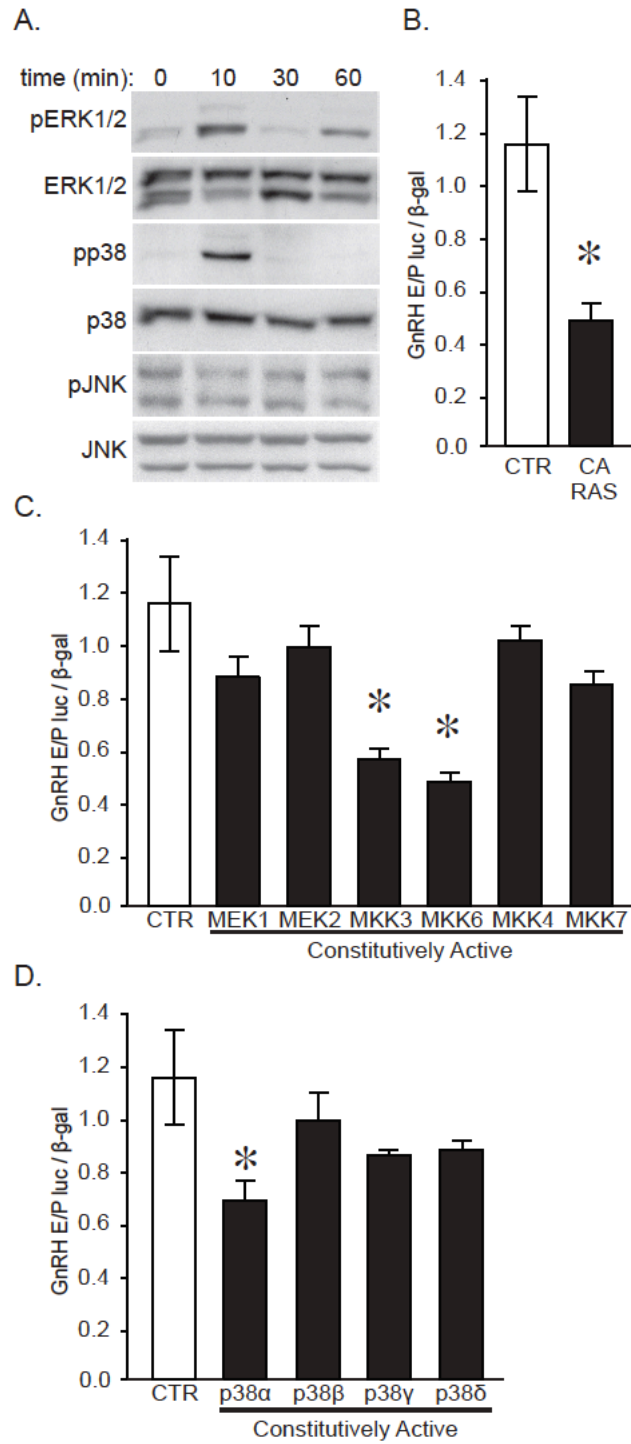


Figure 4.6: p38 α is sufficient to repress GnRH gene expression. A, LIF treatment activates ERK1/2, p38, but not JNK, demonstrated by western blots using whole cells lysate of GT1-7 cells treated with LIF for times indicated above each lane. GnRH-reporter is repressed by co-transfection with B, constitutively active (CA) RAS; C, CA MKK3 and CA MKK6, upstream activators of p38; and D, CA p38 α . Statistical significance, $p < 0.05$, indicated by *, was determined with ANOVA followed by Tukey's post hoc test.

cFOS is induced by LIF in GT1-7 cells and represses GnRH

OCT-1 has been previously identified as an essential regulator of GnRH gene transcription [67]. Western blot analysis revealed that OCT-1 protein level was not affected by LIF treatment in GT1-7 cells, suggesting that OCT-1 may not mediate LIF repression of the GnRH gene. GnRH gene repression by cFOS has previously been shown [291], and thus we next examined cFOS protein levels. Indeed, western blots demonstrated that cFOS was induced by LIF (Fig. 4.7A). GnRH E/P reporter was repressed by 43.4% with overexpression of cFOS (Fig. 4.7B). This indicates that induction of cFOS is likely mediating LIF repression of GnRH.

Several cFOS binding sites were previously identified in the GnRH regulatory region [78, 291, 292]. To identify the site involved in LIF repression of GnRH, GT1-7 cells were transfected with luciferase reporters containing mutation of the putative cFOS sites at -79 in the promoter region (-79m), -1723 (-1723m), -1782 (-1782m), and -1793 (-1793m) in the enhancer and their expression was compared to wild type GnRH E/P (WT). LIF repressed luciferase activity of all reporters used by 61.0%, 61.8%, 65.9%, and 61.6% respectively, except of the -1793 mutant, indicating that the mutation of this site prevents LIF repression of GnRH (Fig. 4.7C). Thus, the -1793 site is involved in LIF repression of GnRH. We confirmed the role of this site by transfecting the mutation of the -1793 site in the GnRH E/RSVp reporter and comparing the repression of the mutant to the wild-type reporter containing the enhancer linked RSV promoter (GnRH E/RSVp).

Similar to what was observed in Fig.4. 7C, LIF repression of GnRH was inhibited with a mutation of the -1793 site (Fig. 4.7D). Therefore, LIF induces cFOS which represses GnRH gene expression via the -1793 site.

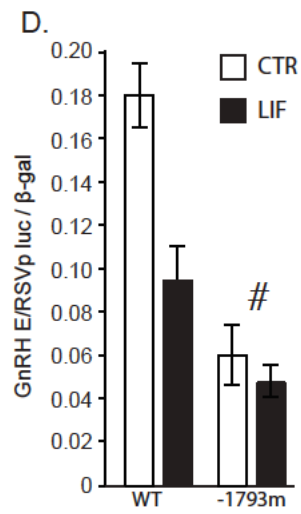
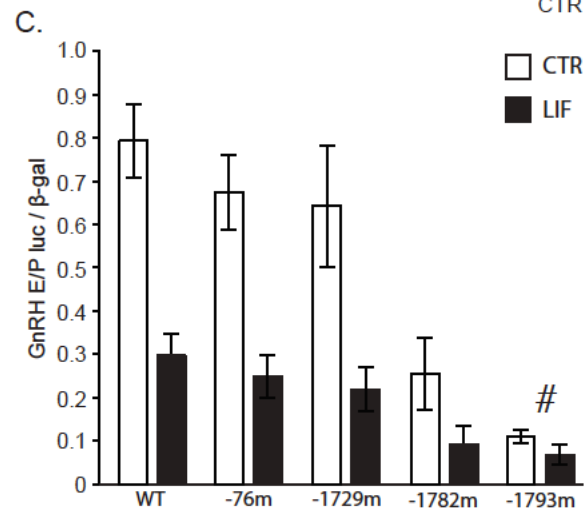
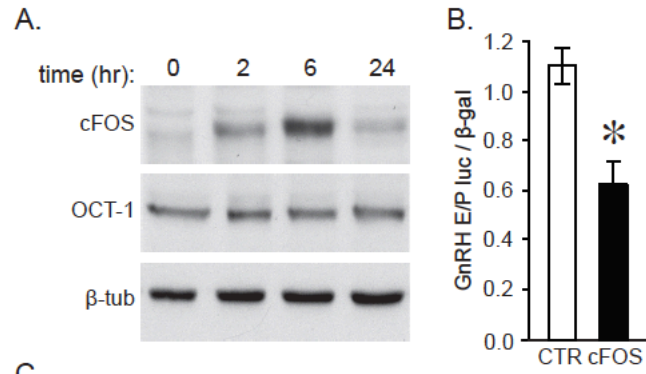


Figure 4.7: cFOS mediates LIF repression of the GnRH gene. A, LIF treatment of GT1-7 cells induces cFOS, but not OCT-1, demonstrated by western blots of nuclear extracts. B, Overexpression of cFOS, by co-transfection of cFOS expression vector, is sufficient to repress GnRH E/P reporter. * indicates significant difference ($p < 0.05$) determined with t-test. C, Mutation of the cFOS binding site at -1793 abolishes LIF repression of GnRH E/P reporter, demonstrated by transient transfections of reporters containing the mutations of the putative cFOS binding sites in GT1-7 cells. D, the same mutation was created in the GnRH E/RSVp reporter and also abrogates the repression by LIF. Statistical significance, $p < 0.05$ indicated by *, was determined with ANOVA followed by Tukey's post hoc test.

cFOS expression is induced with HFD in mice

Since we determined that cFOS mediates LIF repression of the GnRH gene and we observed reduced GnRH mRNA levels in the hypothalami of mice fed HFD, we explored cFOS expression in the hypothalamus. We concentrated specifically in the sections containing OVLT where the largest numbers of GnRH neuron soma are found. In the hypothalamus of GnRH-GFP mice fed HFD, a significant increase in the percent of double labeled green GnRH neurons with cFOS (red) was observed compared to control (CTR) (Fig. 4.8A and 4.8B.1). In CTR fed mice, 17.9% of GnRH neurons expressed cFOS, while in the HFD mice, 36.3% of GnRH neurons expressed cFOS, suggesting that HFD induces cFOS expression in GnRH neurons. To examine specificity of this increase, we also counted cells that express cFOS in two other areas, one proximal to OVLT and one more dorsal from OVLT, using DAPI to identify cell nuclei. We determined that there is an increase in the percent of cFOS positive neurons in the area proximal to the OVLT of mice fed HFD (45.4%) compared to CTR (30.5%) (Fig. 4.8A.2, 4.8B.2). However, in the area dorsal to OVLT, we did not observe a difference in the number of neurons that express cFOS (Fig. 8B.3). Thus, obesity induces cFOS expression in neurons in the proximity to OVLT and in GnRH neurons. Given that cFOS mediates LIF repression of the GnRH gene in GT1-7 cells, and the reduced levels of GnRH mRNA observed *in vivo* coupled with an increased cFOS expression in GnRH neurons following LIF induction by inflammatory stimuli, our data together demonstrate that LIF represses *Gnrh mRNA in vivo* through cFOS. Furthermore, LIF may mediate repression of the *Gnrh* mRNA and reproductive function caused by inflammation.

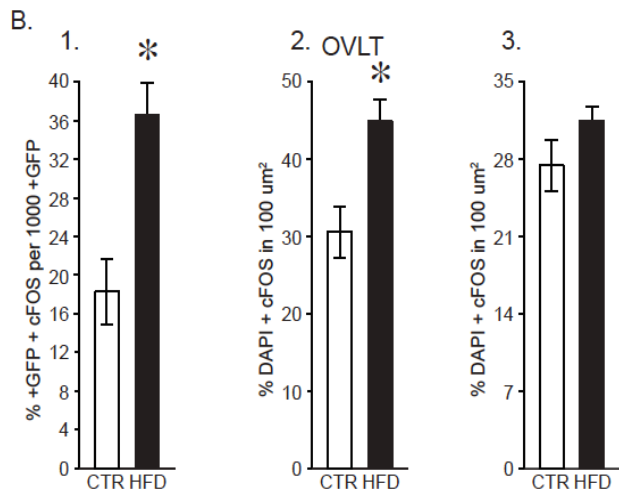
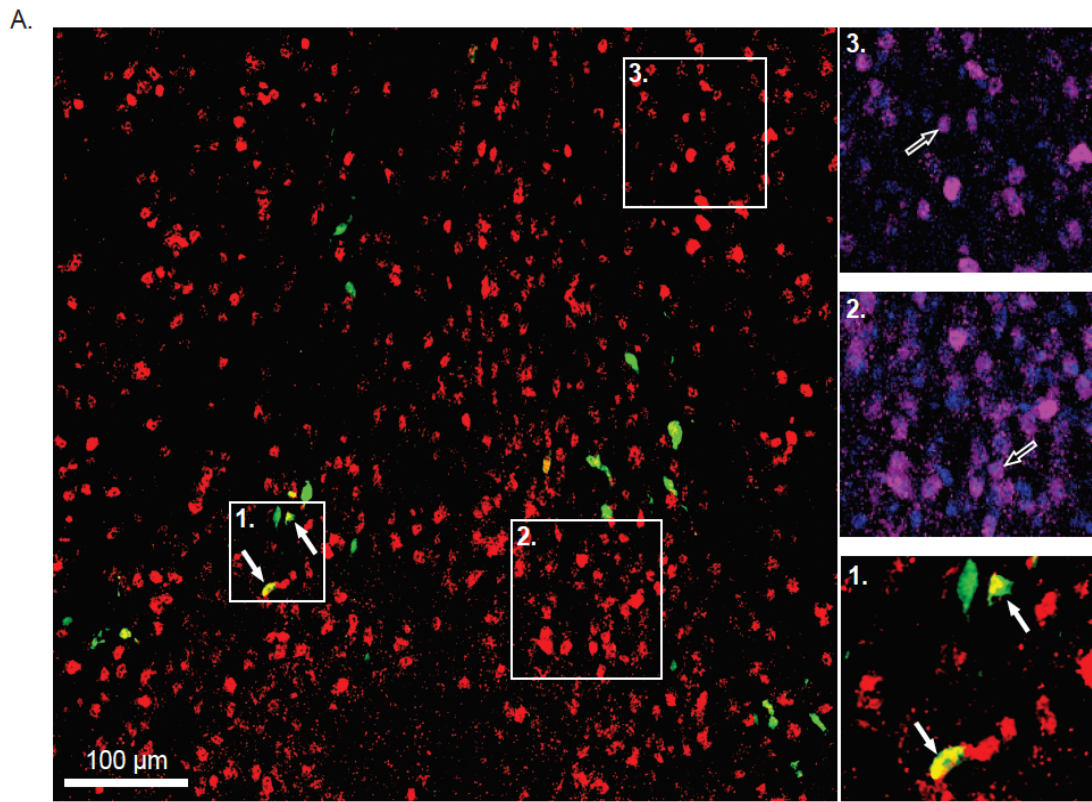


Figure 4.8: More GnRH neurons express cFOS following high fat diet (HFD). A, Coronal sections of the preoptic area in the hypothalamus of GnRH-GFP mice following HFD stained for GFP (green) and cFOS (red). Scale bar represents 100 μ m, white arrows indicate GnRH neurons labeled with GFP and cFOS. Numbered squares correspond to enlarged areas below; 1, GnRH neurons green, cFOS red; Arrows indicate GnRH neurons that express cFOS, an arrowhead points to the GnRH neuron without cFOS; 2-3, DAPI channel is included to facilitate cell count; nuclei blue, cFOS magenta. Arrows indicate cells labeled with cFOS and DAPI. B, quantification of neurons expressing cFOS in control and HFD male mice: 1, increase in the percent of GnRH neurons with cFOS is observed in HFD compared to control. 2, quantification of the neurons that express cFOS, proximal to the OVLT delineated with #2 square; 3, quantification of the neurons that express cFOS, dorsally from the OVLT delineated with #3 square following control and HFD. Three hundred GnRH neurons were counted in each mouse. Statistical significance, $p < 0.05$ indicated by *, was determined with t-test.

Discussion

Both infection and obesity negatively affect hypothalamic function, the GnRH neuronal network and reproduction, but the mechanisms are unknown. We postulated that neuroinflammation is a common characteristic between these conditions and that inflammatory cytokines may mediate impairment of reproductive function by both infection and obesity. Classical pro-inflammatory cytokines, TNF α , IL1 β , and IL-6, are induced in the brain during inflammation, when GnRH and LH expression and secretion are diminished, but direct regulation of GnRH neuron function by cytokines remain inconclusive. In our recently published report [20] and herein, we identified a novel cytokine, leukemia inhibitory factor (LIF), which is induced during these inflammatory states that directly affects GnRH gene expression. In this report, we also determined the precise mechanisms of GnRH gene repression and signaling pathways that are necessary for LIF-mediated effects on GnRH neurons. Finally, we demonstrated that the transcription factor induced by LIF to repress GnRH gene is increased in a location specific manner following HFD *in vivo*. Repression of *Gnrh* mRNA expression is consistently observed during inflammatory states. In agreement with previous reports, acute inflammation, elicited with an injection of LPS and chronic inflammation, caused by high fat diet (HFD)-induced obesity, suppresses GnRH mRNA in our studies. LPS treatment, resulting in inflammation, represses *Gnrh* in ewes [303], birds [304] and rats [305]. Infusion of the pro-inflammatory IL-1 β cytokine into the rodent hypothalamus also represses *Gnrh* expression [211].

We and others reported that obese mice fed HFD, with a low grade chronic inflammation, exhibit lower *Gnrh* mRNA [20, 188, 210]. These reports suggested that repression of *Gnrh* mRNA during inflammation may be a result of increased cytokine concentration. Alternatively, microglia, brain resident immune cells, are involved in the regulation of synaptic transmission and activity-dependent structural remodeling [252]. In neuroinflammation, in response to injury, infection, or disease, microglia engulf damaged synapses [252].

We demonstrated decreased synaptic spine density in GnRH neurons following obesity-mediated neuroinflammation and microglia activation, indicating reduced connectivity [20]. This reduced synaptic activity may regulate *Gnrh* expression in an activity-dependent manner [212, 213]. Some synaptic proteins are regulated in an activity-dependent manner at the transcriptional level [306]. This may be the case with GnRH gene expression as well, since several hypothalamic factors, such as RFamide-related peptide 3 (RFRP-3), a mammalian gonadotropin-inhibitory hormone ortholog; senktide, a neurokinin B receptor agonist; and oxytocin; alter both GnRH secretion and *Gnrh* transcription [214-216]. However, since GnRH neurons express cytokine receptors [157], herein, we investigated if *Gnrh* gene is repressed via activation of cytokine receptor signaling pathways.

We identified LIF as a critical player in the neuroinflammation-induced impairment of GnRH gene expression. In the brain, LIF is involved in neural stem cell maintenance and axonal growth, and in modulation of gene expression [307]. During embryonic development, LIF is expressed in the olfactory placode, which led to the hypothesis that LIF affects GnRH neuron migration [276]. Our results demonstrate that LIF represses GnRH gene expression, which is in agreement with findings that GnRH mRNA is repressed during embryonic GnRH neuron migration [308], in addition to repression during inflammation. To analyze the effects of infection on GnRH, previous studies analyzed classical inflammatory markers, TNF- α , IL-1 β and IL-6, but not LIF. Although LPS, or TNF- α or IL-1 β administration in the ventricle consistently reduces GnRH secretion *in vivo* [154-156, 262], some have found that IL-6 treatment of the hypothalamic slices stimulates GnRH secretion [263, 309]. While IL-1 α and TNF- β are prototypical pro-inflammatory cytokines, IL-6 may function as either a pro- or anti-inflammatory cytokine depending on timing and stimulus. IL-6 synthesis in the hypothalamus is induced by IL-1 β [310], and may be engaged in the negative feedback to dampen inflammation [274]. Thus, results may vary due to the differences between *in vivo* and *in vitro* treatments, since initially-induced cytokines may cause a cascade of other cytokines *in vivo*. For this reason, we concentrated on analyzing the effects of LIF in GT1-7 cells. LIF alone repressed *Gnrh* mRNA and GnRH reporter, similarly to HFD-induced and LPS-elicited repression of GnRH mRNA *in vivo*. LIF is induced by IL-1 β *in vivo*. Therefore, IL-1 β repression of GnRH mRNA, demonstrated in previous studies, may occur via induction of LIF.

We determined that LIF induces cFOS which represses GnRH gene expression. GnRH repression by cFOS has been demonstrated previously and several cFOS binding sites have been identified in the GnRH regulatory region [291, 292]. However, a specific site necessary for GnRH repression has not been identified, until now. GnRH gene is inhibited by PKC signaling pathway, which induces cFOS [76, 290]; and PKC activation by TPA treatment represses GnRH gene via the -1793 site [78]. Here we demonstrate that this same site at -1793 is involved in LIF repression of the GnRH gene. This site is juxtaposed to the OCT-1 binding site, and OCT-1 is the critical transcription factor that regulates GnRH gene expression [67, 70, 78, 311]. Thus, although OCT-1 protein levels do not change with LIF treatment, its interaction with cFOS [312], likely modulates that level of GnRH gene expression.

Signaling pathways involved in the regulation of GnRH gene transcription by cytokines have not been elucidated. GP130, the common signal transducer for IL-6 and LIF family, is expressed in GnRH neurons *in vivo*. LIF treatment activates STAT3 in GT1-7 cells, a model of mature GnRH neurons, as we show herein, and in GN11 cells, a model of immature, migrating GnRH neurons [276]. However, to exert its effect on GnRH gene expression, LIF activates MAPK-p38 pathway, and in particular p38 α is critical for this effect. This is an important finding, since while other p38 isoforms have normal physiological functions, p38 α is involved in inflammatory processes [313]. Moreover, p38 α is the target for several potential therapeutics for chronic inflammatory disorders.

Leptin is necessary for pubertal transition and normal reproductive function [314, 315]. Since leptin is elevated in obese and HFD-fed mice, leptin was previously analyzed as a mediator of obesity-induced impairment of reproductive function [111, 208, 258]. Additionally, a mouse model with leptin overexpression exhibits early puberty and lower GnRH at 21 weeks of age [316], which may correlate with precocious puberty in obese girls and lower *Gnrh* mRNA in obese mice in our study. However, previous studies determined that leptin does not directly affect GnRH neurons in vivo, since deletion of the leptin receptor specifically in GnRH neurons has no effect on fertility [101]. STAT3 signaling molecule, activated by leptin, is required for leptin's role in energy balance, on the other hand, it is not required for leptin's effect on fertility [317, 318]. Therefore, regardless of the metabolic dysfunctions in STAT3 mutant mice, reproductive functioning remained unaffected, demonstrating a clear separation in roles for each signaling pathway. Although leptin receptor is similar to the IL-6 and LIF signaling receptor, GP130, and can activate the same signaling pathways, leptin does not engage GP130 [319]. Taken together, it is unlikely that elevated leptin levels in our studies had direct effect on GnRH gene expression.

We demonstrate that cFOS expression is increased specifically in GnRH neurons and in cells located close to the OVLT, but not in cells located more dorsally, following HFD. Others reported increased cFOS following HFD in specific areas and postulated that the location is specific for the function of the neuronal population. For example, cFOS was increased in the reward circuitry following HFD [320] and in the dorsomedial and lateral hypothalamus [321]. cFOS is similarly induced following LPS treatment in the hypothalamus [322] and this induction is location specific [323]. These studies further postulated that the location is dependent on the function of the neurons. We, on the other hand, posit that it is dependent on the proximity to the vasculature. Previous studies also suggested that cFOS was induced following neuron activation, since cFOS is most commonly used marker of neuronal activity. As an immediate early gene, cFOS is induced via activity-dependent transcription in neurons. cFOS expression is increased in activated GnRH neurons as well, during the preovulatory LH surge and after kisspeptin treatment [324, 325]. The roles of cFOS as a transcription factor, involved in the GnRH gene regulation, and as a marker of neuronal activity, are not mutually exclusive. Thus, we postulate that increased LIF concentration induces cFOS specifically in GnRH neurons to repress GnRH gene expression. Specificity for GnRH neurons may be due to either their proximity to the OVLT or because GnRH processes extend beyond the blood brain barrier into the OVLT.

Our studies delineate the mechanisms of neuroinflammation impairment of GnRH gene expression and may explain repression of reproductive function during infection and in obesity. We identified LIF as the cytokine that mediates these effects and p38 α as a critical signaling molecule. We also postulate that the proximal location of GnRH neurons to the leaky blood brain barrier, where the local concentration of this cytokine may be the highest, permits for repression of GnRH gene expression via cFOS induction. Future studies will determine *in vivo* role of p38 α in neuroinflammation-mediated GnRH repression.

Chapter 5: Conclusion

My dissertation research identified mechanisms of obesity-mediated impairment of reproduction function and identified profound sex differences in responses to high fat diet (HFD). My work determined that male C57BL/6J mice are prone to diet-induced obesity (DIO) with HFD, and experience profound neuroendocrine and inflammatory changes when obese. Male mice exhibit chronic inflammation and high levels of cytokines in the circulation and in the hypothalamus that are locally produced. On the other hand, female mice are resistant to DIO. Loss of ovarian hormones, after ovariectomies, made females susceptible to DIO. This is in agreement with previous studies using rodent and primate animal models and with finding in human population where women gain weight after menopause. My studies determined further that although ovariectomized female mice gain weight similarly to males, they remained resistant to neuroendocrine changes and inflammatory changes, showing that ovarian hormones are not necessary for this protection. My results challenge the long accepted protective role of ovarian estrogen in neuroendocrine and neuroinflammation in females and identify that the female immune system responds differently to DIO compared to males. Females have higher levels of the anti-inflammatory cytokine IL-10 than males at the basal level, and the difference is exacerbated with chronic inflammation in obesity. Thus, females are resistant to chronic inflammation regardless of the gonadal status.

Males, but not females, exhibited reduction in reproductive hormones in all levels of the hypothalamic-pituitary-gonadal axis, lower GnRH expression in the hypothalamus, diminished levels of gonadotropin hormones, LH and FSH, from the pituitary and reduced levels on testosterone from the testes. Since all levels of the HPG are similarly affected, hypothalamus and GnRH neuron network are the likely primary site of dysregulation. My results, indeed demonstrate decrease in the levels of post-synaptic density, 95, protein in the hypothalamus, and reduced spine density in GnRH neurons. Microglial activation and peripheral macrophage infiltration is seen in the hypothalami of males, and both can contribute to synapse elimination in neuroinflammation. As explained in chapter 1, GnRH neuron activation requires stimulation by a pulse generator since they are scattered in the hypothalamus. Therefore, it is possible that the reduced spine density illustrates reduced afferent neuron connectivity to GnRH neurons, which will be analyzed in future studies. Thus, reduced activity of GnRH neuronal network may be responsible for diminished levels of gonadotropin hormones, testosterone, and sperm numbers, which corresponds to the observations in obese humans.

Additionally, neuroinflammation-induced cytokines can directly regulate GnRH gene expression. Previous studies have demonstrated that both states of inflammation, acute, induced with LPS injection or the central administration of cytokines in the mouse, and chronic, observed in obesity, have resulted in reduced levels of GnRH mRNA, but the mechanism was unknown. I determined that LIF cytokine that is increased with both acute inflammation, by LPS injection, and chronic inflammation, by DIO, repressed GnRH gene expression. I examined LIF signaling pathways and mechanisms of GnRH repression *in*

vivo and in GnRH-expressing cell line, GT1-7. LIF activates MAPK pathway, specifically p38 α , and induces cFOS to represses GnRH gene, mediated via the -1793 site in the enhancer region. *In vivo*, following HFD, cFOS is induced in GnRH neurons and neurons juxtaposed to the leaky blood brain barrier of the organum vasculosum of the lamina terminalis, but not in the neurons further away. Our results indicate that the increase in LIF due to neuroinflammation induces cFOS and represses GnRH gene. Therefore, in addition to synaptic changes in GnRH neurons, neuroinflammatory cytokines directly regulate gene expression and reproductive function.

Taken together, our data implicate neuro-immune mechanisms underlying sex-specific differences in obesity-induced impairment of the hypothalamic function with potential consequences for reproduction and fertility.

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