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Glucocorticoids Regulate Kisspeptin Neurons during Stress and Contribute to Infertility and Obesity in Leptin-Deficient Mice

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Glucocorticoids regulate kisspeptin neurons during stress and contribute to infertility and obesity in leptin-deficient mice

Abstract

Stressors generate adaptive responses, including transient suppression of reproductive function. Natural selection depends on successful reproduction, but inhibition of reproduction to survive famine or escape predation allows animals to survive to reproduce at a later time. The cellular locations and mechanisms responsible for inhibiting and reactivating the reproductive axis during and after stress, respectively, are not well understood.

We demonstrated that stress-induced elevation in glucocorticoids affects hypothalamic neurons that secrete kisspeptin (KISS1), an important reproductive hormone. Stressors that stimulated glucocorticoid secretion, as well as glucocorticoid administration itself, inhibited *Kiss1* mRNA expression, while conditions that did not change glucocorticoid secretion did not alter *Kiss1* mRNA expression. In mice lacking glucocorticoid receptor specifically in kisspeptin-containing neurons, *Kiss1* mRNA expression was no longer inhibited during restraint stress despite a rise in corticosterone, and both testosterone and copulatory behaviors showed accelerated recovery in the post-traumatic period.

We also demonstrated that increased glucocorticoid secretion contributed to infertility and obesity in leptin-deficient mice. Leptin deficiency creates a chronic state of perceived starvation, and leptin-deficient mice exhibit elevated plasma glucocorticoid concentrations, morbid obesity, and infertility. Leptin-deficient, glucocorticoid-deficient mice exhibited decreased body weight and fat composition, decreased hyperphagia, and normal fertility. When supplemented with glucocorticoids back to the initial levels present in leptin deficiency, these mice gained weight and became infertile. Thus, leptin is not required for fertility as previously believed, and glucocorticoids can contribute to obesity and suppress fertility independently of leptin signaling.

Together, these findings implicate glucocorticoids in the regulation of obesity and reproductive inhibition during stress, including perceived starvation caused by leptin deficiency. These studies may provide novel mechanisms and molecular targets in the reproductive and metabolic aspects of disorders characterized by glucocorticoid dysregulation, including post-traumatic stress disorder, anorexia nervosa, and mood disorders.

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CHAPTER 1

INTRODUCTION

1.1 STRESS

The world is full of stressors, real or perceived, that challenge self-preservation: predators, food shortages, wounds. Such stressors disrupt homeostasis, and survival depends on the body's ability to generate appropriate stress responses. Adaptations include enhanced cognition, physical strength, and analgesia, along with inhibition of nonessential functions such as reproduction and growth. Natural selection is driven by successful reproduction; yet first and foremost, successful reproduction depends on surviving to reproduce another day. Thus acute stressors generate responses that improve the chances of immediate survival at the expense of immediate reproductive fitness. Multiple stress systems coordinate these central and peripheral adaptive responses.

1.1.1 HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

One important stress response is generated by the hypothalamic-pituitary-adrenal (HPA) axis. A subset of paraventricular nucleus (PVN) neurons in the hypothalamus produces corticotropin-releasing hormone (CRH). Following exposure to stressors, CRH is secreted into the portal circulation and stimulates the pituitary to produce proopiomelanocortin, which is cleaved into adrenocorticotropic hormone (ACTH). ACTH enters the general circulation and stimulates the zona fasciculata of the adrenal cortex to produce glucocorticoids [1]. Chromaffin cells in the adrenal medulla produce the catecholamines epinephrine and norepinephrine, which act as sympathetic nervous system (SNS) hormones [2]. Together, the HPA and SNS react to acute stressors by generating fight or flight responses: increased heart rate, blood flow, oxygen flow, and nutrient release; decreased reproductive, gastrointestinal, and immune activities.

Subsequently, the same hormonal outputs inhibit CRH and ACTH production in an autoregulatory negative feedback loop.

1.1.2 Corticotropin-releasing hormone

CRH is a 41-amino acid peptide that regulates both basal and stress-induced HPA activity [3]. CRH-secreting neurons are located throughout the brain, but primarily concentrated in the PVN. The PVN is a heterogeneous nucleus that comprises magnocellular, dorsal cap, medial parvocellular, and submagnocellular regions. Magnocellular cells secrete vasopressin and oxytocin and send projections to posterior pituitary gland [1]. The dorsal cap and submagnocellular regions project to the brainstem and spinal nuclei to control sympathetic and parasympathetic activity. Parvocellular neurons release CRH into the portal blood system, which activates CRH type I receptors in the anterior pituitary. To date, two CRH receptor genes have been identified in humans and other mammals, with a third additional one being described in the catfish [4]. The CRH type 1 receptor is expressed predominantly in anterior pituitary corticotroph cells, whereas the CRH type 2 receptor is more widely distributed in the brain and periphery [4]. The CRH type 1 receptor mediates fear and anxiety behaviors following stressors, even in CRH-deficient mice [5], suggesting that a CRH-related peptide can mediate fear responses via the CRH type 1 receptor. Transient early-life CRH exposure in the forebrain changes *Crhr1* expression and induces despair-like changes in adulthood [6]. In addition to stimulating ACTH expression and release, CRH can also directly stimulate secretion of glucocorticoids from the adrenal gland [7]. Deletion of *Crh* by homologous recombination in mice results in a phenotype largely a consequence of glucocorticoid deficiency [8] and suggests

that lifelong deficiency of CRH may not have important direct behavioral effects on the central nervous system [5].

Several peptide antagonists to CRH receptors were synthesized in hopes of treating conditions from anxiety to depression, but these peptides were all unable to pass through the blood-brain barrier. In 1996, a CRH type 1 receptor-specific antagonist was developed [9]. CP-154,526 is non-peptide, orally active, and features a central ring core with a basic nitrogen group that modulates the confirmation of an agonist binding site [9]. Male rhesus macaques treated with antalarmin, a related CRH type 1 receptor-specific antagonist, and challenged with an intense social stressor exhibited significantly decreased ACTH and cortisol responses [10]. Additionally, behaviors typically associated with social stress, such as body tremors, grimacing, teeth gnashing, urination, and defecation, were decreased in antalarmin-treated males. Exploratory and sexual behaviors that are typically suppressed during stress were increased in antalarmin-treated primates.

Arginine vasopressin (AVP) is expressed in the supraoptic nucleus and cosecreted by PVN CRH neurons. AVP amplifies the CRH effect at the pituitary [11]. Stress, circadian rhythms, and glucocorticoids also influence CRH release. Afferent inputs to the PVN may mediate the action of stressors by controlling the release of CRH [12]. Sources of neuronal afferents to the hypothalamus include the amygdala, hippocampus, and brainstem regions involved in autonomic functions [12]. Acetylcholine, norephinephrine, angiotensin II, and possibly CRH itself, increase CRH concentrations in the hypophyseal portal plasma. CRH, vasopressin, and glucocorticoids

all inhibit expression of *Crhr1* mRNA, which may limit the effect of these agents during the stress response.

1.1.3 Adrenocorticotropic Hormone

ACTH is derived from a 266-amino acid precursor, proopiomelanocortin (POMC), so named because it encodes opioid, melanotropic, and corticotropic activities [13]. The human *POMC* gene is a single copy gene located on chromosome 2p23, and the murine *Pomc* gene is located on chromosome 12. It and the genes encoding the highly homologous opioid peptides, preproenkephalin A and preproenkephalin B (dynorphin), are all located on different chromosomes.

The human *POMC* gene is 8 kb long. It consists of a promoter of at least 400 bp at the 5' end of the gene, followed by an untranslated 86-bp exon 1, 152-bp exon 2, 833-bp exon 3, and two introns, 3708 and 2886 bp in length. The initiator methionine is located 20 bp into exon 2 and followed by a 26 amino acid hydrophobic signal peptide. Except for the signal peptide and 18 amino acids of the amino-terminal glycopeptide, the majority of the POMC precursor is encoded by exon 3 [13]. Exon 1 of the human and other mammalian POMC genes are less than 50% identical. Exon 2 is close to 90% identical between the *POMC* genes of humans and other mammals. Within exon 3 of *POMC* are all known peptide products of the *POMC* gene, including N-terminal glycopeptide, γ-MSH, joining peptide, ACTH, α-MSH, corticotropin-like intermediate lobe peptide (CLIP), ß-lipotropin (ß-LPH), ß-MSH, and ß-endorphin. The regions encoding the N-terminal glycopeptide, α-MSH, ACTH, and ß-endorphin, are greater than 95% identical between humans and other mammals. In contrast, joining peptide, the region between

the N-terminal glycopeptide and ACTH, is very poorly conserved among mammals, which suggests that it does not encode a biologically important function [13].

The CRH-induced rise in cAMP is responsible for both the increase in *POMC* transcription and peptide synthesis as well as for the rise in intracellular calcium which results in ACTH secretion [14]. CRH mediates its stimulation of *POMC* transcription via the POMC CRH responsive element (PCRH-RE), which binds PCRH-RE binding protein [14]. The negative effect of glucocorticoids upon POMC gene transcription is thought to be mediated by a glucocorticoid-glucocorticoid receptor complex binding to cis-acting DNA sequences within the POMC promoter. The possibility exists that the glucocorticoid receptor complex does not bind directly to the POMC gene, but instead to another protein such as a positive transcription factor, and in this way mediates its negative effect on POMC gene expression. Glucocorticoid stimulates, rather than inhibits, POMC gene expression in the arcuate nucleus of the hypothalamus, the site of α-MSH production.

Release of ACTH from the corticortroph is mediated by second messengers through signal transduction pathways, involving protein kinase A, protein kinase C, glucocorticoids, or the Janus kinase/STAT system. These pathways result in changes in the phosphorylation pattern of specific cellular proteins, and/or in intracellular calcium levels, impacting on ACTH synthesis and release. Circulating ACTH then binds to the G-protein coupled type 2 melanocortin receptors (MC2R) in the adrenal gland, leading to steroid biosynthesis [1].

1.1.4 Glucocorticoid receptor signaling

Glucocorticoids are an important output of the HPA axis, and classical actions of glucocorticoids are exerted through glucocorticoid receptors (GRs) expressed throughout the body and brain. GR is a steroid hormone receptor that is encoded by the *Nr3c1*, or *nuclear receptor subfamily 3, group C, number 1*, gene. GR is part of the nuclear receptor family of transcription factors and is related to the mineralocorticoid, androgen, estrogen, progesterone, vitamin D, and retinoic acid steroid receptors [15]. GRs and related steroid receptors are thought to have originated from gene duplications over 400 million years ago and are highly conserved in mammals [15].

The murine GR gene is located on chromosome 18, and the human GR gene is located on chromosome 5. The gene contains 9 untranslated alternative first exons and 8 translated exons. GR transcription is regulated by the 5' untranslated region, and the multitude of possible first exons is thought to be a mechanism for local fine-tuning of GR levels [16]. GR also has a variable 3' region, which encodes splice variants, including GRα, GRβ, and GR-P [16]. GRα and GRβ are generated by two spliced 3' exons, 9α and 9β. The predominant isoform is GRα, a 777-amino acid protein, whereas GRβ, a 742-amino acid protein, is expressed at much lower levels [15]. GR-P lacks both exons 8 and 9, and the translated protein is a truncated ligand binding domain that is thought to enhance GRα activity.

The endogenous ligands of GR are the glucocorticoids cortisol in humans and corticosterone in rodents. Upon glucocorticoid binding, GR translocates to the nucleus from its inactive cytoplasmic localization and regulates the activity of specific target genes. GR interacts with DNA sequence-specific glucocorticoid responsive elements (GREs) and negative GREs (nGREs)

to cause transcriptional changes in target genes. The DNA-binding domain of GRα contains two zinc finger motifs that bind GREs in the promoter region of target genes [17]. GREs are characterized by a 15-bp consensus sequence 5'-AGAACAnnnTGTTCT-3'. nGREs repress the expression of certain transcripts [18, 19]. Despite the fact that the majority of genes regulated by glucocorticoids are repressed, very few nGREs have been identified in these genes [19]. Known genes with nGREs include CRH, prolactin, proopiomelanocortin, and osteocalcin. In the osteocalcin promoter, the nGRE sequence overlaps with the TATA box, and gene silencing is the result of competition for binding sites with other transcription factors [15]. GR can exert non-genomic effects through protein-protein interactions. Glucocorticoids also bind mineralocorticoid receptors with higher affinity than GR, such that mineralocorticoid receptors are occupied under basal conditions and GRs are only occupied during stress [1].

The PVN is a major site for glucocorticoid negative feedback via GR. Dexamethasone, a potent synthetic glucocorticoid, decreases the amount of basal CRH in hypothalamic explants and CRH responsiveness to stress. Glucocorticoids also decrease *Crh* mRNA expression and prevent the rise in CRH and AVP usually seen after adrenalectomy. Additionally, glucocorticoids increase the amount of GABA in the hypothalamus and thus inhibit CRH release. In the anterior pituitary, glucocorticoid inhibition of ACTH secretion *in vitro* is mediated via GR.

In animal models, acute glucocorticoid exposure can cause chronic changes, including a decrease in neurogenesis as a result of epigenetic changes on gene transcription [20, 21] and long-lasting alterations in calcium influx in the hippocampus [22, 23]. Acutely, brain-specific deletion of GR results in decreased anxiety in forced-swim and dark-light box tests [24]. Removing GR in

dopamine receptor-expressing neurons causes decreased motivation to self-administer cocaine [25]. Deletion of central amygdala GR causes changes in conditioned fear behaviors [26] . Forebrain-specific disruption of GR produces alterations in stress-induced locomotor activation [27]. Inactivation of GR in macrophages and neutrophils abolishes downregulation of inflammatory response [28]. GR inactivation in hepatocytes causes a reduction in body size [29]. T-cell inactivation of GR results in significant mortality after immune activation [30]. Lung epithelial-specific GR deletion leads to impaired epithelial differentiation and reduced viability [31]. Osteoclast-specific GR deletion enhances osteoclast survival but decreases their bone-degrading capacity [32]. GR is essential for life, and global deletion of the gene results in death hours after birth [33].

1.1.5 Afferents and efferents of the stress response

Many areas of the central nervous system are involved in the regulation of stress responses. Afferent inputs diverse mechanisms of action that can blunt or promote the stress response via direct and indirect pathways. The HPA axis, described above, is an important stress output, and in the hypothalamus, CRH neurons of the PVN activate this axis. The PVN receives major direct catecholaminergic inputs from the nucleus of the solitary tract. Catecholamines activate the HPA axis, and destroying ascending norepinephrine or epinephrine neurons reduces the HPA axis response to physical but not psychogenic stressors [34]. Norepinephrine and epinephrine inputs from the A2/C2 region innervate the medial parvocellular area of the PVN [35] . These projections also release neuropeptide Y, glucagon-like peptide 1, inhibin-β, somatostatin, and enkephalin [36-38], which can regulate HPA axis activity.

Serotonin stimulates the HPA axis, and serotonergic fibers from the dorsal and median raphe nucleus project to the PVN [39]. Lesions of the raphe nuclei decrease HPA responses to restraint stress [40]. Serotonin has been shown to stimulate ACTH and corticosterone via 5HT2A and possibly 5HT2B receptors in the PVN. Many serotonergic fibers innervate the peri-PVN region, which is dense in GABAergic cells, as well as forebrain stress-related structures, including the hippocampus, prefrontal cortex, and amygdala.

Projections from the subfornical organ and lamina terminalis target the medial parvocellular PVN neurons and convey information on blood pressure, fluid balance, and electrolyte status [41, 42]. The system is critical in the central regulation of blood pressure by angiotensin II [34]. Direct angiotensin II-containing projections from the subfornical organ reach the medial parvocellular PVN, where they activate the angiotensin II type I receptor [43].

Direct inputs to the PVN from the bed nucleus of the stria terminalis and parastrial nucleus contain predominantly GABAergic neurons [44]. The anteroventral bed nucleus of the stria terminalis activates the HPA axis, and lesions here reduce the activation of the PVN following restraint [45, 46]. The anteroventral region also contains CRH neurons that project to the PVN, supporting a central excitatory role on the HPA axis. By contrast, the posteromedial bed nucleus of the stria terminalis is inhibitory for the HPA axis, and lesions to the posterior bed nucleus of the stria terminalis enhance expression of CRH in the PVN [47]. Regulation of neurons in the bed nucleus of the stria terminalis by dopamingergic pathways may also contribute to CRH-dependent affective states [48].

The PVN may also receive inputs from the thalamic sensory nuclei, including the subparagascicular and posterior intralaminar regions, which are thought to relay audiogenic stressors to the medial parvocellular PVN [49, 50]. This region of the PVN is also innervated by dopaminergic neurons from the anteromedial zona incerta [42, 51, 52], though the role of dopaminergic neurons as a PVN afferent remains controversial. It is thought that PVN neurons are well-positioned to receive direct information from the blood and CSF. This region is clearly able to access blood-brain barrier permeable factors, including steroid hormones, though there is no evidence that the capillary plexus surrounding the PVN is fenestrated.

Physical, systemic stressors are thought to be relayed directly to the PVN, while stressors requiring interpretation by higher brain structures (e.g., psychological stress) are thought to be channeled through the limbic system [53]. The PVN is densely surrounded by GABAergic neurons that provide significant inhibitory tone [44, 54, 55]. These peri-PVN GABAergic neurons receive inputs from the limbic system and modulate both autonomic and HPA axis stress responses.

Multiple indirect pathways connect the limbic structures of the brain to the PVN. The hippocampus is involved in inhibiting the HPA axis response via trans-synaptic mechanisms [56]. Hippocampal lesions lead to elevated basal glucocorticoid levels [57, 58], and hippocampal stimulation decreases glucocorticoid secretion in rats and humans [59, 60]. Both GR and mineralocorticoid receptors are expressed abundantly in the hippocampus and allow the region to modulate negative feedback by stress levels of glucocorticoids [56].

The medial prefrontal cortex (mPFC) also provides negative regulation of the HPA axis. Both c-fos expression and glucose utilization are enhanced in this region after acute exposure to stressors [61-64]. Lesions in the mPFC enhance ACTH and corticosterone responses [65-67]. Raphe efferents to the mPFC are activated by CRH pathways in anxiety [68]. Both the hippocampus and mPFC regulate the duration of the HPA response but not the peak of glucocorticoid secretion [34]. Additionally, the mPFC is thought to modulate chronic stress responses. Chronic stress causes retraction of mPFC dendrites, reduction of prefrontal dopamine, and sensitization to norepinephrine [56].

The amygdala activates the HPA axis. GABAergic cells engrafted into the amygdala of young rats are anxiolytic [69]. Electrical stimulation of the amygdala increases glucocorticoid secretion in rats, monkeys, and humans [70-72]. Ablation of the amygdala produces reduced HPA responsiveness [58, 73]. Damage to the amygdala can suppress ACTH secretion following adrenalectomy [74, 75]. Discrete regions within the amygdala mediate different stressor-specific responses: the central nucleus of the amygdala is sensitive to systemic, physical stressors, like hemorrhage and inflammation, but not restraint; the medial nucleus of the amygdala is responsive to restraint, forced swim, and noise, but not inflammation; and the basolateral nucleus of the amygdala is activated by psychological stressors [34]. The amygdala does not send many projections to the PVN and is thought to affect the PVN predominantly through indirect pathways involving the peri-PVN GABAergic neurons.

Not all stress responses are relayed through the CRH neurons of the PVN. Glucocorticoids exert a relatively protracted secretory effect, but the autonomic nervous system is able to respond to

stressors within seconds. The autonomic nervous system is closely coupled with sensory systems that monitor homeostatic disruptions. The brainstem, for example, receives information about major homeostatic disruptions, such as blood loss, pain, and respiratory distress [34]. Sympathetic responses involve reflex arcs that communicate with the medulla and preganglionic sympathetic neurons in the spinal cord [34]. The medulla and spinal cord systems also communicate with higher-order autonomic sites in the raphe pallidus, parabrachial nucleus, Kölliker-Fuse nucleus, midbrain, and forebrain. These autonomic pathways, independent of hormonal inputs, exert integral stress responses that can generate important behavioral changes. The autonomic nervous system, hypothalamic-pituitary-adrenal axis, and possibly other uncharacterized sites generate stress responses, including the suppression of reproductive function, in response to homeostatic disruptions.

1.2 Reproduction

Stress is a well-documented inhibitor of reproduction. Offspring are unlikely to survive under conditions of food shortage or heightened predation, and in supporting pregnancy, parents may jeopardize their own chances of surviving to reproduce again under more favorable environmental conditions. In juveniles, reproductive inhibition can manifest as delayed onset of puberty. Even after sexual maturation, stress exerts control over reproduction, causing decreased reproductive hormone production, abnormal estrous cycling or amenorrhea in females. How the reproductive axis senses extrinsic stressors remains a mystery. Though it is clear that stress correlates with decreases in every reproductive hormone, it is unclear what stressors target in the first place and how the reproductive axis recovers in the post-traumatic period. The discovery of

kisspeptin, an upstream and global regulator of the reproductive axis, raises the exciting possibility that it is through kisspeptin that stress interacts with the reproductive axis.

1.2.1 Hypothalamic-pituitary-gonadal axis

For decades, the hypothalamic-pituitary-gonadal (HPG) reproductive axis was thought to consist of hypothalamic gonadotropin-releasing hormone (GnRH), which is secreted via the portal circulation to stimulate luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the anterior pituitary. The gonadotropins LH and FSH enter general circulation and stimulate the gonads to produce the sex steroids testosterone in males and estrogen in females. The HPG axis is also carefully controlled by negative feedback loops: sex steroids inhibit further GnRH, LH, and FSH release. GnRH has two modes of secretion: tonic secretion maintains follicle development and steroidogenesis, while cyclical GnRH surges are responsible for the LH surge that leads to ovulation. GnRH is low in childhood and begins to rise immediately before puberty, when heightened reproductive hormone levels coincide with sexual maturation. As late as the 1990s, experts in the field described GnRH as “the central core of the hypothalamic-pituitary-gonadal axis” [76]. While GnRH may be a regulator in the HPG axis, it has become clear that an upstream molecule, kisspeptin, controls GnRH.

1.2.2 Role of kisspeptin in reproductive function

In 1996, cancer researchers in Hershey, Pennsylvania, discovered the *KiSS-1* gene, a suppressor sequence that had anti-metastatic function in tumor cells. By subtractive hybridization, *KiSS-1* was found to be upregulated nearly 10-fold in tumorigenic cells that never metastasized [77]. Herein referred to as *KISS1*, the human gene was mapped to chromosome 1q32 and contains 4

exons [78]; the transcript that derives from exons 3 and 4 encodes a 145-amino acid precursor to the kisspeptin peptide. The precursor is cleaved into several active C-terminus products, including kisspeptin-54, -14, -13, and -10, and the decapeptide is the most potent activator of the HPG axis.

At the same time, independent researchers cloned a galanin receptor-like protein, GPR54 [79]. The five-exon *GPR54* gene localized to chromosome 19p13.3 and encoded a 396-amino acid G-protein coupled receptor. Despite its 45% homology to known galanin receptors, GPR54 did not bind galanin and remained an orphan receptor until groups discovered its remarkable binding affinity for kisspeptin [80-82]. In 2003, *GPR54* (renamed *KISS1R*) became prominent in reproductive biology, when two independent groups discovered hypogonadotropic individuals with *KISS1R* mutations in large, consanguineous families [83, 84]. In the two families studied, an L148S mutation and 155-base pair deletion in the *KISS1R* gene, respectively, rendered affected individuals hypogonadotropic, prepubescent, and infertile. Inactivating mutations in the *KISS1* gene also rendered affected individuals infertile [85], and *KISS1R* gain-of-function mutations slowed receptor desensitization and resulted in precocious puberty [86]. Kisspeptin has also been identified as an HPG axis regulator in mice [83], rats [87], monkeys [88], fish [89], guinea pigs [90], sheep [91], pigs [92], and cows [92].

Kiss1R knockout mice exhibited hypogonadotropic hypogonadism, failure to undergo puberty, low reproductive hormones, small gonads, and infertility [83, 93]. Inactivating mutations in the *Kiss1* gene also caused infertility in mice [94, 95], though hypogonadism was less severe in *Kiss1* knockout than in *Kiss1R* knockout animals. In *Kiss1* but not *Kiss1R* knockout mice,

infusion of kisspeptin restored puberty and sexual maturation [94]. In wild-type rats, kisspeptin infusion in juvenile females caused advanced puberty [87]. While *Kiss1R* transcript levels were similar in juvenile and adult mice, *Kiss1* transcript levels reached maximum levels at puberty in both males and females [87]. The number of *Kiss1* mRNA-positive neurons increased postnatally until puberty [96].

In adult men, kisspeptin caused a dose-dependent increase in plasma LH and testosterone, while in adult women, kisspeptin caused a dose-dependent increase in plasma LH, with the greatest kisspeptin effect observed during the preovulatory phase; [97, 98]. Likewise in adult female rats, the maximal LH response to kisspeptin occurred at estrous [99].

In wild-type mice and rats, the stimulatory effects of kisspeptin were blocked by pretreatment with the GnRH antagonist, acyline [100-103]. This suggests that kisspeptin acts on GnRH neurons to stimulate LH/FSH in the HPG axis. *Kiss1R* is expressed in GnRH neurons [104], and kisspeptin-positive fibers were found in close apposition to GnRH-positive neurons in the preoptic area and median eminence [91, 96, 105]. Direct electrophysiological recordings of GnRH neurons showed that kisspeptin causes increased firing in prepubertal males and proestrous females [106, 107]. In juvenile mice, kisspeptin activated roughly 30% of GnRH neurons, and this number increased during the prepubertal period; by adulthood, kisspeptin administration elicited depolarization of >90% of GnRH neurons in both males and females [106]. Exogenous GnRH or LH/FSH infusion increased downstream HPG output, but did not affect kisspeptin expression or activity [108]. Continuous administration of kisspeptin-10 led to LH peaks after 2-3 h and receptor desensitization thereafter in rhesus monkeys; desensitization

was associated with decreased LH and pituitary response to GnRH injection [109]. Tracing experiments have not provided conclusive evidence as to which population(s) of kisspeptin neurons project to GnRH neurons.

Kisspeptin transcript and protein have been found in several places in the hypothalamus, including the anteroventral periventricular (AVPV), periventricular (PeN), anterodorsal preoptic nucleus, and arcuate nuclei [100, 110]. In the AVPV but not arcuate nucleus, kisspeptin expression is sexually dimorphic; females have more than 10-fold higher *Kiss1* transcript [96]. The AVPV and arcuate nuclei are differentially regulated by sex steroid feedback. Castrated males and ovariectomized females, which have absent sex steroids, had increased arcuate and decreased AVPV nucleus *Kiss1* transcript levels [111, 112]. Replacement testosterone or estradiol decreased arcuate and increased AVPV nucleus kisspeptin expression. Taken together, sex steroids negatively regulate the arcuate and positively regulate the AVPV nucleus. Arcuate nucleus kisspeptin neurons are thought to mediate traditional HPG axis negative feedback. Given the higher expression of *Kiss1* mRNA in female AVPV nucleus and the positive feedback from sex steroids, AVPV nucleus kisspeptin neurons are thought to be responsible for the preovulatory surge in LH release [105, 113]. In mice, AVPV nucleus *Kiss1* expression peaks while arcuate levels reach a nadir during proestrous, which coincides with the estrogen- and progesterone-induced LH surge [114]. AVPV nucleus but not arcuate kisspeptin neurons express c-Fos during the proestrous surge. Thus, arcuate nucleus kisspeptin neurons are posited to underlie the tonic secretion of GnRH in males and females, while AVPV nucleus kisspeptin neurons may control the proestrous surge in females [115, 116]. Kisspeptin is the most upstream

regulator of the HPG axis known to date, and we hypothesize that the stress-induced elevation in glucocorticoids during stress regulates kisspeptin neurons (**Figure 1**).

1.2.3 Stress-induced inhibition of reproductive function

Environmental factors like food availability, predator population, photoperiod, and mate availability cause powerful reproductive changes, and even modify genetically programmed behaviors such as the timing of puberty or ovulation. In female rodents, the presence of male pheromones accelerated puberty and promoted lordosis behavior [117]. In males, the presence of female pheromones led to increased testosterone, LH, and copulatory behaviors [117]. When housed only with other males, male mice delayed pubertal onset. Mated females spontaneously aborted pregnancies when novel males were introduced.

In humans, delayed puberty was observed in elite runners, ballet dancers, gymnasts, and girls with anorexia nervosa, who had elevated serum cortisol [118-122]. Even in healthy girls, elevated glucocorticoid levels in the high-normal range correlated with delayed puberty [123]. A study of rugby players found that cortisol rose and testosterone fell during exercise and both returned to normal after five days [124].

Corticosterone pellets implanted in neonatal rats at P3, 6, 12, or 18 caused females to exhibit decreased lordosis behavior, prolonged estrous cyclicity, and decreased insemination by males [125, 126]. Neonatal treatment with ACTH and hypothalamic treatment with cortisol produced decreased sexual behaviors in adulthood [125, 126]. Treatment with a GR antagonist [127] and adrenalectomy [128] prevented stress-induced decreases in plasma LH.

Following immobilization stress, intratesticular and serum testosterone levels and cAMP content in Leydig cells fell more quickly than plasma LH levels do [129]. Inhibition of testosterone by corticosterone was due to increased apoptosis of Leydig cells [130]. Immobilization stress produced decreased plasma testosterone concentrations, and this effect was partially blocked by pre-treatment with the glucocorticoid receptor antagonist, RU486 [131]. Glucocorticoids act directly on GRs in testicular interstitial cells to suppress the testicular response to gonadotropins *in vitro* [131].

CRH administration inhibited the secretion of GnRH [132] and synthesis of LH [133]. β-endorphin inhibited GnRH secretion, and central CRH can regulate arcuate nucleus β-endorphin release [132]. CRH neurons indirectly regulate the HPG axis via downstream glucocorticoid production and directly regulate the HPG axis via synapses on GnRH neurons [132]. Stress-induced reproductive inhibition by undernutrition was partially reversed by the administration of the CRH antagonist, astressin B [134].

Throughout life, animals are confronted by stressors that inhibit the reproductive axis and must overcome this inhibition in order to reproduce subsequently. The ability to adapt after acute stress promotes subsequent reproductive success, while dysregulation of the response can cause maladaptive changes, including long-term infertility. Major depression, post-traumatic stress disorder (PTSD), and anorexia nervosa are all characterized by increased cortisol secretion, dysregulated HPA axis feedback, and suppression of fertility [132]. Reproductive inhibition during stress is not mediated by the HPA axis alone. Glucocorticoids are not the primary mediator of cold stress, for example, in which norepinephrine is thought to mediate adaptive

thermoregulatory changes [135]. Restraint stress caused suppression of LH in rats, which was ameliorated by lesions in the medial amygdala [136]. The relationship between stress and reproduction is not unidirectional. Testosterone can affect basal and stress-dependent HPA function, and several stress responses rely on the sex hormone milieu and are absent in ovarectomized rats [133].

1.2.4 Sex steroid-dependent reproductive behaviors

Males and females of many species display strikingly different behavioral repertoires, especially in reproduction. Many areas of the brain exhibit sexually dimorphic gene expression [137-141]. Sex steroids, genetic programming, and social experience all contribute to the coordination of these dimorphisms. Arnold Berthold was one of the earliest proponents of the hypothesis that sex steroid hormones organize and activate male- versus female-specific sexual behaviors. Using roosters, he demonstrated that castrated juvenile males exhibited decreased mating, aggression, and crowing as adults. The effects of castration were reversed if the testes of another male were implanted in the body cavity of the juvenile [117]. Female guinea pigs exposed to perinatal androgens displayed increased male copulatory behaviors and failed to develop female sexual behaviors [142].

The presence or absence of the Y chromosome determines gonadal sex in mammals. Sex-specific gonadal differentiation is determined by the *SRY* gene on the Y chromosome, which directs undifferentiated gonads to form testes. In mice, the sex of the gonad is specified by E14.5, and the testes begin to secrete testosterone during the remaining days of gestation [143, 144]. Sex-specific traits in other tissues are then determined by sex steroids produced by the

developing gonad. The rise in testosterone is responsible for the sexual differentiation of external genitalia in most mammals, and removing undifferentiated gonads of genetically male rabbit embryos resulted in the birth of female offspring [145]. By contrast, replacing the undifferentiated gonads with a testis in either genetically male or female embryos resulted in the birth of offspring with male genitalia [145]. Investigation of SRY-independent development was made possible by the generation of transgenic mice in which the sex of the brain is independent of the gonadal sex. Animals that lacked the *Sry* gene on the Y chromosome were gonadally female but genetically male (XY). These animals exhibited different mating and sniffing behaviors compared to genetically XY males that also had testes [146].

Gonadectomy and sex steroid replacement in development and adulthood has been used to determine the role of hormones in the development of sex-specific behaviors. Castrated male rat pups treated with testosterone displayed masculine behavior as adults only if testosterone was given during the first four days of life [147]. By contrast, males given testosterone after this critical period failed to show male sexual behavior as adults. Female pups treated with either testosterone or estradiol exhibited masculine adult behavior [143]. Castrated male pups treated with estradiol showed partial restoration of normal male sexual behaviors [143]. Testosterone is aromatized to estradiol during fetal development [148], and fetal estradiol is thought to be responsible for sexual differentiation in the brain. Both fetal males and females have aromatase expression in the preoptic area, a sexually dimorphic nucleus. Concentrations of aromatase peaked with the critical period of sexual differentiation, and males had higher aromatase activity than females [149, 150]. Estrogen exerts its effects through estrogen receptors, the manipulation of which affects both male and females. Estrogen receptor α (ERα) knockout males and females

were infertile, and females exhibited no lordosis [143, 151], and males had decreased mounting behavior, no ejaculation, and decreased preference for females in estrous [143].

Male sexual behavior is thought to be dependent on central actions of androgens acting through the estrogen receptors, whereas peripheral androgens exert effects through androgen receptors [143]. In castrated males, non-aromatizable androgens used in conjunction with estradiol were more effective at restoring male-specific sexual behaviors than administration of estradiol alone [152]. Central administration of an androgen receptor antagonist inhibited the restoration of male copulatory behaviors after castration and testosterone replacement [153]. Castrated males lost the preference for females in estrous, and testosterone replacement restored this preference [154]. Not only are male-specific copulatory behaviors restored in males treated with either estradiol or testosterone, ovariectomzied females that are treated with testosterone exhibited male-specific mounting and thrusting behaviors [143, 155].

The medial preoptic nucleus is a site of sexually dimorphic kisspeptin expression. In rodents, the medial preoptic nucleus is larger in adult males than in females, and progesterone receptor expression here is higher in males than in females, which may mediate the dimorphic development of the nucleus [137, 138]. Aromatization of testosterone into estradiol is critical for differential progesterone receptor expression [138], and progesterone receptor signaling is critical to the size of the adult preoptic nucleus [137]. Progesterone receptor sexual differentiation is controlled by gonadal and not genetic sex [140]. The Bax protein is required for cell death in developing neurons, a sexually dimorphic process, and Bax knockout mice did not exhibit sex differences in the normally dimorphic bed nucleus of the stria terminalis and

preoptic area [141] Deletion of Bax did not diminish sex differences in kisspeptin expression in the AVPV, though arcuate kisspeptin expression was significantly increased in these mice [156]. Bax knockout males exhibited normal aggression in resident/intruder tests, but did not exhibit the same preference for female-soiled bedding as wild-type males [157]. Together, these results demonstrate the importance of sex steroids in governing complex sexual behaviors in juveniles and adults of both sexes.

1.3 LEPTIN

Leptin is a 167-amino acid adipokine with a four-helix bundle motif similar to that of cytokines [158] and is produced by adipocytes in proportion to adipose mass [159]. Circulating leptin also fluctuates acutely with caloric intake and short-term fasting [160, 161]. Leptin binds at least six isoforms of the leptin receptor (ObR): ObRa, ObRb, ObRc, ObRd, ObRe, and ObRf. These isoforms have homologous extracellular but different intracellular domains due to alternative splicing [162]. The long isoform, *ObRb*, is primarily responsible for leptin signaling, while the short isoforms, *ObRa* and *ObRc*, are thought to function in the transport of leptin across the blood-brain barrier [163]. Leptin binding activates the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signal transduction pathway. Activation of STAT3 stimulates *POMC* mRNA transcription in the arcuate nucleus of the hypothalamus. Disruption of STAT3 signaling via the intracellular tyrosine residue Tyr1138 of ObRb caused hyperphagia and obesity, but not infertility [164].

Leptin conveys peripheral information about fat stores to central regulators of metabolism in the hypothalamus. The hormone targets subsets of neurons in the arcuate nucleus of the

hypothalamus that produce POMC, neuropeptide Y (NPY), and agouti related-peptide (AGRP). When POMC neurons are activated by leptin, *POMC* is transcribed and cleaved into α-melanocyte stimulating hormone (α-MSH), an anorexigenic hormone. NPY/AGRP neuron activation promotes orexigenic behavior [165]. Both POMC and NPY/AGRP neurons project to the PVN and target melanocortin 4 receptors (MC4R) , but the peptides α-MSH and AGRP have antagonistic effects [166, 167]: α-MSH stimulates while AGRP is an inverse agonist of MC4Rs. MC4R is a G-protein coupled receptor that mediates anorexigenic effects [168]. The MC4R knockout was severely overweight [169], and conditional activation of MC4R in the PVN caused decreased food intake and body weight [170].

1.3.1 Leptin deficiency

Naturally-occurring murine mutations in the leptin gene ($Lep^{Ob/Ob}$) and leptin receptor gene ($LepR^{Db/Db}$) cause obesity and infertility [171, 172]. In 1949, researchers at The Jackson Laboratories in Bar Harbor, Maine, described a naturally-occurring strain of obese mice that was dubbed "ob," short for obese. Two theories were postulated to explain the morbid obesity. First, it was possible that these obese animals had an extra hormone compared to normal mice. The extra hormone would cause the obese animal to increase its food intake and gain weight. By contrast, it was possible the obese animals lacked a hormone that normal mice produced. This hormone would normally inform a mouse to stop eating when it had had enough. In the 1950s and 1960s, researchers used a technique called parabiosis to conjoin pairs of animals, and when *ob/ob* mice were conjoined with WT mice, they began to eat less and eventually became lean [173-175]. Scientists concluded the mystery mutation impeded the production of a "stop eating" hormone in obese mice, later identified by positional cloning as leptin [171].

Human leptin mutations were first identified in a large, consanguineous Pakistani family [176]. One child was 190 pounds at the age of eight and had undergone liposuction at the age of seven, while her cousin was 63 pounds at the age of two. The eight-year old patient received one injection of leptin every morning for a year at a dose that would equal 10% the blood leptin levels of a normal, healthy child [177]. The first meal after treatment was 42% less than the patient's usual food intake, and she went on to lose nearly five pounds of fat each month [177].

Leptin deficiency was not only associated with morbid obesity but with infertility and hypercortisolemia as well. Leptin treatment restored fertility in $Lep^{Ob/Ob}$ mice and elicited advanced puberty in WT female mice [178]. As a result, leptin was widely considered to be required for fertility. Leptin stimulated LH secretion *in vitro* and *in vivo,* but GnRH neurons did not express *ObRb* [179-182]. Some studies implicated preoptic area neurons that send afferent projections to GnRH neurons as mediators of leptin-induced activation of the reproductive axis [183]. Strain differences can also be important in the regulation of fertility, and leptin-deficient BALB/cJ mice were more fertile than leptin-deficient C57BL/6J mice [184]. Lack of leptin action causes a failure to stimulate anorexigenic α-MSH and suppress orexigenic AGRP/NPY, and $Lep^{Ob/Ob}$ animals are understandably obese. But why are they infertile?

1.3.2 Fertility

Food availability is a dramatic regulator of fertility. When food is plentiful, both parents and offspring have better chances of surviving the pregnancy. When food is scarce and the energetically costly pregnancy is unlikely to result in healthy offspring, however, females inhibit reproductive function. Anorexic women classically become amenorrheic, and pubertal delay is seen in children who undergo severe stress. Adult female mice develop abnormal estrous cycling and juveniles delay vaginal opening after severe stress. Thus energy metabolism is a potent regulator of reproduction, yet the means by which energy balance is communicated to the HPG axis remains unknown.

For many years, investigators have tried to identify mechanistic connections between energy metabolism and reproduction. Leptin is a crucial signal of full energy stores, and many have correlated decreased leptin with decreased fertility. Despite the insights that have been achieved in leptin action and HPG signaling independently, no direct molecular pathways have been implicated to connect starvation and infertility. Several groups have hypothesized a direct interaction between kisspeptin and leptin. Food deprivation induced a decrease in rat hypothalamic *Kiss1* mRNA transcripts [185]. Exogenous kisspeptin administration did not affect food intake after food deprivation but did elicit vaginal opening in females despite undernutrition [185]. Others hypothesized that leptin receptors were present in kisspeptin neurons based on evidence that fasting inhibited the HPG axis along with kisspeptin expression and that both $Lep^{Ob/Ob}$- and fasting-induced inhibition of gonadotropin secretion were rescued by administration of kisspeptin [186]. Smith et al. reported coexpression of *Kiss1* and *ObR* mRNAs in 40% of neurons in the arcuate nucleus of the hypothalamus by double-label *in situ*

hybridization [186], but so far no other groups have repeated this finding. Additionally, these experiments were conducted in tissues collected from gonadectomized animals, in which arcuate nucleus *Kiss1* mRNA was significantly upregulated, possibly in cells that would not express kisspeptin under intact conditions. Decreased *Kiss1* mRNA expression in these $Lep^{Ob/Ob}$ mice was reported to be reversed by leptin infusion [186]. Selective deletion of leptin receptors in kisspeptin neurons had no effect on fertility or body weight, and it was reported that only 4% of kisspeptin neurons in non-gonadectomized conditions expressed leptin receptor. It is likely that interactions between energy metabolism and reproduction are more complex than direct *ObR* expression in kisspeptin neurons.

Using $Lep^{Ob/Ob}$ and diet-induced obese animals, others have found no changes in hypothalamic *Kiss1* mRNA expression; leptin infusion in $Lep^{Ob/Ob}$ mice, however, induced an increase in *Kiss1* mRNA levels [187]. One group reported that *Kiss1* mRNA was present in adipose tissue [188]. Given its potent role in stimulating the HPG axis, kisspeptin is still being explored as a potential candidate for translating nutritional status to reproductive function. But is food deprivation simple a metabolic cue, or does it represent stress in general? The $Lep^{Ob/Ob}$ mouse is not only obese and infertile but hypercortisolemic as well. Could increased glucocorticoid secretion cause infertility or obesity in $Lep^{Ob/Ob}$ mice?

1.3.3 Hypercortisolemia

Cushing's syndrome

Reproductive disturbances, including menstrual cycle abnormalities and loss of libido, often occur in patients with Cushing's syndrome, a disease characterized by elevated secretion of

cortisol. Men with Cushing's syndrome due to adrenal hyperplasia or adenoma exhibit low plasma testosterone, and a majority of these men complain of impotence or loss of libido [189]. Adrenalectomy restores the decreased plasma testosterone levels to normal [189]. Men with Cushing's syndrome have subnormal testosterone production rates, concomitant with endogenous hypercortisolism [190]. No differences in testosterone production are observed in women with Cushing's syndrome, suggesting that gonadal testosterone production is suppressed by glucocorticoids [190]. Patients with Cushing's disease also have increased risk of developing major depressive disorder, which may be related to elevated plasma cortisol levels [191, 192]. Chronic hypercortisolemia in Cushing's syndrome does not appear to directly affect plasma leptin levels [193].

Type 2 diabetes

Plasma cortisol is increased in patients with type 2 diabetes [194, 195]. Hypogonadotropic hypogonadism is common in type 2 diabetes, and LH, FSH, and testosterone are lower in male patients [196]. Testosterone correlates negatively with BMI, though not with cortisol [197], and several studies have found that higher baseline levels of leptin are correlated with increased risk of type 2 diabetes [198, 199].

Anorexia nervosa

One diagnostic criterion of anorexia nervosa, a psychiatric illness characterized by severe self-imposed malnutrition, is amenorrhea, or the cessation of menstruation. Amenorrhea in anorexia nervosa may be a protective adaptation that prevents pregnancy in times of insufficient nutrition. Patients with anorexia nervosa maximize the stress response to chronic starvation and exhibit

high levels of glucocorticoids [200]. In its maladaptive form, anorexia-induced infertility causes long-term infertility even after weight restoration, although most patients do regain fertility [201].

Elite athletes

Hypogonadotropic hypogonadism is common in male and female elite athletes, and cortisol is significantly elevated in these athletes during exercise [118]. Both LH pulse frequency and amplitude are suppressed, and the LH response to GnRH is decreased in these athletes [118]. 19% of female Olympic marathon runners are amenorrheic, and these amenorrheic runners have higher basal serum cortisol compared to eumenorrheic marathon runners [202]. Menstrual frequency in atheletes is negatively correlated with glucocorticoid levels [200]. Cortisol is increased and testosterone decreased immediately after running a marathon in men, and while cortisol returns to baseline after 24 h, testosterone only partial recovers after 24 h [203]. In non-elite athletes monitored during the Athens marathon, serum cortisol was increased and testosterone decreased 1 h after the race, and both returned to baseline 1 week thereafter [204]. Ultra-marathon runners monitored at the start of a race, 33 km, 75 km, and after completion of 110 km had increased cortisol and β-endorphin levels, while testosterone and LH decreased throughout the race [205, 206]. Both chronic and acute increases in cortisol appear to suppress reproductive axis function. Elite marathoners also exhibit very low levels of serum leptin [207].

Post-traumatic stress disorder

PTSD affects 70% of prisoner of war survivors and 30% of combat veterans, and nearly 70-80% of these individuals report impaired libido [208]. In a study of Operation Iraqi Freedom veterans

with PTSD, 39 of 53 patients reported diminished libido; 26 reported erectile dysfunction; and 8 reported ejaculatory dysfunction [209]. Elite soldiers participating in psychologically stressful exercises have lower testosterone levels immediately after training [210]. British and Australian veterans of the first Gulf war have increased risk of infertility and longer time to conception [211], and individuals with PTSD have lower levels of testosterone compared to healthy controls [212]. The mechanisms by which acute stress can cause these consequences long after the initial stressor has subsided are not well-understood.

In patient populations, it is difficult to isolate cortisol as a causal rather than correlative feature in these disorders. Many of the diseases associated with dysregulated cortisol secretion have complex, interrelated symptoms and etiology. Cushing's syndrome is associated with increased obesity; type 2 diabetes is associated with metabolic syndrome; endurance athletes have extraordinarily low fat mass; and anorexia nervosa is a psychiatric disorder characterized by extreme weight loss. It is clear that elevated cortisol, whether associated with weight gain or loss, correlates with suppressed reproductive function. Using genetically engineered mouse models to disrupt glucocorticoid secretion or signaling, we aimed to study the contribution of glucocorticoids to these disorders, specifically the role of glucocorticoids in the regulation of kisspeptin neurons during stress and infertility in normal mice, and of infertility and obesity in leptin-deficient mice.

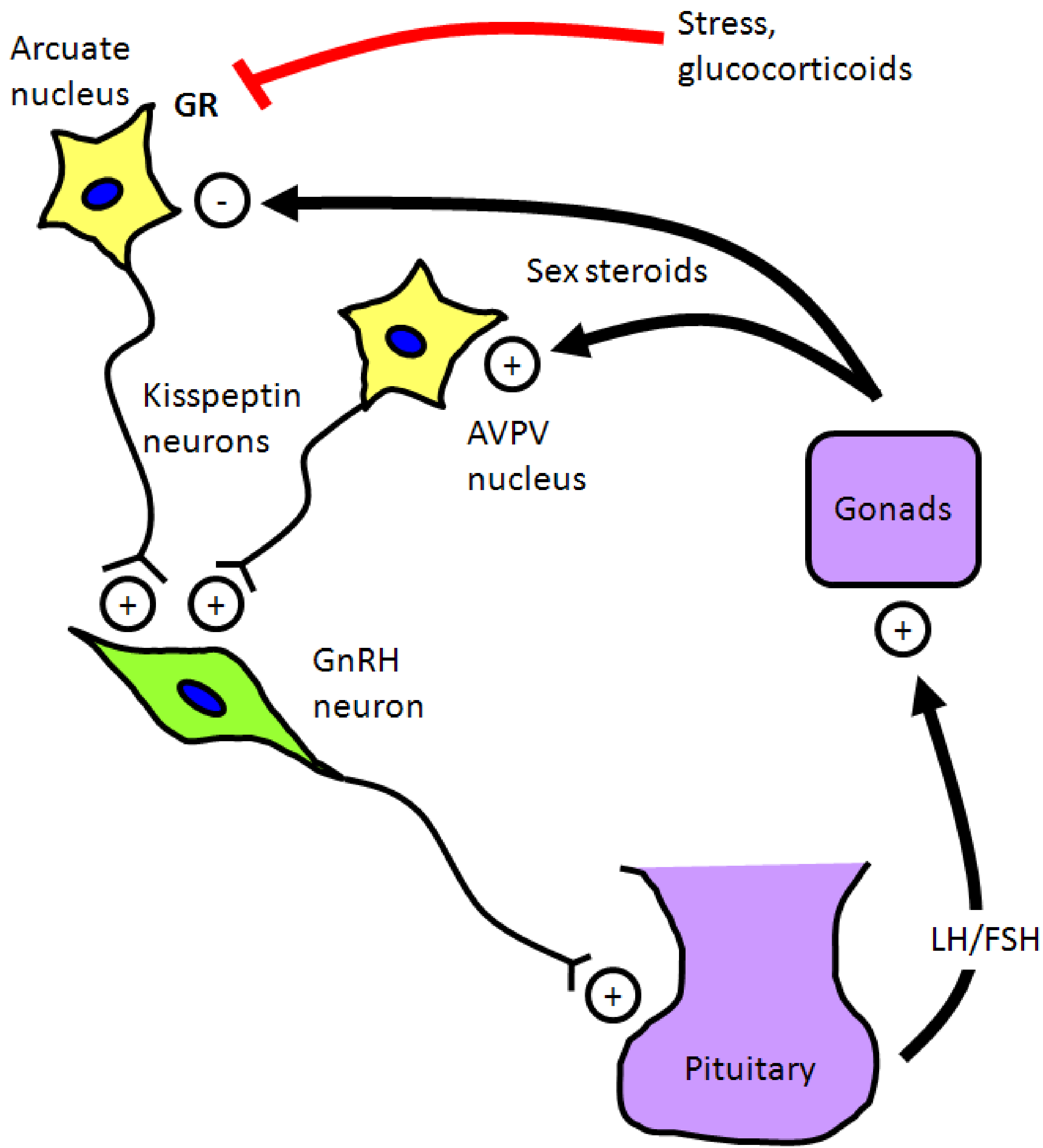


Figure 1
In the hypothalamus, kisspeptin is produced in the anteroventral periventricular (AVPV) and arcuate nuclei. Kisspeptin stimulates gonadotropin-releasing hormone (GnRH) release, which causes the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pituitary. Circulating LH and FSH stimulate the release of sex steroids from the gonads. Sex steroids feed back to inhibit kisspeptin neurons in the arcuate nucleus and to stimulate kisspeptin neurons in the AVPV.

CHAPTER 2

STRESS-INDUCED GLUCOCORTICOID RECEPTOR SIGNALING REGULATES KISSPEPTIN NEURONS

This chapter is based on:

Oulu Wang, Anne Lanjuin, Juliana Basko-Plluska, Louis Muglia, Catherine Dulac, and Joseph Majzoub. Disruption of glucocorticoid receptor signaling in kisspeptin neurons accelerates the recovery of reproductive function in the post-traumatic stress period. *In preparation*, 2012.

Drs. Lanjuin and Dulac generated the *Kiss1*^{CreBAC} transgenic mouse. Dr. Basko-Plluska investigated the use of restraint as a psychological stressor. Dr. Muglia generated the *GR*$^{flox/flox}$ mouse.

2.1 Abstract

Stressors generate adaptive responses to facilitate the return to homeostasis. Dysregulation of this process can cause maladaptive responses, including cessation of reproductive function that persists long after the stressor has subsided, through mechanisms that are not well understood. Kisspeptin (KISS1) is required for the activation of the hypothalamic-pituitary-gonadal reproductive axis in humans and mice. We hypothesized that acute stress in mice, acting through the stress hormone, corticosterone, transiently inhibits kisspeptin neurons and downstream reproductive capacity, and that the restoration of kisspeptin signaling is necessary for normal reactivation of the reproductive axis. We examined the response of hypothalamic *Kiss1* mRNA expression and hormones of the reproductive and stress axes to different stressors. Stressors that stimulated glucocorticoid secretion, as well as glucocorticoid administration itself, inhibited *Kiss1* mRNA expression, while conditions that did not change glucocorticoid secretion did not alter *Kiss1* mRNA expression. In mice lacking glucocorticoid receptor specifically in kisspeptin-containing neurons, *Kiss1* mRNA expression was no longer inhibited during restraint stress despite a rise in corticosterone, and both testosterone and copulatory behaviors showed accelerated recovery in the post-traumatic stress period. Blockade of glucocorticoid receptor signaling in kisspeptin neurons during stress accelerates the recovery of reproductive function during the post-traumatic stress period, a finding that may have therapeutic implications in humans with post-traumatic stress disorders.

2.2 INTRODUCTION

Stress responses to acute stressors improve the chances of immediate survival, even at the expense of immediate reproductive fitness [213]. Stressors can generate adaptive stress responses that prepare for the return to homeostasis, but also maladaptive responses, including the cessation of reproductive function long after the stressor has subsided. In patients with post-traumatic stress disorder (PTSD), reproductive inhibition can persist long after the initial trauma [208, 209]. The mechanisms by which acute stress can result in these maladaptive, post-traumatic consequences are not well understood.

In 2003, two independent groups discovered hypogonadotropic individuals with kisspeptin receptor mutations in large, consanguineous families [83, 84], and in humans and mouse models, loss-of-function mutations in either the kisspeptin receptor or ligand block the onset of puberty [83, 85, 93-95]. Conversely, gain-of-function mutations in the human receptor result in precocious puberty [86], and administration of kisspeptin accelerates the onset of puberty in juvenile rats [214]. Kisspeptin-secreting neurons in the arcuate and anteroventral periventricular nuclei of the rodent hypothalamus stimulate gonadotropin-releasing hormone neurons, which promote the release of luteinizing hormone (LH) and follicle-stimulating hormone in the anterior pituitary [96, 100, 106, 215]. These gonadotropins stimulate the production of testosterone in males and estrogen in females. Acute diphtheria toxin-mediated ablation of kisspeptin neurons in adult mice inhibits fertility, indicating that kisspeptin neurons continue to regulate reproduction in adults [216].

The impact of acute disruption of kisspeptin activity in adulthood may have a physiologic correlate in the acute stress response. We hypothesized that acute stress transiently inhibits kisspeptin neurons and the downstream hypothalamic-pituitary-gonadal (HPG) axis, and that reactivation of kisspeptin signaling is necessary to reactivate the HPG axis, much as kisspeptin is needed to turn on the HPG axis during puberty. Because activation of the adrenal axis is an important part of the mammalian stress response, and adrenal steroids are known to inhibit the HPG axis [125, 129, 189, 190, 217], we further proposed that this stress-induced inhibition of kisspeptin expression was caused by the concomitant elevation of glucocorticoids, with their subsequent fall after stress restoring kisspeptin expression and reproductive function. To test our hypothesis, we examined the response of kisspeptin expression, plasma corticosterone, and the HPG axis to different stressors in mice, and we generated mice lacking glucocorticoid receptor (GR) specifically in kisspeptin-containing neurons.

2.3 MATERIALS AND METHODS

Animals and tissue preparation

All experiments were conducted in compliance with the Institutional Animal Care and Use Committee guidelines of Children's Hospital Boston. Adult C57BL/6J male mice were purchased from The Jackson Laboratory (Bar Harbor, ME; 000664) and tested at 10-14 weeks of age. All experiments concluded between 2-4PM. Animals were maintained on a 12 h light/dark cycle with access to chow and water *ad libitum* and tested between 10-14 weeks of age. Before experiments, animals were transferred to a quiet procedure room and allowed to acclimate for 7 d. Retroorbital blood samples were collected from unanesthetized animals within 1 min of cage handling in all conditions and centrifuged at 3,000 rpm for 10 min at 4°C. Retroorbital phlebotomy was used to collect sufficient volumes of blood in non-terminal experiments. Animals were sacrificed by rapid decapitation without anesthesia, and dissected brains were embedded in OCT and stored at -80°C. Brains were sectioned coronally at 20 µm from the anteroventral periventricular nucleus to mammillary bodies (**Figure 2.1**) in four sets, thaw-mounted onto 25 mm x75 mm slides, and returned to -80°C until further processing.

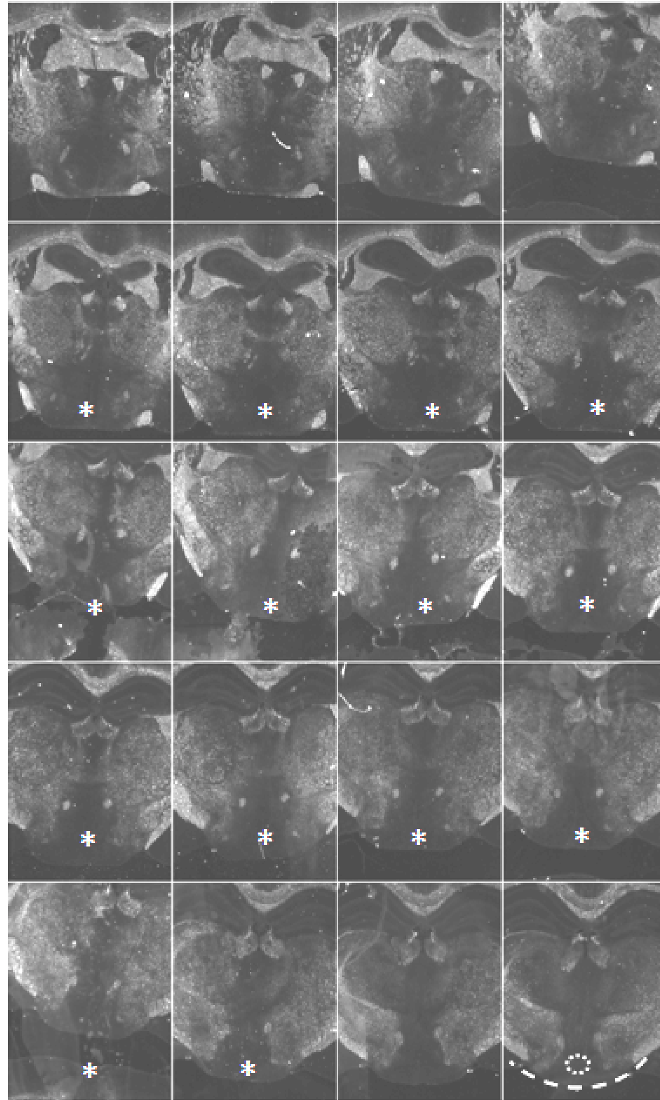


Figure 2.1
Anatomical landmarks of the arcuate nucleus by darkfield microscopy (no staining for kisspeptin was performed). Asterisks denote sections that were collected for analyses of kisspeptin expression, beginning with sections in which the optic chiasm and subsequent bifurcation were visible and ending with sections in which the mammillary bodies were visible.

Luteinizing hormone was assayed by the UVA Center for Research in Reproduction Ligand Assay and Analysis Core. Plasma corticosterone and testosterone were measured by radioimmunoassay (MP Biomedicals) with the following modifications: to minimize the amount of plasma used in the corticosterone radioimmunoassay, we generated 1:200 dilutions using either 1 μL of plasma with 199 μL of steroid diluent or 5 μL of plasma with 995 μL of steroid diluent. In general, aliquots containing 1 μL were more variable in corticosterone concentrations, likely due to pipetting error, and unless blood volume was a major constraint, we used 5 μL of plasma for corticosterone assays. The dynamic range for the corticosterone assay was 25 ng/mL to 1,000 ng/mL. To minimize the amount of plasma used in the testosterone radioimmunoassay, we used 25 μL of plasma in singlet. In pilots, this volume was as effective in identifying stress-induced testosterone suppression as 50 μL of plasma in duplicate. The dynamic range for this assay was 0.1 ng/mL to 10 ng/mL.

Transgenic mice and breeding strategy

To study the effects of GR deletion in kisspeptin neurons, we generated $Kiss1^{CreBAC}::GR^{flox/flox}$ mice as well as $Kiss1^{CreBAC}$, $GR^{flox/flox}$, and WT controls. $GR^{flox/flox}$ animals were previously validated and generously provided by Louis Muglia [30]. Briefly, loxP sites were targeted upstream of exon 1C and in intron 2 of the GR gene, *Nr3c1*. Exon 2 contains the ATG initiation site, and the exon and start sequence are excised by Cre-mediated recombination. $GR^{flox/flox}$ animals were on a C57B background. To genetically target kisspeptin neurons, Drs. Lanjuin and Dulac generated a $Kiss1^{CreBAC}$ mouse line using a BAC transgenic approach [218]. Briefly, Cre cDNA sequences including a bGH polyA tail were recombined after the *Kiss1* translational start ATG on BAC RP23-240P23. The modified BAC was confirmed to be free of gross

rearrangements, linearized with Not1 to release a 102kb fragment containing the modified *Kiss1*^{CreBAC} locus (including 65kb of upstream sequences), and injected into B6/CBA oocytes. Only one of six founder lines that we obtained showed expression in accordance with reported sites of endogenous *Kiss1* expression by double-label *in situ* hybridization. *Kiss1*^{CreBAC} mice were on a C57BL/CBA mixed background.

All animals used in experiments were males on a C57BL/CBA mixed background. Crosses from *Kiss1*^{CreBAC}*::GR*$^{flox/+}$ x *GR*$^{flox/+}$ breeders yielded *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$, *Kiss1*^{CreBAC}, *GR*$^{flox/flox}$ and WT male littermates at frequencies of 6.25% each. Because these yields were insufficient to power our study, we crossed F1 littermates, *Kiss1*^{CreBAC} x WT, and *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$ x *GR*$^{flox/flox}$, to produce all the F2 littermates used in this study. Thus, in the F2 generation, *Kiss1*^{CreBAC} and WT mice were littermates, *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$ and *GR*$^{flox/flox}$ mice were littermates, and all four genotypes from the F2 generation were related, because all F1 breeders were littermates.

Stressors

Restraint causes minimal physical harm and is considered a psychological stressor [219]. Animals were placed in ventilated restraint tubes for 5 h. For food deprivation experiments, animals were placed in cages without food for 48 h, but had access to bedding and *ad libitum* water. For cold exposure experiments, animals were housed at 4°C for 24 h with bedding, food, and water. For intraperitoneal corticosterone injections, corticosterone (Sigma C2505, St. Louis, MO) was administered at 40 mg/kg body weight, and following decapitation, tissues were collected 5 h post-injection for the detection of mRNA changes in the hypothalamus. In restraint

experiments, 14 male mice were tested per treatment (restrained or unrestrained). In food deprivation experiments, 12 male mice were tested per treatment (food-deprived or fed). In cold exposure experiments, 6 male mice were tested per treatment (housed at 4ºC or room temperature). In i.p. corticosterone experiments, 7 male mice were tested per treatment (corticosterone or saline).

In situ hybridization

The *Kiss1* mRNA probe was generously provided by Robert Steiner [100]. Sense and antisense *Kiss1* probes spanning bases 76-486 of the murine *Kiss1* gene were generated from a linearized pAMP1 plasmid containing *Kiss1*, SP6- and T7-binding sequences. Radiolabeled probes were synthesized using ^{33}P-UTP, and *in situ* hybridization was performed as previously described [100], with the following modifications. Briefly, tissues were washed in 4% paraformaldehyde, acetic anhydride, 2X SSC, chloroform, and graded ethanols, then incubated in 12.7 million dpm/mL probe for 16 h at 55°C. Slides were subsequently washed with 4X SSC, RNase, 2X SSC, 0.5X SSC at 62°C, and graded ethanols in ammonium acetate, then dipped in autoradiographic silver emulsion (Kodak NTB 8895666). Silver grains in the hypothalamus were visualized by darkfield microscopy on the 10X objective of a Nikon Eclipse E800. Using NIH ImageJ software (http://imagej.nih.gov/ij/), cells with ten-fold higher silver than background were identified as *Kiss1* mRNA-positive by a blinded observer using a semi-automated program (**Figure 2.2**).

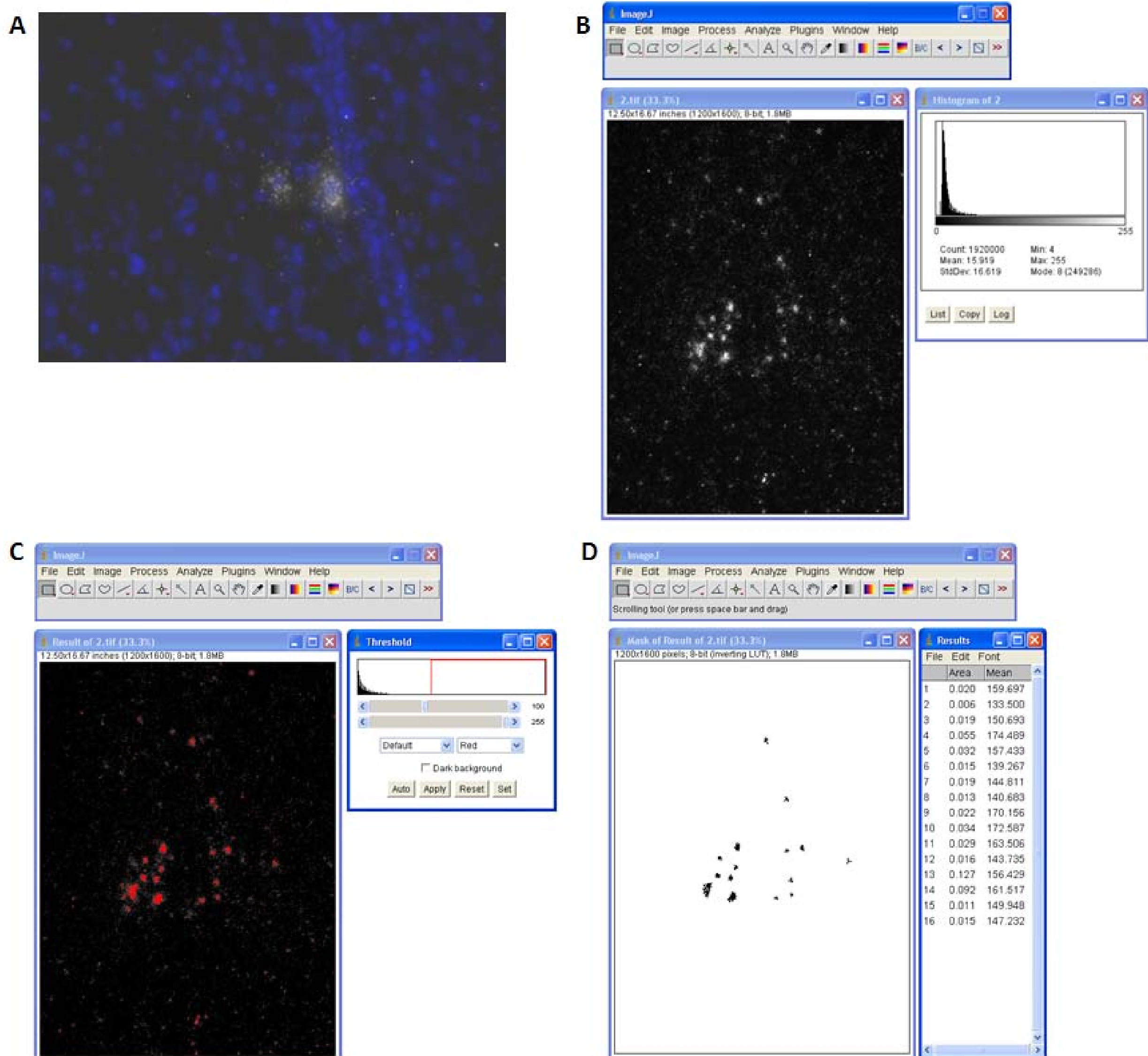


Figure 2.2
(A) *Kiss1* mRNA-positive silver grains were visualized by darkfield microscopy on a Nikon Eclipse E800. Using NIH ImageJ software (http://imagej.nih.gov/ij/), we coded a semi-automated program to identify cells with ten-fold higher silver than background as *Kiss1* mRNA-positive. **(B)** Background grayscale intensity from an area of the brain lacking silver cells was calculated. **(C)** The background mean was subtracted from the original image, and binary thresholding was performed. **(D)** Cells with intensity values above threshold, size larger than background noise particles, and corrected for empirically-determined single cell size were identified.

Immunohistochemical analyses

Brains from *Kiss1*^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$ animals were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and frozen in OCT. 10 µm coronal brain sections through the anteroventral periventricular and arcuate nuclei of the hypothalamus were stained using kisspeptin antiserum at a dilution of 1:5,000 for 7 d at 4ºC. *Kiss1*^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$ and *Kiss1*^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$*::GR*$^{flox/flox}$ brain sections treated in the same manner were stained with rabbit antiserum for mouse/rat glucocorticoid receptor (Santa Cruz 1004) at a dilution of 1:2,000 overnight at 4ºC. We validated the antiserum for glucocorticoid receptor by using a previously validated Cre construct to generate *Sim1*^{Cre}*::GR*$^{flox/flox}$ mice, in which GR is selectively deleted in the paraventricular nucleus. GR staining was robust in the PVN of WT mice and absent in the PVN of *Sim1*^{Cre}*::GR*$^{flox/flox}$ mice, demonstrating that the rabbit antiserum is specific to GR (**Figure 2.3**). Rabbit antiserum for mouse/rat/ovine kisspeptin was previously validated and generously provided by Alain Caraty [110]. In our experiments, kisspeptin staining was only detected in the arcuate nucleus, AVPV, and medial amygdala. Images were captured using Nikon Eclipse E800, Zeiss LSM700 laser scanning, and Perkin Elmer Ultra*VIEW* VoX spinning disk microscopes. Colocalization was analyzed by calculating the overlap coefficient for each hypothalamic slice using NIH ImageJ software [220].

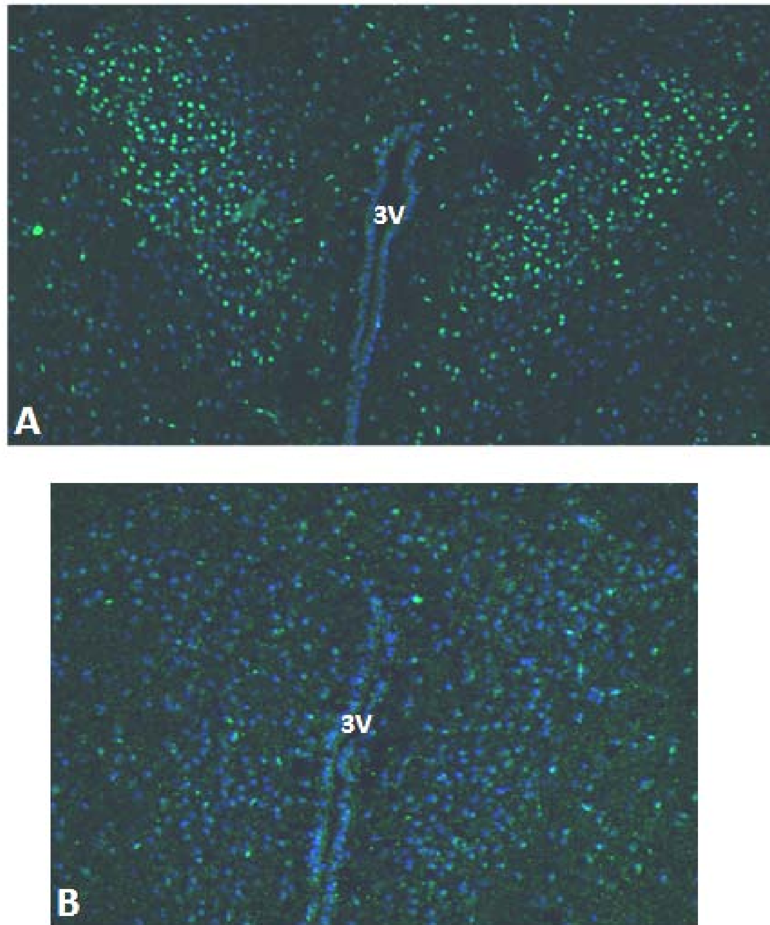


Figure 2.3
(A) Staining for glucocorticoid receptor expression in the paraventricular nucleus of the hypothalamus using rabbit antiserum for mouse/rat glucocorticoid receptor (Santa Cruz 1004). GR staining was robust in the PVN of WT mice. **(B)** We used a previously validated Cre construct to generate $Sim1^{Cre}::GR^{flox/flox}$ mice, in which GR is selectively deleted in the PVN. GR staining was absent in the PVN of $Sim1^{Cre}::GR^{flox/flox}$ mice, demonstrating that the rabbit antiserum is specific to GR.

Behavioral analyses

To examine copulatory behavior, each male was paired with one female with previously proven fertility. Behavior was filmed using a Kodak Zx3 HD camera for 5 min starting at the time the female was introduced into the home cage of the male. The amount of time the male spent sniffing the female and the incidence of copulatory behaviors were analyzed at 30 frames/s using VideoPad® software by a blinded observer. Open-field testing was filmed in a 45 cm x 45 cm arena before stress, immediately after 5 h of restraint, and after 1 week of recovery. Each animal was monitored for 5 min, and movement was tracked and analyzed using the Noldus EthoVision® XT system.

Statistical analysis

Two-tailed two-sample t-tests were performed to compare hormone (corticosterone, LH, testosterone) and *Kiss1* mRNA expression levels between WT unstressed and restrained, food-deprived, cold-exposed, or corticosterone-treated males. Linear mixed model analyses were performed to compare changes in corticosterone, *Kiss1* mRNA expression, and testosterone over time. Linear mixed model analyses were performed and marginal means calculated for sniffing, mounting, thrusting, locomotion, and open-field behaviors over time. Post-restraint, each of these parameters was compared across genotypes by using one-way ANOVA with *post hoc* least square difference (LSD) pairwise tests.

Genotyping

A list of genotyping primers and polymerase chain reaction protocols are detailed here for the mouse strains used in these experiments: $Kiss1^{CreBAC}$, $GR^{flox/flox}$, tdTomato, mTomato/mGFP.

Kiss1^{CreBAC} [221]

Cre forward primer 5' - CGT ACT GAC GGT GGG AGA AT - 3'

Cre reverse primer 5' - TGC ATG ATC TCC GGT ATT - 3'

94°C for 2’, (94°C for 30”, 55°C for 30”, 72°C for 1’) x 30, 72°C for 5’, 4°C

Cre band is 800 bp.

GR$^{flox/flox}$ [30]

GR forward primer 5' - AAT CAG AAT TGC TCA CTC ACA A - 3'

GR reverse primer 5' - CAG TGT TAC TAC TTC CAG TTC - 3'

LoxP reverse primer 5' - TGC TAT ACG AAG TTA TCA GTA C - 3'94°C for 3’, (52°C for 30”, 72°C for 30”, 95°C for 30”) x 30, 52°C for 1’, 72°C for 5’, 4°C

WT band is 200 bp, and floxed band is 290 bp.

tdTomato [222]

WT forward primer 5’ - AAG GGA GCT GCA GTG GAG TA - 3'

WT reverse primer 5’ - CCG AAA ATC TGT GGG AAG TC - 3'

tdTomato forward primer 5’ - CTG TTC CTG TAC GGC ATG G - 3'

tdTomato reverse primer 5’ - GGC ATT AAA GCA GCG TAT CC - 3'

95°C for 5’, (94°C for 30”, 56°C for 30”, 72°C for 1’) x 32, 72°C for 10’, 4°C

WT band is 300 bp, and tdTomato band is 200 bp.

mTomato/mGFP [223]

Because mTomato is expressed in cells throughout the body, we determined mTomato/mGFP-positive genotypes by visualizing mTomato fluorescence in a drop of blood, collected when obtaining tail biopsies, rather than by PCR. The following primers may be used for genotyping by PCR:

5' - TCA ATG GGC GGG GGT CGT T - 3'

5' - CTC TGC TGC CTC CTG GCT TCT - 3'

5' - CGA GGC GGA TCA CAA GCA ATA - 3'

WT band is 330 bp, and mT/mG band is 250 bp.

2.4 RESULTS

Effects of stress on the reproductive axis

To test the effects of stress on *Kiss1* mRNA expression, we subjected adult male mice to 5 h of restraint, which produces psychological trauma without causing physical pain [219]. Compared to unstressed controls, restrained animals had increased plasma corticosterone concentrations (**Figure 2.4a**), concomitant with suppression of *Kiss1* mRNA expression in the arcuate nucleus of the hypothalamus (**Figure 2.4b-d**). LH and testosterone were inhibited following restraint (**Figure 2.4e-f**). *Kiss1* mRNA expression in the anteroventral periventricular nucleus and medial amygdala remained unchanged (**Figure 2.5**). Food deprivation also inhibits reproductive function [224]. Body weight was measured to confirm food deprivation in each animal (**Figure 2.6**). When mice were food deprived for 48 h, plasma corticosterone was increased compared to in fed controls (**Figure 2.7a**), concomitant with suppression of *Kiss1* mRNA expression in the arcuate nucleus (**Figure 2.7b-d**), a trend towards suppression of LH, and significant suppression of testosterone (**Figure 2.7e-f**). Restraint and food deprivation induced similar increases in plasma corticosterone and decreases in *Kiss1* mRNA expression.

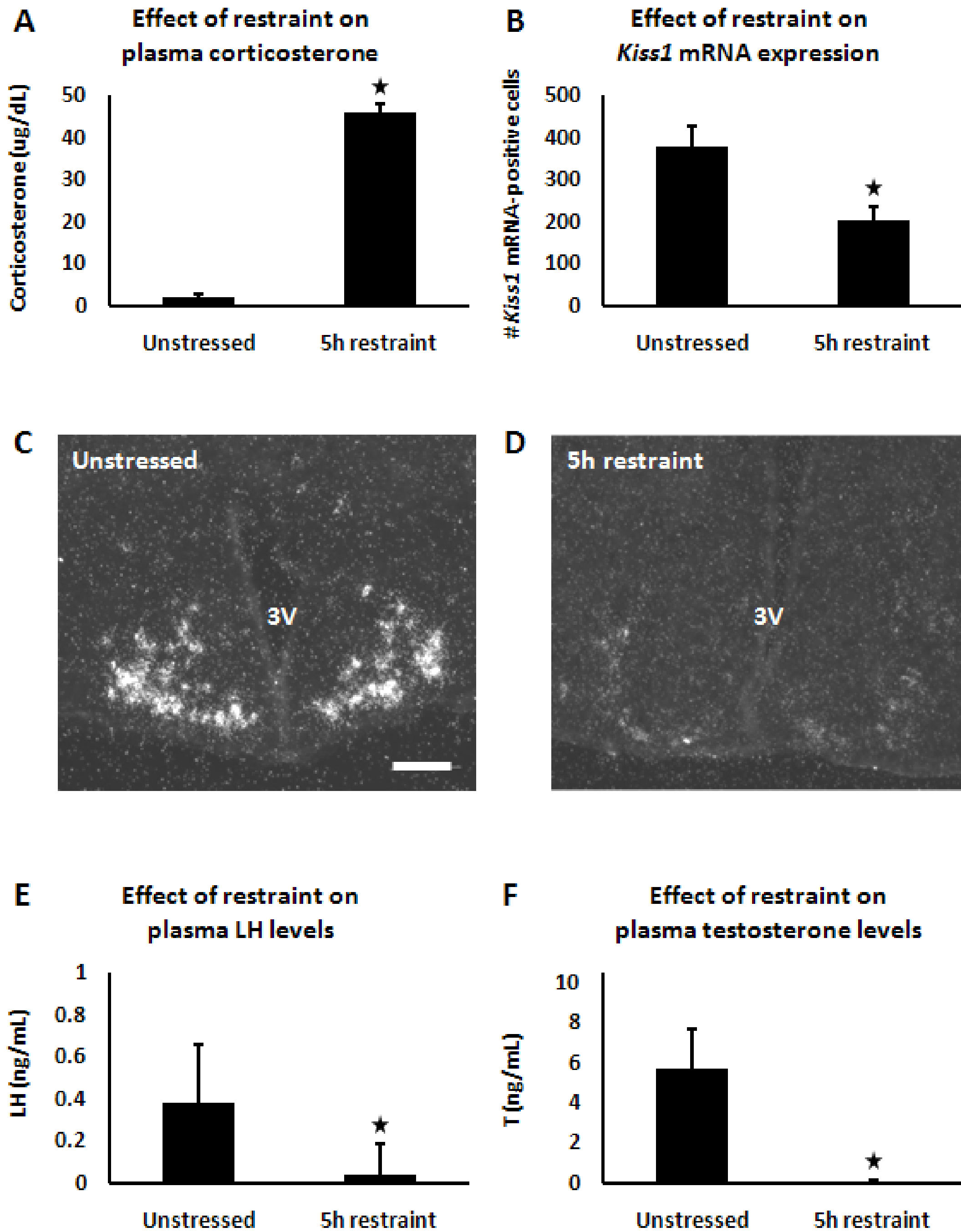


Figure 2.4
Adult male mice were subjected to 5 h of restraint (n=14 per treatment). **(A)** Plasma corticosterone levels were increased after restraint ($p=1.1x10^{-15}$). **(B-D)** *Kiss1* mRNA expression was quantified by *in situ* hybridization, and darkfield autoradiographs of *Kiss1* mRNA-positive silver cells showed decreased expression in the arcuate nucleus of restrained males (p=0.018). **(E-F)** LH (p=0.04) and testosterone (p=0.002) levels were decreased after restraint. Error bars, s.e.m; scale bar, 100µm; 3V, third ventricle.

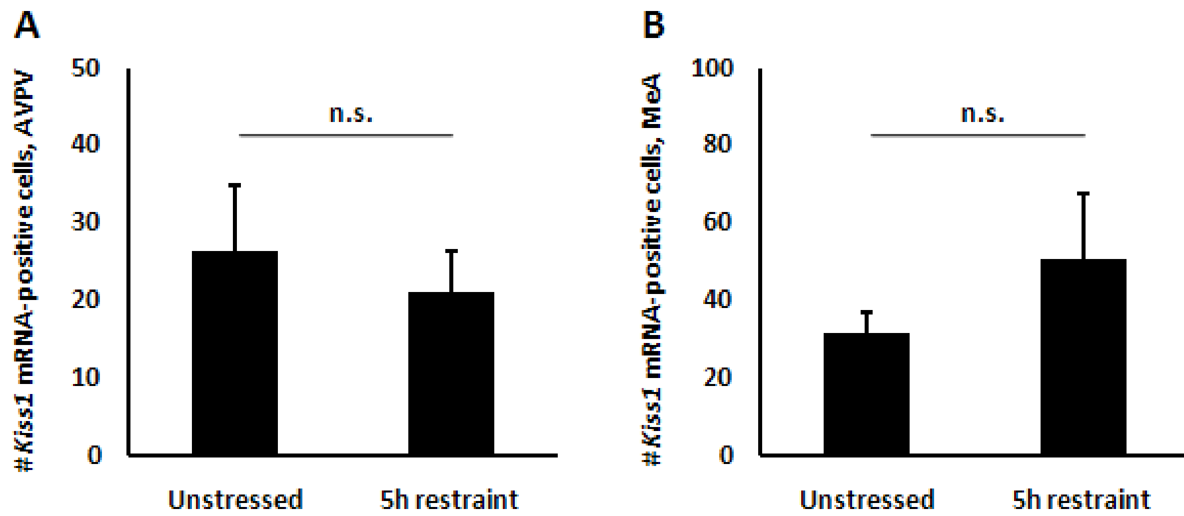


Figure 2.5
Adult male mice were subjected to 5 h of restraint. *Kiss1* mRNA expression was quantified by *in situ* hybridization, and darkfield autoradiographs of *Kiss1* mRNA-positive silver cells showed **(A)** no change in *Kiss1* mRNA expression in the anteroventral periventricular nucleus (p=0.31) after restraint and **(B)** no change in *Kiss1* mRNA expression in medial amygdala (p=0.60) after restraint.

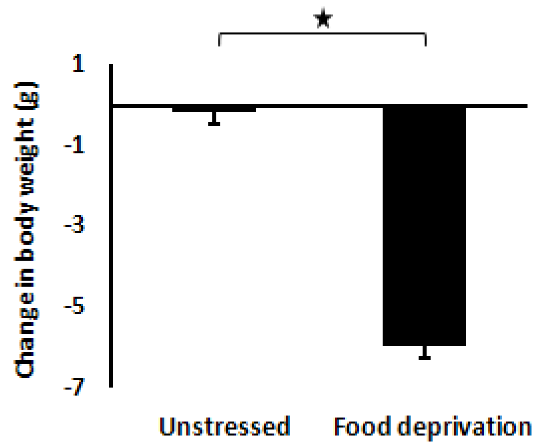


Figure 2.6
The efficacy of food deprivation was examined by measuring change in body weight. Animals were placed in cages without food for 48 h, but had access to bedding and *ad libitum* water. Decreased body weight ($p=7.7x10^{-8}$) was observed in the food-deprived animals compared to fed controls.

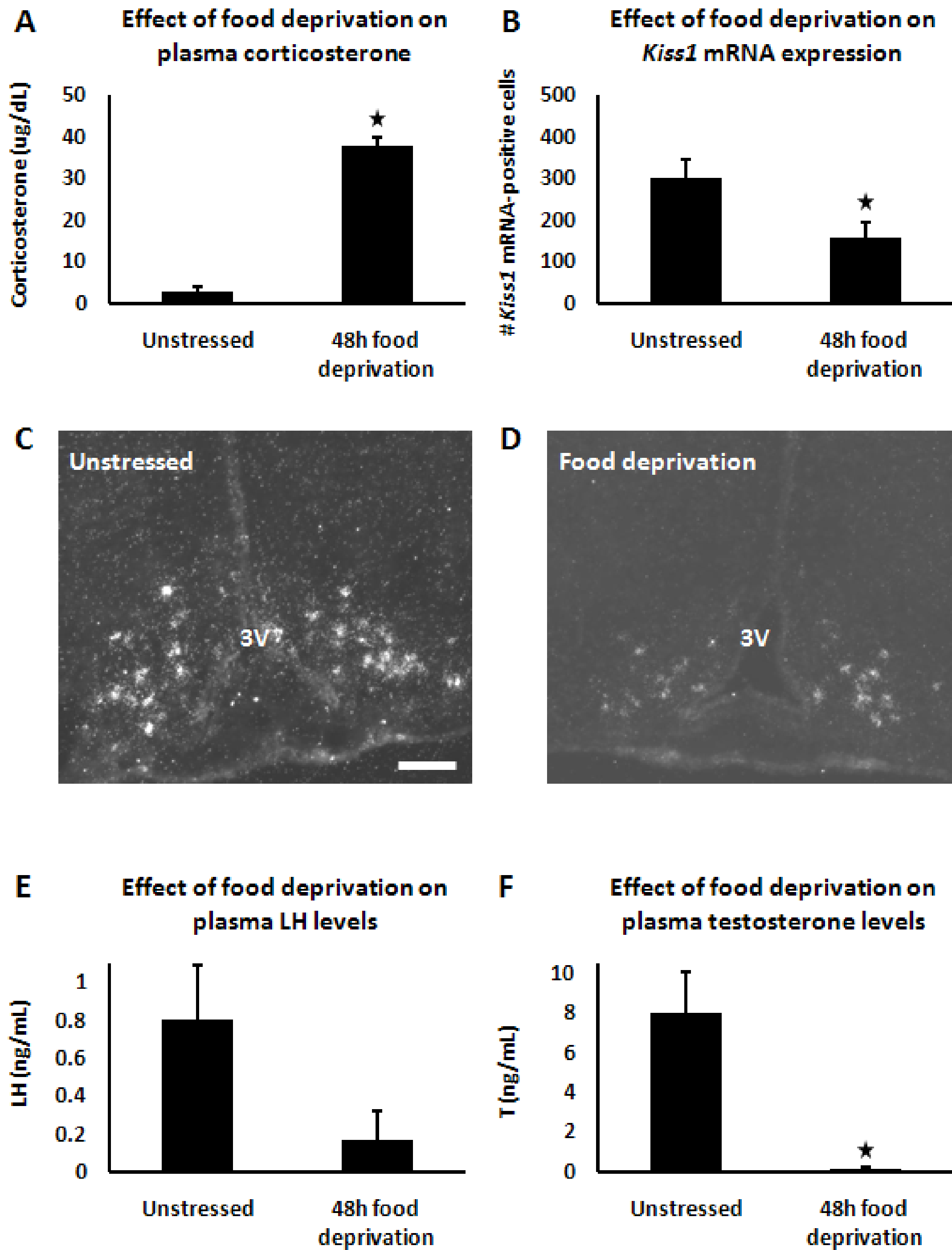


Figure 2.7
Adult male mice were subjected to 48 h of food deprivation (n=12 per treatment). **(A)** Plasma corticosterone levels were elevated after food deprivation ($p=6.7\times10^{-13}$). **(B-D)** *Kiss1* mRNA expression was significantly decreased following food deprivation (p=0.03). **(E-F)** LH trended downward (p=0.06), and testosterone was significantly suppressed (p=0.001) after food deprivation. Error bars, s.e.m; scale bar, 100µm; 3V, third ventricle.

To determine whether inhibition of *Kiss1* mRNA expression occurs in the absence of stress-induced elevation of glucocorticoids, we subjected mice to 24 h of cold (4°C) exposure, because this condition does not induce an increase in plasma corticosterone levels [225]. Core body temperature was measured by rectal probe to confirm cold exposure in each animal (**Figure 2.8**). After 24 h of cold exposure, mice had no significant change in plasma corticosterone compared to controls housed at room temperature (**Figure 2.9a**), and *Kiss1* mRNA expression was unchanged (**Figure 2.9b-d**). LH trended downward and testosterone decreased significantly following cold exposure (**Figure 2.9e-f**). Thus with cold exposure, inhibition of the reproductive axis occurs without an elevation in corticosterone or a suppression of *Kiss1* mRNA expression.

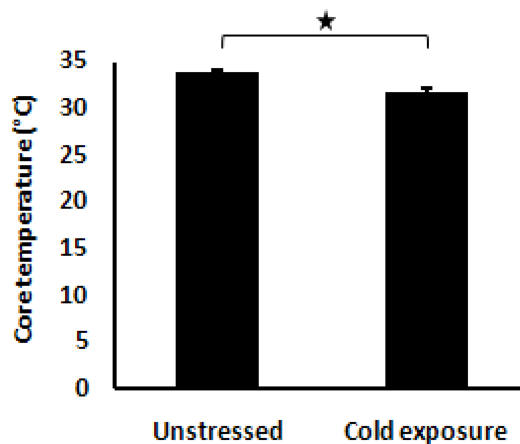


Figure 2.8
The efficacy of cold exposure was examined by measuring change in core body temperature. For cold exposure experiments, animals were housed at 4°C for 24 h with bedding, food, and water. Core body temperature, measured by rectal probe, was significantly decreased (p=0.006) in cold-exposed animals.

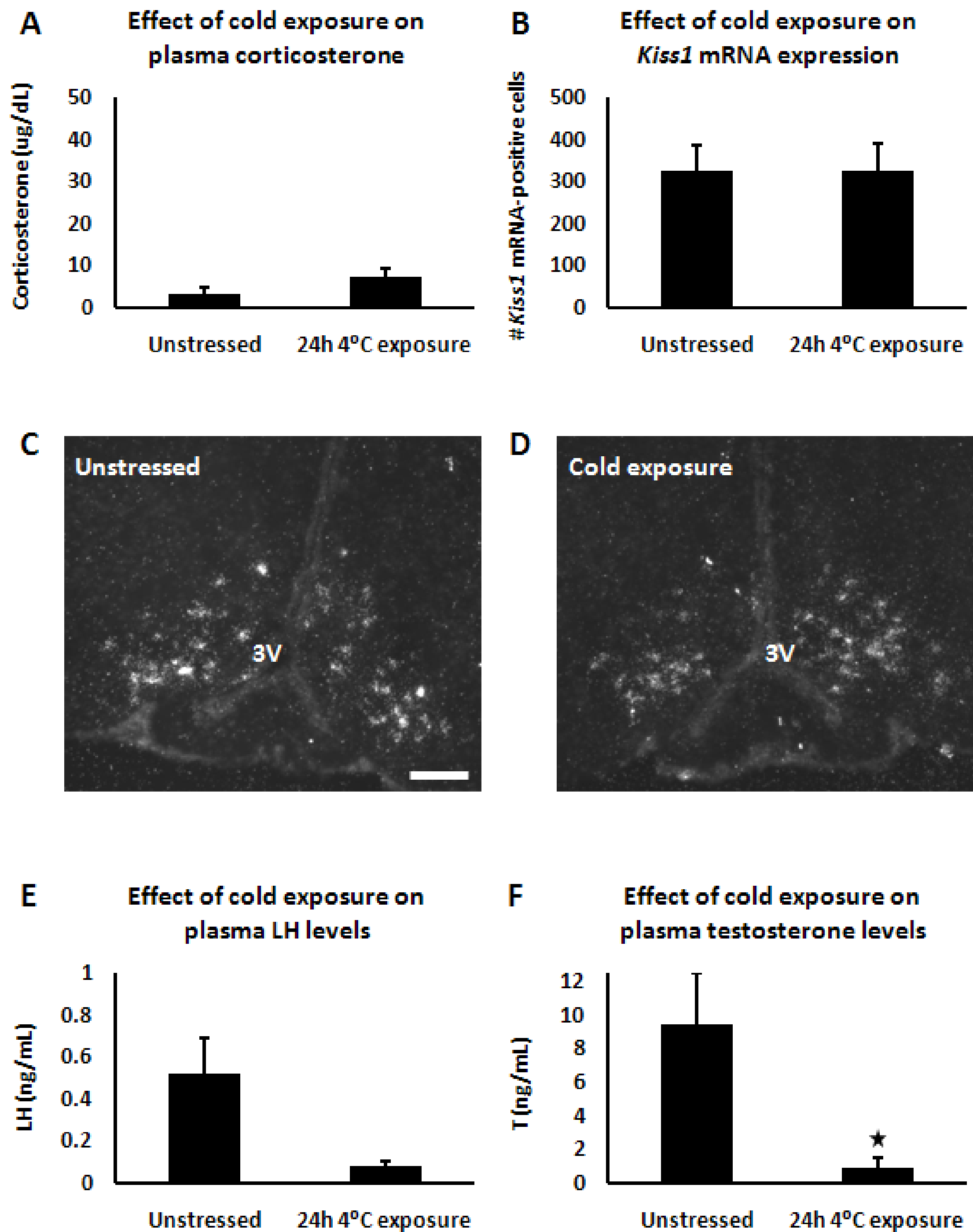


Figure 2.9
Adult male mice were subjected to 24 h of cold exposure (n=6 per treatment). **(A)** Cold exposure did not produce a significant increase in corticosterone levels after 24 h (p=0.23). **(B-D)** *Kiss1* mRNA expression was also unchanged after 24 h of cold exposure (p=0.99). **(E-F)** LH was unchanged (p=0.35), and testosterone was significantly decreased in cold-exposed males (p=0.02). Error bars, s.e.m; scale bar, 100µm; 3V, third ventricle.

With the above three stressors, the consistent relationship between a rise in corticosterone and fall in *Kiss1* mRNA expression suggested that elevated corticosterone inhibits *Kiss1* expression. To test this directly, we injected adult male mice with a stress level dose of corticosterone [226] or saline. To mimic the time course of mRNA changes in the restraint condition, we analyzed tissues 5 h post-injection. As expected, corticosterone levels were elevated (**Figure 2.10a**), and administration of corticosterone alone was sufficient to inhibit *Kiss1* mRNA expression (**Figure 2.10b-d**). LH trended downward, and testosterone decreased following corticosterone administration (**Figure 2.10e-f**). This suppression of *Kiss1* mRNA expression indicates that a rise in plasma glucocorticoid concentration can affect kisspeptin neurons, either directly or indirectly, and may cause the inhibition of the reproductive axis after exposure to psychological trauma and food withdrawal stressors, but not cold exposure.

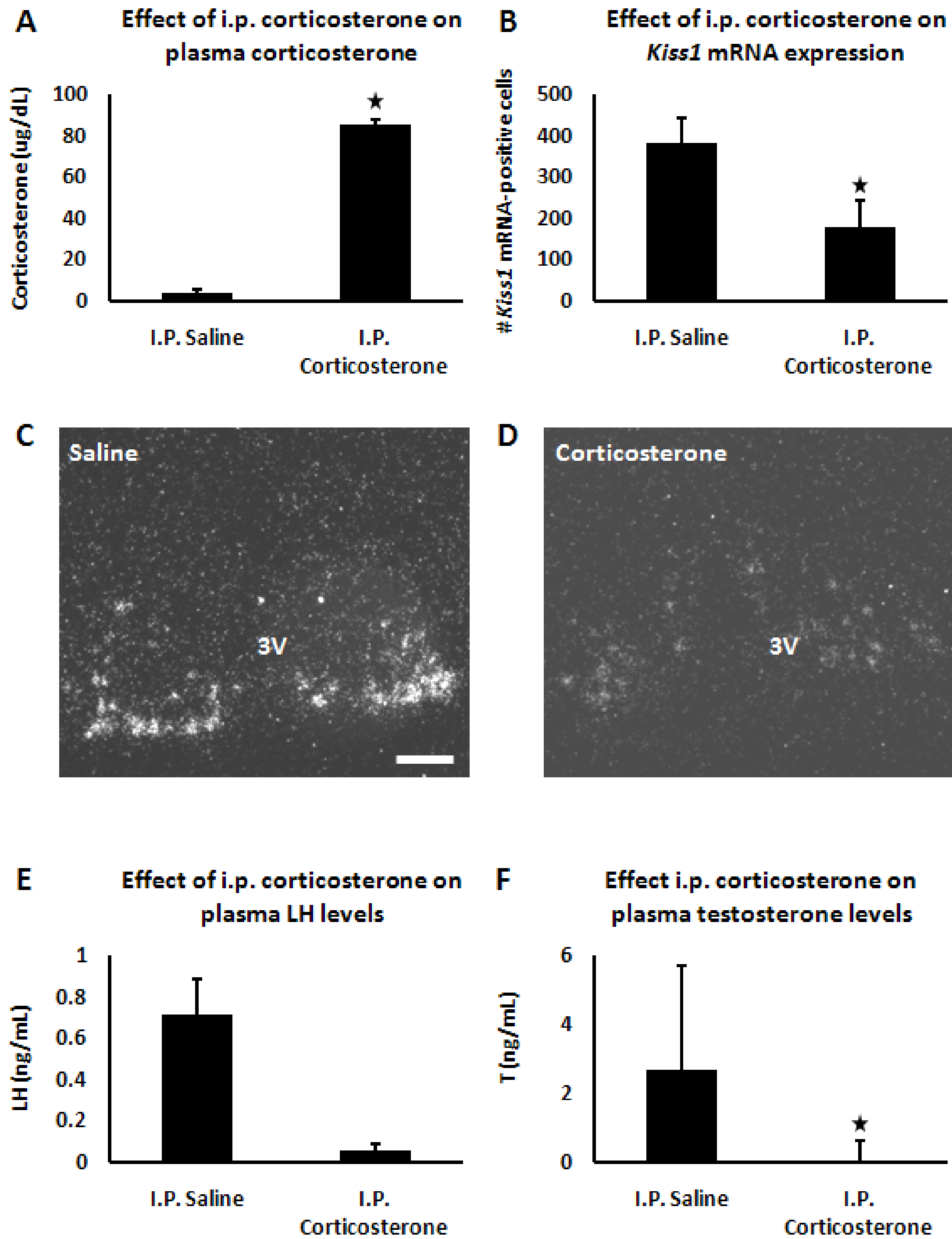


Figure 2.10
Adult male mice were subjected to I.P. corticosterone (n=7 per treatment). **(A)** I.P. corticosterone injection caused an increase in glucocorticoid levels compared with levels in I.P. saline injection ($p=9.5x10^{-11}$). **(B-D)** Administration of corticosterone was sufficient to inhibit *Kiss1* mRNA expression (p=0.036). **(E-F)** LH trended downward (p=0.08), and testosterone decreased significantly in corticosterone-treated males (p=0.04). Error bars, s.e.m; scale bar, 100µm; 3V, third ventricle.

Kisspeptin neuron-specific deletion of glucocorticoid receptors

To directly evaluate the role of GR signaling in kisspeptin neurons, we selectively deleted GR from these neurons in $Kiss1^{CreBAC}::GR^{flox/flox}$ animals. $GR^{flox/flox}$ mice [227] were bred to $Kiss1^{CreBAC}$ mice [221]. In $Kiss1^{CreBAC}::R26^{flox\text{-}stop\text{-}tdTomato}$ reporter mice, in which tdTomato expression is induced by Cre-mediated recombination, and $Kiss1^{CreBAC}::R26^{mTomato/mGFP}$ reporter mice, in which mGFP expression is induced by Cre-mediated recombination, greater than 95% of hypothalamic kisspeptin neurons identified by immunostaining coexpressed tdTomato (**Figures 2.11, 2.12**). In $Kiss1^{CreBAC}::R26^{flox\text{-}stop\text{-}tdTomato}$ animals, 72% of tdTomato neurons in the arcuate nucleus expressed GR (**Figure 2.13a, c**), whereas in $Kiss1^{CreBAC}::R26^{flox\text{-}stop\text{-}tdTomato}::GR^{flox/flox}$ animals, less than 6% of tdTomato neurons expressed GR (**Figure 2.13b, c**). The overall number of kisspeptin neurons in the arcuate nucleus was similar in animals with or without GR in kisspeptin neurons (**Figure 2.13d**), indicating that their ontogeny was not affected. Cre expression was limited to the AVPV, arcuate, and medial amygdala (**Figures 2.14, 2.15**).

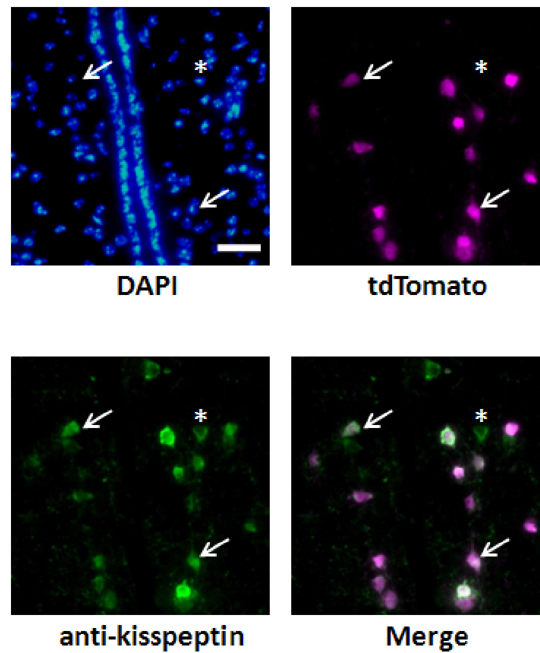


Figure 2.11
In *Kiss1*^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$ reporter animals, 95% (±0.8%) of kisspeptin neurons (anti-kisspeptin, green) in the hypothalamus expressed tdTomato (magenta). Arrows indicate examples of kisspeptin-positive and tdTomato-positive cells. Asterisk indicates example of kisspeptin-positive, but tdTomato-negative cell. Scale bar, 25 µm.

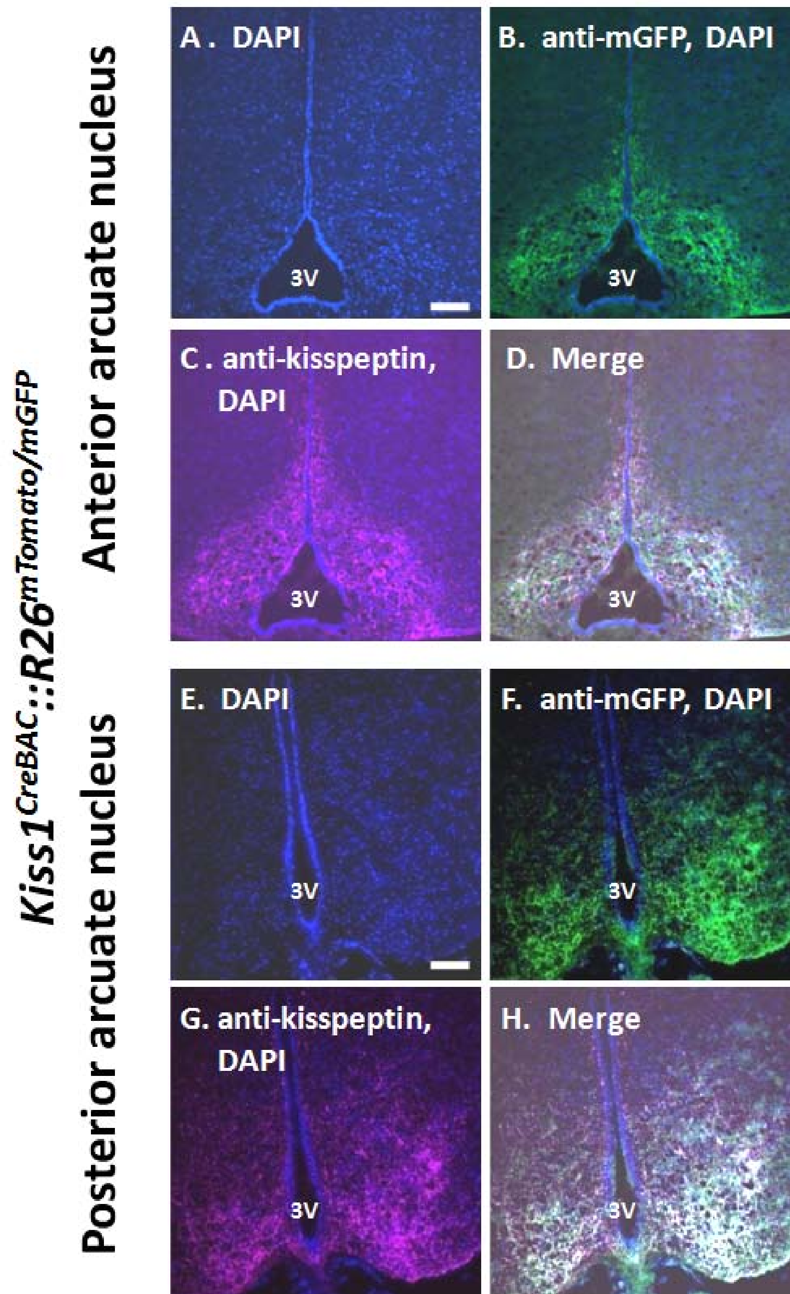
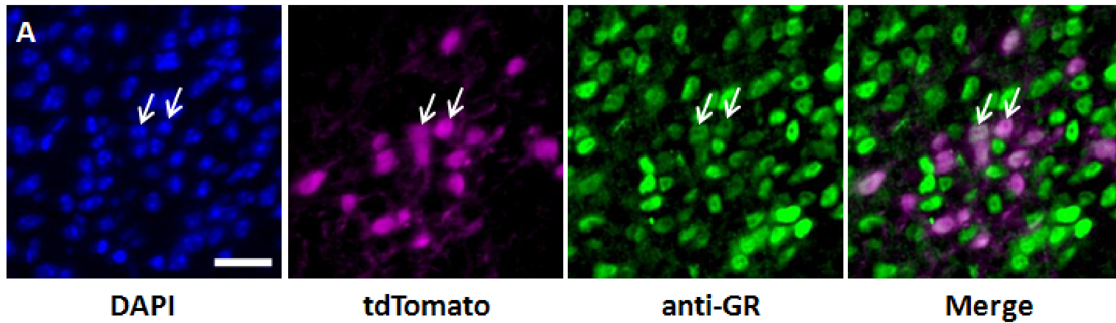
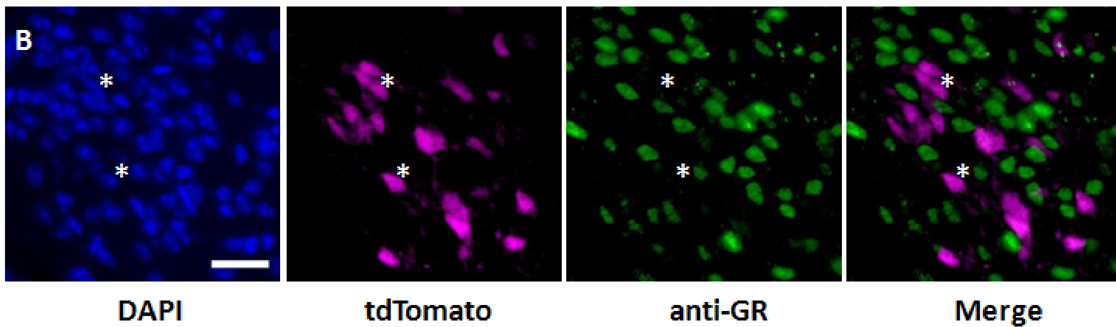


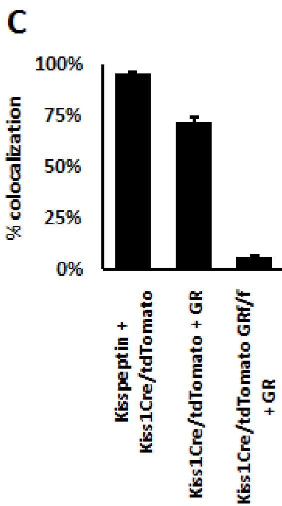
Figure 2.12
Kisspeptin staining in the arcuate nucleus preferentially stains fibers, so *Kiss1*^{CreBAC}*::R26*$^{mTomato/mGFP}$ reporter animals (green) were generated to evaluate colocalization (white) with endogenous kisspeptin (magenta). Greater than 95% of kisspeptin fibers (magenta) in the arcuate nucleus expressed driven mGFP (green). Representative images of the anterior arcuate nucleus **(A-D)** and posterior arcuate nucleus **(E-H)** from two *Kiss1*^{CreBAC}*::R26*$^{mTomato/mGFP}$ animals are shown. Scale bar, 100μm; 3V, third ventricle.

Kiss1^{CreBAC}**/tdTomato**



Kiss1^{CreBAC}**/tdTomato *GR***$^{flox/flox}$





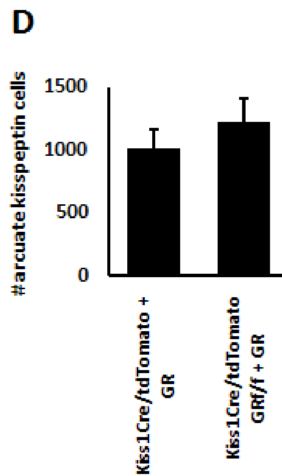


Figure 2.13
Kiss1^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$ reporter animals were generated to evaluate efficiency of GR deletion. **(A, C)** 72% (±2.6%) of tdTomato neurons (magenta) in the hypothalamus also expressed GR (green). Arrows indicate examples of tdTomato- and GR-positive cells. **(B, C)** In *Kiss1*^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$*::GR*$^{flox/flox}$ animals, 6% (±0.9%) of tdTomato neurons (magenta) expressed GR (green). Asterisks indicate examples of tdTomato-positive and GR-negative cells. **(D)** The total number of kisspeptin cells per arcuate nucleus was similar in *Kiss1*^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$ and *Kiss1*^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$*::GR*$^{flox/flox}$ hypothalami. Scale bars, 25µm.

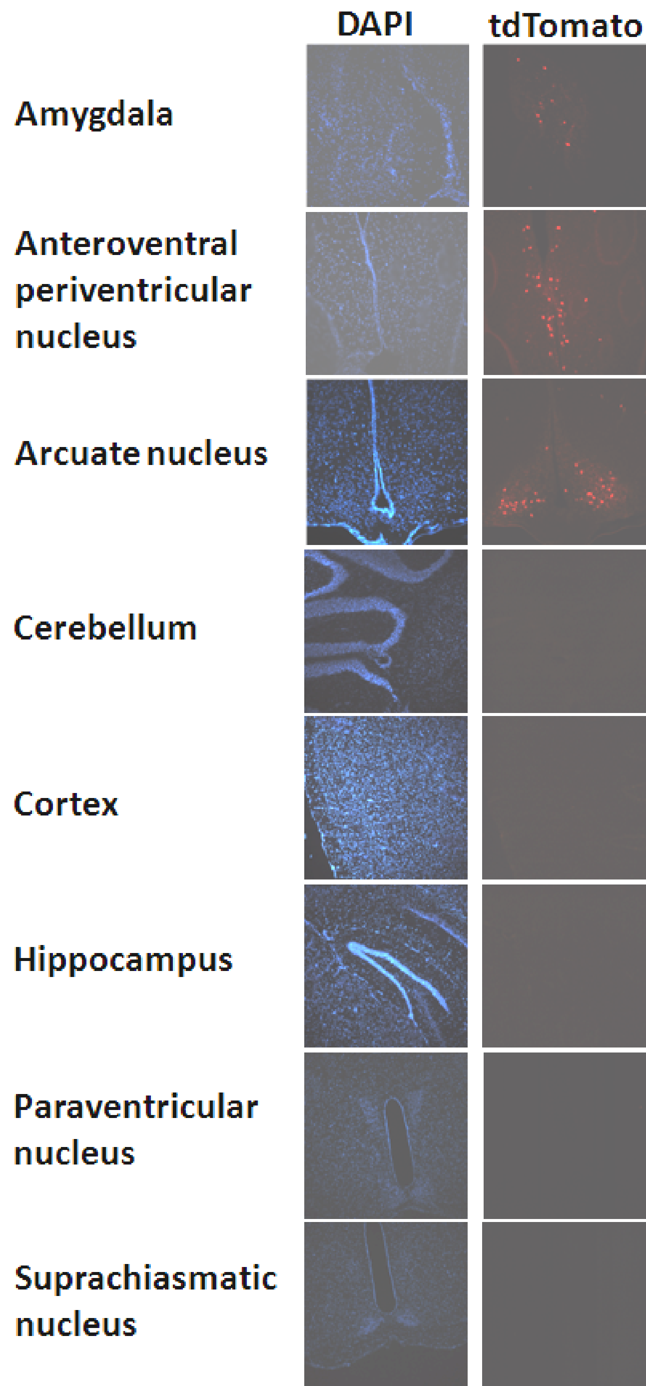


Figure 2.14
Kiss1^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$ reporter animals were generated to evaluate the distribution of Cre expression. tdTomato was expressed in the AVPV and arcuate nuclei of the hypothalamus and medial amygdala, as previously reported. In the brain, no tdTomato expression was observed in the cerebellum, cortex, hippocampus, paraventricular nucleus, suprachiasmatic nucleus.

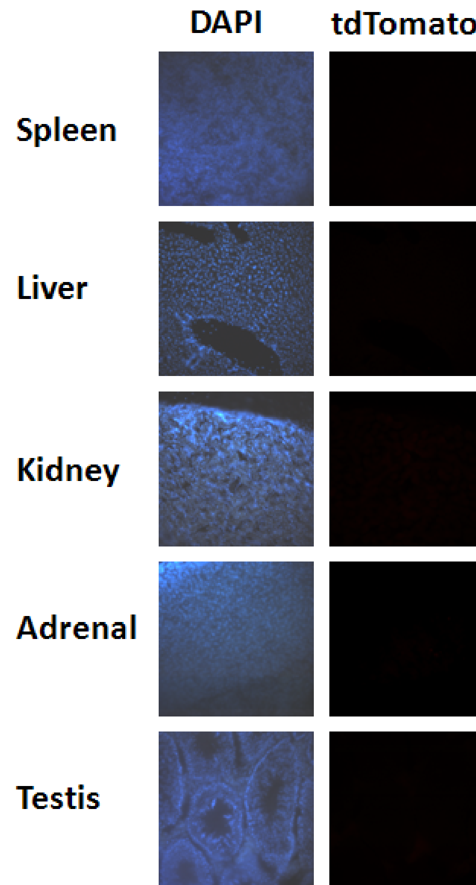


Figure 2.15
Kiss1^{CreBAC}*::R26*$^{flox\text{-}stop\text{-}tdTomato}$ reporter animals were generated to evaluate the distribution of Cre expression. In peripheral tissues, no tdTomato expression was observed in the adrenal gland, kidney, liver, spleen, or testis.

HPG axis function in the absence of GR signaling in kisspeptin neurons

Since arcuate nucleus *Kiss1* mRNA expression is inhibited by elevated glucocorticoid concentrations, we asked whether GR deletion in kisspeptin neurons affects the HPG axis, either during the acute exposure to psychological trauma or during the recovery of the axis from stress in the post-traumatic period. In the former case, the reproductive function of $Kiss1^{CreBAC}::GR^{flox/flox}$ animals should be preserved during and after acute stress, whereas in the latter case, reproductive function of $Kiss1^{CreBAC}::GR^{flox/flox}$ animals should decline during stress but recover more rapidly thereafter.

$Kiss1^{CreBAC}::GR^{flox/flox}$, $GR^{flox/flox}$, $Kiss1^{CreBAC}$, and WT males were subjected to 5 h of restraint and monitored after release. All genotypes had comparable concentrations of plasma corticosterone throughout the experiment, including at baseline, during the rise throughout restraint, the decrease after release, and the return to baseline after 1 week (**Figure 2.16a**). *Kiss1* mRNA levels were comparably high at baseline in all genotypes (**Figure 2.16b**). After 5 h of restraint, *Kiss1* mRNA levels in WT, $GR^{flox/flox}$, and $Kiss1^{CreBAC}$ controls were significantly decreased (**Figure 2.16b**), consistent with our previous results (**Figure 2.4**), while expression remained unsuppressed in $Kiss1^{CreBAC}::GR^{flox/flox}$ mice. After 1 week of recovery, *Kiss1* mRNA levels had increased in all animals (**Figure 2.16c**). Thus, GR signaling in kisspeptin neurons causes the fall in *Kiss1* mRNA expression during restraint stress. Testosterone concentrations were comparable at baseline, fell precipitously at 1 h and 5 h of restraint, and began to increase similarly 4 h after release in all genotypes (**Figure 2.16c**). However, 1 week following restraint, testosterone concentrations had recovered to normal only in $Kiss1^{CreBAC}::GR^{flox/flox}$ animals (**Figure 2.16c**). Thus, GR signaling in kisspeptin neurons is not required for the acute

suppression of the HPG axis during stress, but is required for the regulation of reproductive recovery following stress.

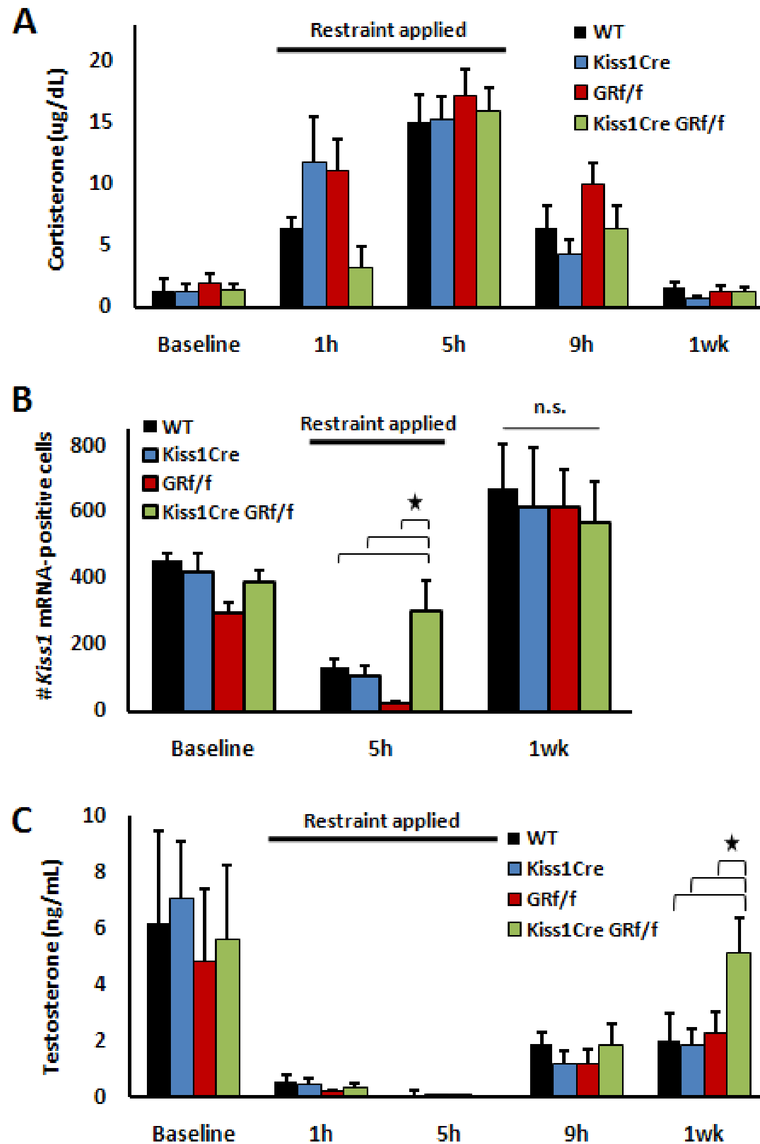


Figure 2.16
(A) All males showed an increase in corticosterone levels that peaked at 5 h of restraint. Corticosterone levels began to decrease after release from restraint, and after 1 week of recovery, these had returned to baseline in all animals. Changes over time were significant ($p=3.3x10^{-36}$), and no significant differences were observed between genotypes. **(B)** *Kiss1* mRNA levels were comparably high at baseline in all animals. *Kiss1* mRNA levels were not suppressed in *Kiss1*^{CreBAC}::*GR*$^{flox/flox}$ mice at 5 h of restraint (one-way ANOVA, p=0.008), compared to *GR*$^{flox/flox}$ (p=0.001, LSD *post hoc* test), *Kiss1*^{CreBAC} (p=0.013, LSD *post hoc* test), and WT (p=0.027, LSD *post hoc* test) controls. After 1 week, *Kiss1* mRNA levels were comparably elevated in all animals. **(C)** Testosterone levels were high at baseline and fell precipitously at 1 h and 5 h of restraint in all genotypes. After 4 h of recovery, testosterone levels began to recover in all genotypes. Changes were significant over time (p=0.001). 1 week after restraint, testosterone levels in *Kiss1*^{CreBAC}::*GR*$^{flox/flox}$ males were no longer suppressed (one-way ANOVA, p=0.04) compared to *GR*$^{flox/flox}$ (p=0.03, LSD *post hoc* test), *Kiss1*^{CreBAC} (p=0.01, LSD *post hoc* test), and WT (p=0.02, LSD *post hoc* test) controls.

Effects of stress on reproductive behaviors in the absence of GR signaling in kisspeptin neurons

To determine the impact of kisspeptin neuron-specific GR deletion on reproductive behaviors during and after stress, we examined copulatory behaviors before and after 5 h of restraint. Immediately after restraint, all genotypes exhibited comparable suppression of the copulatory behaviors, mounting and thrusting, compared to baseline (**Figure 2.17a, b**), consistent with the decrease in testosterone observed in all genotypes (**Figure 2.16c**). In $Kiss1^{CreBAC}$::$GR^{flox/flox}$ mice, however, both mounting and thrusting behaviors were fully recovered 1 week after restraint, while copulatory activity remained low at this time in $GR^{flox/flox}$, $Kiss1^{CreBAC}$, and WT controls (**Figure 2.17a, b**). From these studies of $Kiss1^{CreBAC}$::$GR^{flox/flox}$ mice, we conclude that immediately following restraint stress, GR signaling within kisspeptin neurons causes the fall in *Kiss1* mRNA, though not the inhibition of hormonal and behavioral components of the HPG axis. GR-dependent inhibition of kisspeptin neurons during acute stress is required for regulates the time course of recovery from stress-induced reproductive inhibition.

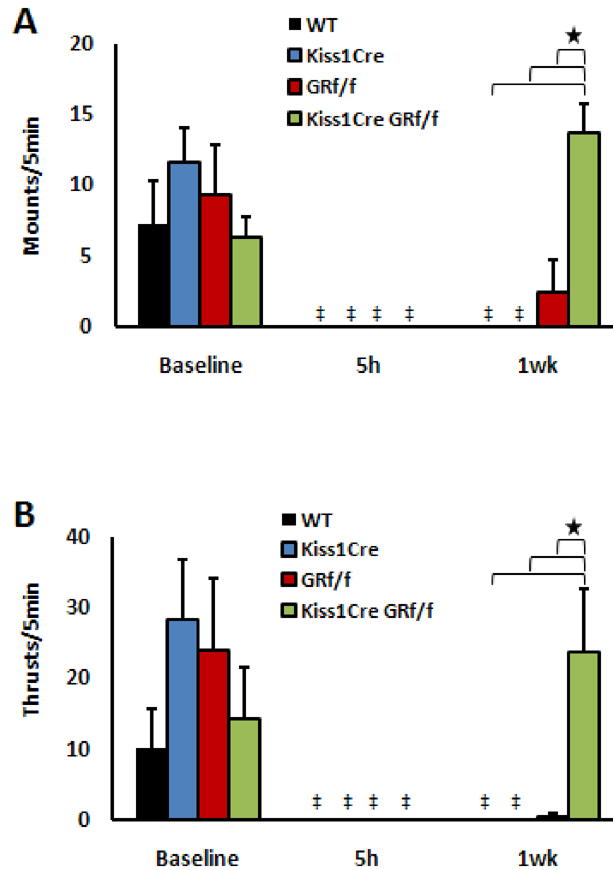


Figure 2.17
(A) Immediately after restraint, all genotypes exhibited a comparable decrease in mounting behavior (p=5.0x10^{-5}). In *Kiss1^{CreBAC}::GR$^{flox/flox}$* mice, mounting was fully recovered after 1 week (p=0.017, one-way ANOVA), compared to *GR$^{flox/flox}$* (p=0.018, LSD *post hoc* test), *Kiss1^{CreBAC}* (p=0.007, LSD *post hoc* test), and WT (p=0.011, LSD *post hoc* test) controls in which the incidence remained low. **(B)** All genotypes exhibited decreased thrusting behavior after restraint (p=7.0x10^{-5}). In *Kiss1^{CreBAC}::GR$^{flox/flox}$* mice, thrusting was fully recovered after 1 week (p=0.012, one-way ANOVA), compared to *GR$^{flox/flox}$* (p=0.006, LSD *post hoc* test), *Kiss1^{CreBAC}* (p=0.007, LSD *post hoc* test), and WT (p=0.011, LSD *post hoc* test) controls in which the incidence remained low.

Finally, we examined the specificity of the behavioral consequences of deleting GR from kisspeptin neurons. While testosterone and copulatory behaviors recovered more quickly after stress in *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$ males, behaviors such as sniffing, locomotor activity, and general anxiety were unaffected by deletion of GR. *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$, *GR*$^{flox/flox}$*, Kiss1*^{CreBAC}, and WT animals spent similar amounts of time sniffing females at baseline, and all mice exhibited decreased sniffing behavior immediately after restraint, but recovered this behavior at 1 week (**Figure 2.18a**). Likewise, all genotypes exhibited decreased locomotor activity after 5 h restraint compared to baseline and recovered after 1 week (**Figure 2.18b**). All animals spent less time in the center of an open-field test immediately after restraint, a sign of increased general anxiety [228], which was not fully recovered after 1 week (**Figure 2.18c**), though this may have been due to increased time spent in the center by WT males. Thus, deletion of GR in kisspeptin neurons selectively impacts reproductive behaviors.

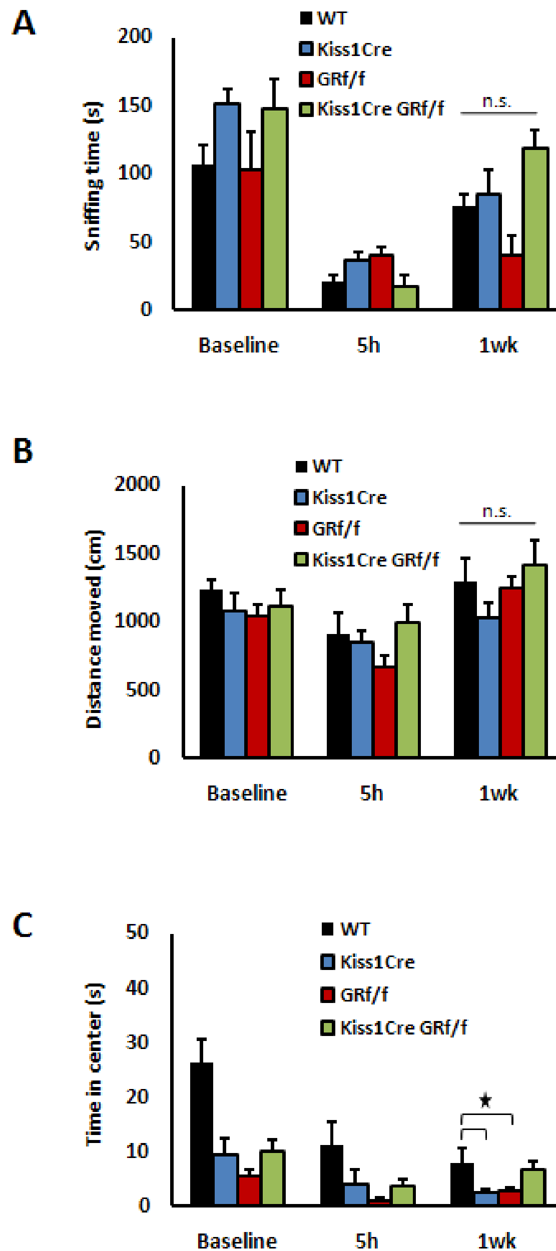


Figure 2.18
(A) All animals spent similar amounts of time sniffing females at baseline. Sniffing decreased immediately after restraint and recovered after 1 week in all animals ($p=1.9x10^{-11}$). **(B)** All animals exhibited decreased locomotor activity after restraint compared to baseline and recovered after 1 week ($p=1.8x10^{-6}$). **(C)** All animals spent less time in the center of an open-field test immediately after restraint, which did not recovery after 1 week ($p=2.5x10^{-6}$), though this may be due to increased time spent in the center by WT males.

2.5 DISCUSSION

Natural selection depends on successful reproduction, but inhibition of reproduction to survive famine or escape predation allows animals to survive to reproduce at a later time. The cellular locations and mechanisms responsible for inhibiting and reactivating the HPG axis during and after stress, respectively, are not well understood. We examined the suppression of the HPG axis to multiple stressors in male mice: restraint, food deprivation, cold exposure, and corticosterone administration. In all cases, there was an inverse relationship between plasma corticosterone and *Kiss1* mRNA content, but even with cold exposure, when plasma corticosterone and *Kiss1* mRNA were not affected, plasma testosterone was acutely inhibited.

We generated mice lacking GR specifically in kisspeptin-containing neurons. In these animals, *Kiss1* mRNA expression was no longer inhibited during restraint stress, even though corticosterone was increased, and both testosterone and copulatory behaviors showed accelerated recovery in the post-traumatic stress period. Neither systemic glucocorticoid levels nor general anxiety were affected in these animals, demonstrating the hormonal and behavioral specificity of the targeted disruption of GR signaling in kisspeptin neurons.

Stress, and glucocorticoids in particular, are known to inhibit the HPG axis. Men with Cushing's syndrome, a condition characterized by hypercortisolemia, and men treated with exogenous glucocorticoid therapy experience decreased testosterone levels and loss of libido [189, 190, 217]. PTSD and other psychiatric disorders are characterized by HPA axis hyperactivity and HPG axis suppression [208, 209]. Stress-induced elevation of corticosterone in male mice can lead to decreased testosterone secretion [129], and male mice treated with exogenous corticosterone exhibit decreased copulatory behaviors [125]. Food deprivation suppresses *Kiss1*

mRNA expression in rats and macaques [185, 229-231], but undernutrition delays puberty without affecting *Kiss1* mRNA expression [232]. Although stressful conditions correlate with decreased *Kiss1* mRNA expression, GR signaling in kisspeptin neurons is unlikely to be the sole "stop" signal to the HPG axis, as there are multiple other glucocorticoid-dependent and -independent stress pathways that inhibit the reproductive axis acutely [233-235].

While multiple stress pathways and stress-responsive cells can turn off the HPG axis during stress, kisspeptin neurons, which are critical to activate the HPG axis during puberty, may also serve in adults as the primary reactivation switch to turn on the HPG axis after the termination of stress. Removing GR signaling from kisspeptin neurons during stress, therefore, may not protect the HPG axis from inhibition, since multiple non-GR and non-kisspeptin mechanisms also suppress reproductive function. However, preservation of kisspeptin signaling during stress may permit faster kisspeptin neuron-mediated reactivation of the reproductive axis after stress, allowing for testosterone to rise and copulatory behaviors to recover. Thus, while kisspeptin is not the sole reproductive "stop" signal during stress, it is a critical "go" signal for the HPG axis during the recovery from stress.

We have rendered kisspeptin neurons insensitive to GR signaling and demonstrated that this abolishes stress-induced inhibition of *Kiss1* mRNA expression, but we cannot exclude the possibility that the acceleration of reproductive recovery after stress is conferred by other gene products within the neuron rather than by kisspeptin itself. Kisspeptin neurons in the hypothalamus co-express dynorphin, an endogenous opioid, and neurokinin B, a tachykinin peptide [236, 237]. Stress responses are known to interact with endogenous opioid systems, and

dynorphin, in particular, is responsive to stress and activates κ-opioid receptors in the amygdala, nucleus accumbens, dorsal raphe, and hippocampus [238-242]. It is possible that these gene products are also protected from stress-induced GR signaling in *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$ mice and contribute to the recovery of the reproductive axis.

In *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$ mice, *Kiss1* mRNA expression was maintained at normal levels despite elevated glucocorticoid levels during restraint stress. After 5 h of restraint, elevated *Kiss1* mRNA expression in *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$ mice did not immediately restore HPG axis function. One week after restraint, testosterone and copulatory behaviors recovered only in *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$ but not control mice, even though *Kiss1* mRNA expression had returned to normal levels in all animals at this time. By two weeks after restraint, copulatory behaviors were recovered in all animals, including those with intact GR signaling (OW and JM, unpublished observations). During adulthood, humans experience a similar delay in reproductive restoration after stress. Testosterone is suppressed during athletic competition and returns to baseline only after five to seven days [124, 204]. During puberty in mice, the gradual increase in hypothalamic kisspeptin precedes pubertal onset and HPG axis activation [96]. In animal models of pubertal onset and recovery from stress, and possibly in humans, an increase in *Kiss1* mRNA expression precedes activation of the HPG axis, consistent with the former contributing to the latter.

Acute stress responses are generally transient, with the short-term elevation in glucocorticoid secretion subsiding with the stressor. Exaggerated stress responses, including decreased reproductive function after a traumatic stressor has subsided, are clinically important features of

PTSD [208, 209], which may be mediated by persistently elevated glucocorticoid secretion [243]. Our findings indicate not only that kisspeptin neurons can be protected from stress by the removal of GR signaling, but that doing so has the ability to orchestrate a repertoire of complex behaviors leading to more rapid return of reproductive function, possibly by the downstream regulation of sex steroid secretion. The requirement for glucocorticoid receptor signaling to regulate kisspeptin neurons during the acute stress response suggests molecular targets for the treatment of reproductive dysfunction in PTSD, such as the blockade of glucocorticoid receptor signaling in kisspeptin neurons.

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CHAPTER 3

LEPTIN IS NOT REQUIRED FOR FERTILITY

This chapter is based on:

Oulu Wang, Satoru Sakihara, Kolbein Gudmundsson, and Joseph Majzoub. Leptin is not required for fertility *In preparation*, 2012.

Dr. Gudmundsson created CRHOB mice on a mixed background, and Dr. Sakihara initiated studies of metabolic phenotypes in the CRHOB mouse. Oulu Wang created CRHOB mice on a B6 background and documented fertility and metabolic effects on this background.

3.1 ABSTRACT

Leptin is widely considered to be required for fertility. Leptin deficiency causes obesity and infertility, and both conditions are ameliorated by leptin treatment. Leptin-deficient mice also exhibit elevated glucocorticoid secretion, and excess glucocorticoids, independent of leptin signaling, are associated with suppression of reproductive function. We hypothesized that elevated glucocorticoids inhibit fertility in leptin-deficient mice. To evaluate the role of glucocorticoids in leptin deficiency, we generated mice that were genetically leptin-deficient and glucocorticoid-deficient. Leptin-deficient, glucocorticoid-deficient mice were fertile and exhibited decreased body weight, fat composition, and food intake. When supplemented with corticosterone to physiologic levels, only the leptin-deficient, glucocorticoid-deficient mice became infertile. When corticosterone was removed, these mice regained reproductive capacity. Rescuing hypercortisolemia in leptin-deficient mice was sufficient to restore reproductive function, and thus, leptin is not required for fertility. These finding may provide novel mechanisms of glucocorticoids affecting fertility, not only in leptin deficiency, but also in multiple disorders characterized by hypercortisolemia, such as Cushing's disease, anorexia nervosa, and mood disorders.

3.2 Introduction

Leptin deficient *Lep*$^{Ob/Ob}$ mice are morbidly obese and infertile [171]. *Lep*$^{Ob/Ob}$ mice pair-fed to achieve wild-type body weights do not regain reproductive function [244]. Leptin replacement restores fertility in *Lep*$^{Ob/Ob}$ [244] and elicits advanced puberty in wild-type [178] mice, giving rise to the tenet that leptin is required for fertility.

Lep$^{Ob/Ob}$ mice are also hypercortisolemic [245]. Leptin deficiency creates a state of perceived starvation, and starvation is known to induce glucocorticoid secretion [246] (**Figure 2.7**). The hypothalamic-pituitary-adrenal stress axis comprises corticotropin-releasing hormone (CRH) neurons that stimulate pituitary adrenocorticotropic hormone (ACTH) release, which causes adrenal glucocorticoid secretion. Negative feedback by glucocorticoids then suppresses CRH and ACTH production. Excess glucocorticoid concentrations, independent of leptin signaling, are associated with reproductive inhibition in Cushing's disease, anorexia nervosa, exercise-induced amenorrhea, glucocorticoid therapy, and other conditions characterized by glucocorticoid dysregulation [132, 189, 217]. Adrenalectomy ameliorates obesity in *Lep*$^{Ob/Ob}$ mice [247], a phenomenon that was ascribed to disinhibition of CRH, itself an anorectic hormone [248]. Adrenalectomy did not reverse sterility in *Lep*$^{Ob/Ob}$ mice [249]. Using surgical adrenalectomy to study the effects of glucocorticoids is confounded by upregulation of CRH, surgical stress, and the regenerative capacity of adrenal tissue.

We hypothesized that glucocorticoids contribute to infertility in *Lep*$^{Ob/Ob}$ mice. We generated *Lep*$^{Ob/Ob}$ mice that were genetically CRH- and glucocorticoid-deficient (CRHOB) to study the role of glucocorticoids on fertility. Compared to *Lep*$^{Ob/Ob}$ controls, CRHOB mice displayed

normal fertility, normal reproductive organs and hormone secretion, and decreased body weight, fat composition, and hyperphagia. When supplemented with glucocorticoids to levels present in leptin deficiency, CRHOB but not wild-type mice became infertile, and regained reproductive function after the termination of glucocorticoid treatment. Thus, leptin is not directly required for fertility as previously postulated, and glucocorticoids contribute to infertility and obesity downstream of leptin signaling. These studies may provide novel molecular targets in the reproductive and metabolic aspects of disorders characterized by glucocorticoid dysregulation, including leptin deficiency, Cushing's disease, anorexia nervosa, and mood disorders.

3.3 Material and Methods

Animal husbandry and breeding strategy

All experiments were conducted in compliance with the Institutional Animal Care and Use Committee guidelines of Children's Hospital Boston. Animals were maintained on a 12 h light/dark cycle with access to chow and water *ad libitum* and tested between 5-20 weeks of age. $Crh^{-/-}$ mice were previously generated in our laboratory [8]. $Lep^{Ob/+}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Crosses from ♀ $Crh^{+/-}$ $Lep^{Ob/+}$ x ♂ $Crh^{-/-}$ $Lep^{Ob/+}$ breeders yielded $Crh^{-/-}$ $Lep^{Ob/Ob}$ and $Crh^{-/-}$ progeny at a frequency of 12.5% each. Crosses from $Lep^{Ob/+}$ x $Lep^{Ob/+}$ breeders yielded $Lep^{Ob/Ob}$ and WT progeny at a frequency of 25% each. Thus, $Crh^{-/-}$ $Lep^{Ob/Ob}$ and $Crh^{-/-}$ progeny were littermates, and $Lep^{Ob/Ob}$ and WT progeny were littermates. All animals were backcrossed >10 generations onto a C57BL/6 background. 5-10 males and 5-10 females per genotype (WT, $Crh^{-/-}$, $Lep^{Ob/Ob}$, and $Crh^{-/-}$ $Lep^{Ob/Ob}$) were generated and analyzed.

Tissue preparation

Retroorbital blood samples were collected from unanesthetized animals within 1 min of cage handling in all conditions and centrifuged at 3,000 rpm for 10 min at 4°C. At 7AM (lights on) at 6 weeks of age, blood samples were collected for analysis of corticosterone, triglycerides, and testosterone. At 7PM (lights off) at 20 weeks of age, blood samples were collected for analysis of corticosterone, luteinizing hormone, and estradiol. Animals were sacrificed by rapid decapitation without anesthesia at 20 weeks, and dissected brains were embedded in OCT and stored at -80°C. Brains were sectioned coronally at 10 µm through the hypothalamus in eight sets, thaw-mounted onto 25 mm x75 mm slides, and returned to -80°C until further processing,

and slide mounted for *in situ* hybridization. Testes, ovaries, uteri, liver, pancreas, and adrenal glands were fixed in 10% formalin and embedded in paraffin. 10 μm midline sections were obtained for each tissue and stained by hematoxylin and eosin (Harvard Medical School, Rodent Histopathology Core). Histological sections were visualized and analyzed by brightfield microscopy on the 2X, 4X, and 10X objective of a Nikon Eclipse E800.

Luteinizing hormone was assayed by the UVA Center for Research in Reproduction Ligand Assay and Analysis Core. Plasma triglycerides were analyzed by ELISA (Crystal Chemical). Plasma corticosterone and testosterone were measured by radioimmunoassay (MP Biomedicals) with the following modifications: to minimize the amount of plasma used in the corticosterone radioimmunoassay, we generated 1:200 dilutions using either 1 μL of plasma with 199 μL of steroid diluent or 5 μL of plasma with 995 μL of steroid diluent. In general, aliquots containing 1 μL were more variable in corticosterone concentrations, likely due to pipetting error, and unless blood volume was a major constraint, we used 5 μL of plasma for corticosterone assays. The dynamic range for the corticosterone assay was 25 ng/mL to 1,000 ng/mL. To minimize the amount of plasma used in the testosterone radioimmunoassay, we used 25 μL of plasma in singlet. In pilots, this volume was as effective in identifying stress-induced testosterone suppression as 50 μL of plasma in duplicate. The dynamic range for this assay was 0.1 ng/mL to 10 ng/mL.

In situ hybridization

The *Kiss1* mRNA probe was generously provided by Robert Steiner [100], and the *Crh* mRNA probe was previously synthesized in our laboratory [5]. Radiolabeled probes were synthesized

using ^{33}P-UTP, and *in situ* hybridization was performed as previously described [5, 100], with the following modifications. Briefly, tissues were washed in 4% paraformaldehyde, acetic anhydride, 2X SSC, chloroform, and graded ethanols, then incubated in 12.7 million dpm/mL probe for 16 h at 55°C. Slides were subsequently washed with 4X SSC, RNase, 2X SSC, 0.5X SSC at 62°C, and graded ethanols in ammonium acetate, then exposed to autoradiographic film (Kodak XAR 1651579) or silver emulsion (Kodak NTB 8895666). Silver grains in the hypothalamus were visualized by darkfield microscopy on the 10X objective of a Nikon Eclipse E800. Silver-positive cell counts were analyzed by using NIH ImageJ software (http://imagej.nih.gov/ij/), as described in Chapter 2 (**Figure 2.2**). Film densitometry was analyzed using NIH ImageJ software: autoradiographic films were scanned at 2,400 dpi, and a region-of-interest (ROI) was cropped from images of the hypothalamus (**Figure 3.1a**). Intensity was maintained within a linear dynamic range (0.002 - 3.58 nCi/mg) by comparing our *in situ* hybridization slides a control ^{14}C standard slide (American Radiolabeled Chemicals 0146E) such that low intensities were identifiable and high intensities were not oversaturated. An adjacent section without probe labeling ("background") was also cropped (**Figure 3.1b**). The mean intensity of the background section was measured using NIH ImageJ (**Figure 3.1c**) and applied uniformly to a newly generated image (**Figure 3.1d**). This background (**Figure 3.1d**) was subtracted from the ROI (**Figure 3.1a**) to yield a resultant background-corrected image (**Figure 3.1e**). The background-corrected image can be inverted for visual contrast (**Figure 3.1e'**). Pixels with ten-fold higher intensity than background were identified (**Figure 3.1f**). Multiple sections from two brains were obtained (**Figure 3.1g, h**). Brain H had noticeably higher background intensity than Brain G. After background subtraction, these ROIs were comparable

in intensity (**Figure 3.1g' , h'**). Densitometric measurements were calculated based on the [mean intensity within ROI] x [area of ROI] and summed across all brain sections.

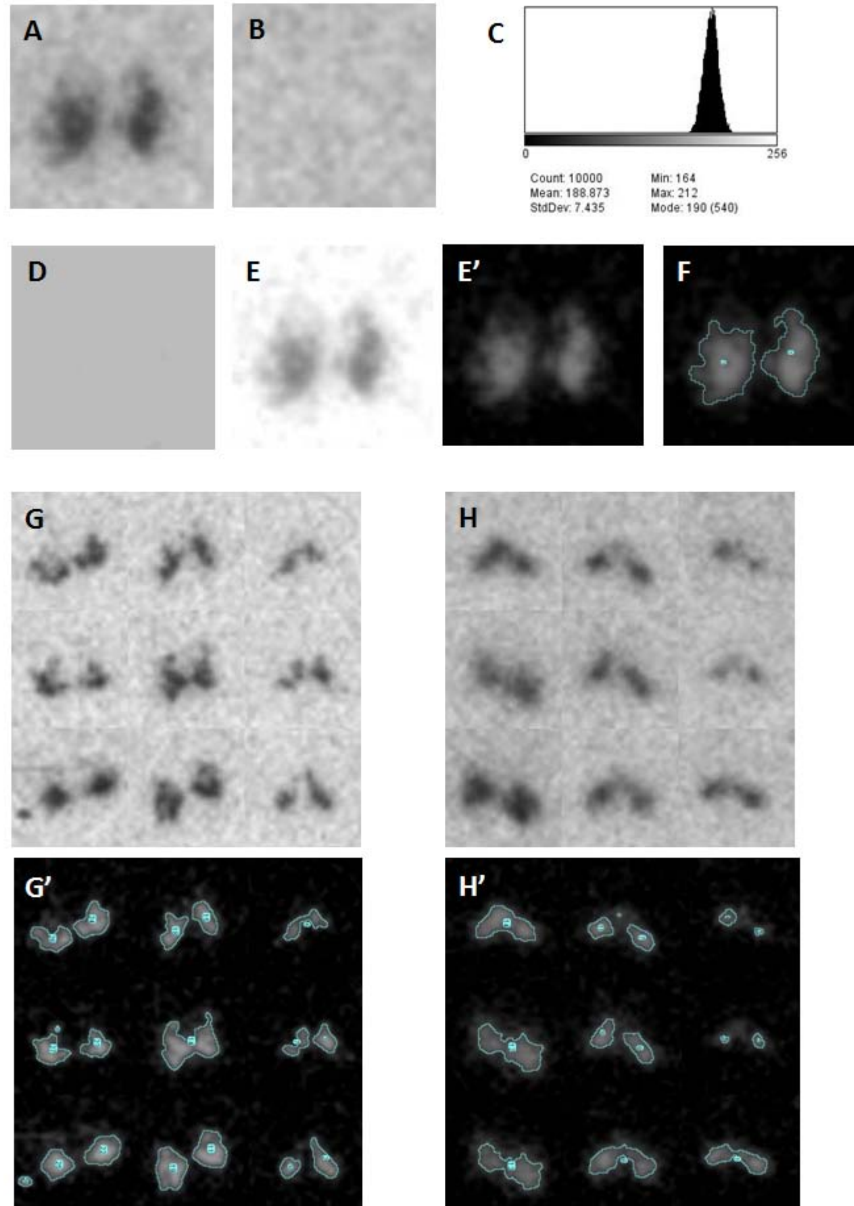


Figure 3.1
To develop a method for automated analysis of film densitometry, we performed *in situ* hybridization for *Kiss1* mRNA expression. Autoradiographic films were scanned at 2,400 dpi, and a region-of-interest (ROI) was cropped from the hypothalamus **(A)**. An adjacent section without *Kiss1* mRNA labeling (background) was also obtained **(B)**. The mean intensity of the background section **(C)** was measured using NIH ImageJ and applied uniformly to a newly generated section **(D)**. The background **(D)** was then subtracted from the ROI **(A)** to yield a resultant background-corrected image **(E)**. The background-corrected image can be inverted for visual contrast **(E')**. Pixels with ten-fold higher intensity than background were identified **(F)**. Multiple sections from two brains were obtained **(G, H)**. **(H)** had noticeably higher background intensity than **(G)**. After background subtraction, these ROIs were comparable in intensity **(G' and H')**. Densitometric measurements were calculated based on the [mean intensity within ROI] x [area of ROI] and summed across all brain sections.

We compared quantification of mRNA expression by autoradiographic emulsion versus film. We used *Kiss1* mRNA expression to validate the densitometric quantification methodology. By autoradiographic emulsion, *Kiss1* mRNA levels were decreased 3.3-fold after stress (**Figure 3.2a**), and by film densitometry, *Kiss1* mRNA levels were decreased 2.8-fold after stress (**Figure 3.2b**). We also used a physiologic system to study the validity of densitometric quantification. The AVPV nucleus is sexually dimorphic, and the number of kisspeptin neurons in the AVPV is higher in females than in males [96]. We performed *in situ* hybridization for *Kiss1* mRNA in the AVPV of male and female mice and quantified expression by autoradiographic film densitometry. Expression of *Kiss1* mRNA was significantly higher in the female compared to male AVPV nucleus (**Figure 3.2c**).

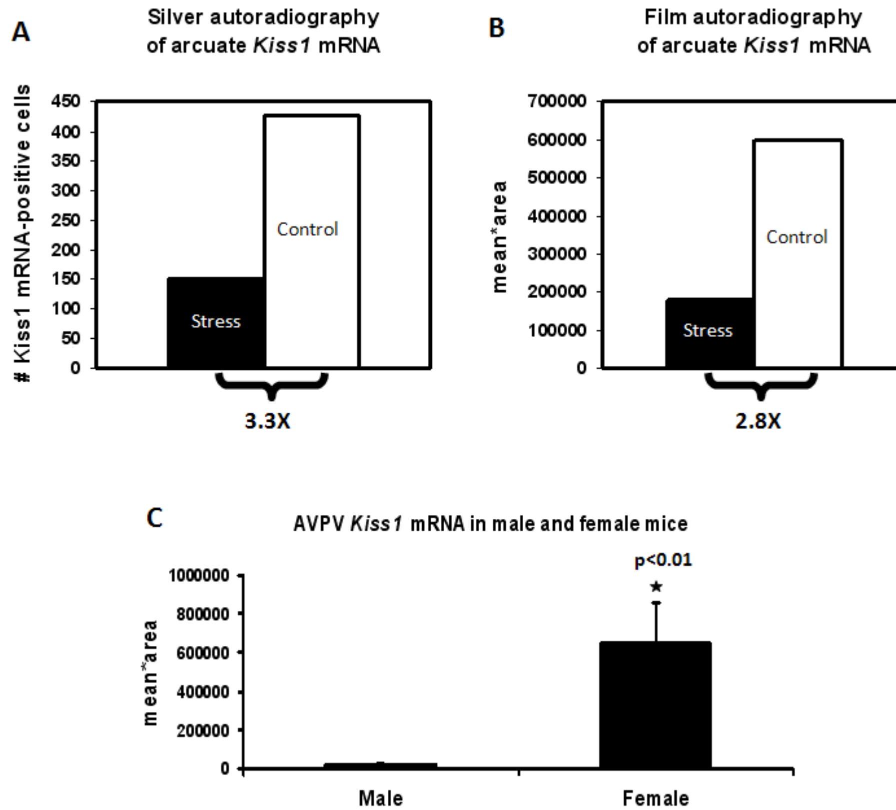


Figure 3.2
We used quantitative densitometry to measure *Kiss1* mRNA expression by *in situ* hybridization and verified the technique by using two physiologic models. **(A)** By autoradiographic emulsion, a well-established technique for quantification of *Kiss1* mRNA, *Kiss1* mRNA levels were 3.3 times lower in stressed compared to unstressed mice. **(B)** By quantitative densitometry, *Kiss1* mRNA was 2.8 times lower in stressed compared to unstressed mice. **(C)** Kisspeptin expression is sexually dimorphic, and kisspeptin expression is significantly higher in the female AVPV. By quantitative film densitometry, *Kiss1* mRNA expression was significantly higher in female than male AVPV.

Fertility assays

Testosterone was assyed at 6 weeks of age in males. Because of the large plasma volume required for LH and estradiol assays required terminal experiments, phlebotomies were conducted at 20 weeks. Vaginal lavages were obtained from adult females, slide mounted, and Geimsa stained (Fisher 22122911) for estrous cycle analysis. At 10 weeks of age, each experimental animal was paired 1:1 with a WT mate that had previously proven fertile. Parturition was monitored daily, and pups were euthanized at P1-3 and genotyped to confirm parentage.

Metabolic assays

Body weight was monitored from 5-18 weeks in males and 5-10 weeks in females. Because mating assays were conducted at 10 weeks of age and females gained weight during pregnancy thereafter, female body weight was not analyzed after 10 weeks. We also assayed body weight in adrenalectomized mice. We performed bilateral adrenalectomy on leptin-deficient (OBADX) and WT mice at 4 weeks of age, obtained plasma at 6 weeks of age, and sacrificed animals at 10 weeks of age. Dual-energy X-ray absorptiometry scans were performed on mice anesthetized by isoflurane at 5 and 10 weeks, and body fat composition was analyzed by using Lunar PIXImus software. Food intake was measured over a 24 h period at 5 and 10 weeks of age. Rebound hyperphagia is a phenomenon whereby an animal eats more than typical after a bout of food deprivation. In pilot studies, we measured baseline food intake in CRHOB and OB mice for 24 h, fasted animals for 24 h, then measured food intake for 24 h in the rebound period [250]. Animals were tested at 10 weeks of age.

In pilot studies, we examined energy metabolism by using the Comprehensive Laboratory Animal Monitoring System (CLAMS), which monitors oxygen consumption, carbon dioxide release, food and water consumption, and beam break activity. Monitoring occured in single-animal metabolic cages, and mice were acclimated to single housing in home cages for 3 d, then acclimated to metabolic cages for 2 d, and monitored subsequently for 48 h. Measurements were binned into and averaged as 12 h light and 12 h dark periods. We assessed energy metabolism by indirect calorimetry based on calculations of the respiratory exchange ratio (RER) for carbohydrate versus fatty acid oxidation:

Carbohydrate oxidation

$6\ O_2 + C_6H_{12}O_6 \rightarrow 6\ CO_2 + 6\ H_2O + 38\ ATP$

$RER = V_{CO2}/V_{O2} = 6\ CO_2/6\ O_2 = 1.0$

Fatty acid oxidation

$23\ O_2 + C_{16}H_{32}O_2 \rightarrow 16\ CO_2 + 16\ H_2O + 129\ ATP$

$RER = V_{CO2}/V_{O2} = 16CO_2/12O_2 = 0.7$

Corticosterone treatment

From 6 – 8 weeks, male mice were administered 30 μg/mL corticosterone (Sigma C2505, St. Louis, MO) in drinking water. After 1 week of corticosterone treatment, plasma was collected by retroorbital phlebotomy at 7AM (lights on) in unanesthetized animals and analyzed for corticosterone and triglyceride concentrations. Body weight was measured before and after corticosterone treatment. Males treated with corticosterone were paired 1:1 with a females that

had previously proven fertile. After corticosterone treatment was terminated, animals were again paired 1:1 with a WT mate. Parturition was monitored daily, and pups were euthanized at P1-3 and genotyped to confirm parentage.

Transgenic mice and breeding strategy

To study the role of GR in kisspeptin neurons in leptin deficiency, we generated $Kiss1^{CreBAC}::GR^{flox/flox}::Lep^{Ob/Ob}$ mice. Generation and validation of $Kiss1^{CreBAC}::GR^{flox/flox}$ mice were described in Chapter 2. All animals used in experiments were on a C57BL/CBA mixed background. Crosses from $Kiss1^{CreBAC}::GR^{flox/flox}::Lep^{Ob/+}$ x $GR^{flox/flox}::Lep^{Ob/+}$ breeders yielded $Kiss1^{CreBAC}::GR^{flox/flox}::Lep^{Ob/Ob}$ males and females at frequencies of 6.25% each. 6 male $Kiss1^{CreBAC}::GR^{flox/flox}::Lep^{Ob/Ob}$ and 2 female $Kiss1^{CreBAC}::GR^{flox/flox}::Lep^{Ob/Ob}$ mice were generated and studied.

To study the role of GR in the brain in leptin deficiency, we generated $Blbp^{Cre}::GR^{flox/flox}::Lep^{Ob/Ob}$ mice. $Blbp^{Cre}$ mice, in which Cre recombinase expression is driven by the *brain lipid binding protein* promoter, were generously provided by David Gutmann [251]. All animals used in experiments were on a C57BL background. Crosses from $Blbp^{Cre}::GR^{flox/flox}::Lep^{Ob/+}$ x $GR^{flox/flox}::Lep^{Ob/+}$ breeders yielded $Blbp^{Cre}::GR^{flox/flox}::Lep^{Ob/Ob}$ males and females at frequencies of 6.25% each. 4 male $Blbp^{Cre}::GR^{flox/flox}::Lep^{Ob/Ob}$ and 6 female $Blbp^{Cre}::GR^{flox/flox}::Lep^{Ob/Ob}$ mice were generated and studied.

Statistical tests

One-way ANOVA with *post hoc* least significant difference (LSD) pairwise testing was performed to compare adrenal cortex width, plasma corticosterone, plasma leptin, food intake, body fat composition, testicular weight, plasma testosterone, corpora lutea number, uterine width, plasma LH, and plasma estradiol. Linear mixed model analyses were performed to compare changes in body weight over time by genotype. Two-tailed two-sample t-tests were performed to compare plasma corticosterone in corticosterone-treated versus untreated mice. Fisher's exact test was performed to compare fertility in males, females, and corticosterone-treated males.

Genotyping

Kiss1^{CreBAC} and GR$^{flox/flox}$ PCR protocols were described in Chapter 2. CRHKO and OB genotyping primers and polymerase chain reaction protocols are described:

CRHKO

Crh$^{+/+}$ forward primer 5' - GAG CTT ACA CAT TTC GTC C - 3'

Crh$^{+/+}$ reverse primer 5' - GCT CAG CAA GCT CAC AGC - 3'

Crh$^{-/-}$ reverse primer 5' - ATC GCC TTC TTG ACG AGT T- 3'

96°C for 1', (96°C for 30", 62°C for 30", 72°C for 1'30") x 34, 72°C for 5', 4°C

WT band is 450 bp, and CRHKO band is 600 bp.

OB

Ob forward primer 5’ - ACT GGT CTG AGG CAG GGA GCA - 3'

Ob reverse primer 5’ - TGT CCA AGA TGG ACC AGA CTC - 3'

94°C for 3’, (94°C for 30”, 62°C for 1”, 72°C for 45”) x 36, 72°C for 2’, 10°C

Amplicon is digested with the restriction enzyme Dde I overnight at 37°C.

WT band is 155 bp, and OB band is 100 bp and 55 bp.

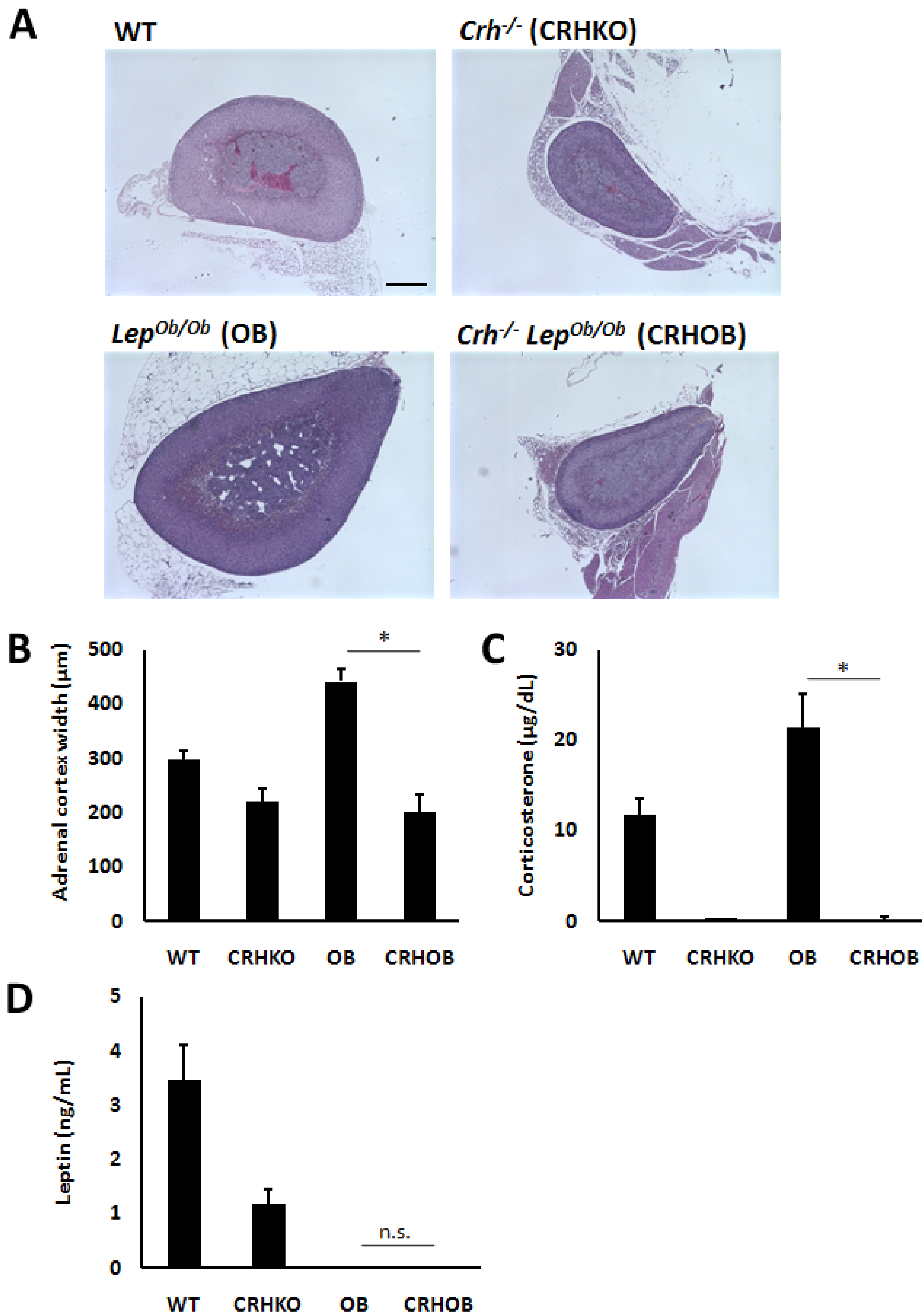
3.4 RESULTS

To study the role of glucocorticoids in leptin deficiency, we generated CRH-, glucocorticoid-, and leptin-deficient *Crh*$^{-/-}$ *Lep*$^{Ob/Ob}$ (CRHOB) mice for comparison to wild-type (WT), *Crh*$^{-/-}$ (CRHKO), and *Lep*$^{Ob/Ob}$ (OB) controls. Compared to WT mice, the adrenal cortex was hypertrophied in OB mice (**Figure 3.3a, b**), consistent with hypercortisolism in these mice (**Figure 3.3c**). Adrenal cortex size and plasma corticosterone concentrations were comparable in CRHKO and CRHOB mice and significantly decreased compared to OB and WT mice (**Figure 3.3a-c**). Plasma leptin was undetectable in OB and CRHOB and significantly decreased compared to WT and CRHKO mice (**Figure 3.3d**).

Figure 3.3

Adrenal development, plasma glucocorticoids, and plasma leptin were evaluated in WT, CRHKO, OB, and CRHOB mice. **(A)** Midline adrenal sections of the adrenal gland were stained by H&E. Scale bar, 250 µm. **(B)** Adrenal cortex width was significantly decreased in CRHKO ($p=0.02$, LSD *post hoc* test) and CRHOB ($p=0.03$, LSD *post hoc* test) compared to WT mice ($p=6.5 \times 10^{-6}$, one-way ANOVA) and significantly increased in OB mice ($p=1.9 \times 10^{-4}$, LSD *post hoc* test). Adrenal cortex width was significantly decreased in CRHOB compared to OB mice ($p=2.2 \times 10^{-5}$, LSD *post hoc* test). **(C)** Glucocorticoid concentrations were significantly decreased in CRHKO ($p=0.003$, LSD *post hoc* test) and CRHOB ($p=0.01$, LSD *post hoc* test) compared to WT mice ($p=3.0 \times 10^{-5}$, one-way ANOVA) and significantly increased in OB mice ($p=0.006$, LSD *post hoc* test). Plasma corticosterone was significantly decreased in CRHOB compared to OB mice ($p=8.5 \times 10^{-5}$, LSD *post hoc* test). **(D)** Plasma leptin was undetectable in OB ($p=7.0 \times 10^{-6}$, LSD *post hoc* test) and CRHOB ($p=3.0 \times 10^{-5}$, LSD *post hoc* test) compared to WT animals (1.2×10^{-5}, one-way ANOVA) and not different from one another ($p=1.0$, LSD *post hoc* test).

Figure 3.3, continued



Patients with Cushing's disease, a condition characterized by hypercortisolemia, and those treated with exogenous glucocorticoids experience weight gain, and we hypothesized that elevated glucocorticoids in OB mice contribute to obesity. Body weight was significantly decreased in CRHOB compared to OB males and females (**Figure 3.4a, b**). Body weight in CRHOB males was comparable to that of adrenalectomized OB males (**Figure 3.5**). Hyperphagia was reduced in CRHOB compared to OB males and females (**Figure 3.6a, c**). Body fat composition was reduced in CRHOB compared to OB males and females (**Figure 3.6b, d, e**). Body weight, food intake, and body fat composition were comparable in WT and CRHKO animals, and CRHOB animals did not completely normalize to WT and CRHKO levels.

The weight loss observed in CRHOB animals was due, at least in part, to decreased food intake, and in pilot studies, we examined whether basal metabolic rate, estimated by the respiratory exchange ratio (RER), was normalized as well. Because sample sizes were small (n=2-3) and not all genotypes were represented, we did not perform statistical analyses for these pilot studies. Mice are nocturnally active, and during the night, preferentially oxidize carbohydrates as an energy source (i.e., RER $\approx$ 1). During the day, mice shift towards fatty acid oxidation (i.e., RER $\approx$ 0.7). Control CRHKO animals exhibited this circadian change in energy utilization, but OB animals did not (**Figure 3.7a**). In OB mice, energy utilization was skewed towards fatty acid oxidation (RER $\approx$ 0.7) in the nocturnal activity period (**Figure 3.7a**), consistent with lower RER observed in leptin receptor-deficient *db/db* animals during the dark period [252]. CRHOB mice were more similar to CRHKO controls, oxidizing carbohydrates in the activity period and fatty acids in the quiescent period (**Figure 3.7a**). Locomotor activity at night, assayed as the sum of beam breaks in the x-, y-, and z-axes, was significantly lower in OB mice compared to in

CRHKO controls (**Figure 3.7b**). CRHOB animals had slightly elevated locomotor activity compared to OB but not CRHKO animals. These preliminary results suggested that the amelioration of body weight in CRHOB mice was due in part to changes in basal energy metabolism.

Rebound hyperphagia is a phenomenon whereby an animal eats more than typical after a bout of food deprivation. Food deprivation is known to cause a rise in plasma corticosterone (**Figure 2.7**), and we hypothesized that increased glucocorticoid and decreased leptin levels together facilitate rebound hyperphagia. Thus, the leptin-deficient animal may be considered to be in a chronic state of perceived starvation, which would elicit chronic rebound hyperphagia. In CRHOB animals with decreased leptin but without increased glucocorticoids, we predicted that rebound hyperphagia would be ameliorated, both at baseline and following food deprivation. This diminution of rebound hyperphagia, perhaps as a result of reduced glucocorticoid secretion, would partly explain the body weight reduction in CRHOB animals. In WT mice, 24 h food intake after a 24 h fast was increased by 50% compared to baseline [250]. In pilot studies, we found that in leptin-deficient mice with intact glucocorticoid secretion, rebound feeding was also 50% higher than baseline (**Figure 3.8**). In leptin-deficient, glucocorticoid-deficient mice, rebound feeding was only 28% higher than at baseline (**Figure 3.8**). Rebound hyperphagia is reduced in CRHOB mice, and glucocorticoid-deficiency may ameliorate hyperphagia in leptin-deficient animals. Because sample sizes were small (n=1-3) and not all genotypes were represented, we did not perform statistical analyses for these pilot studies.

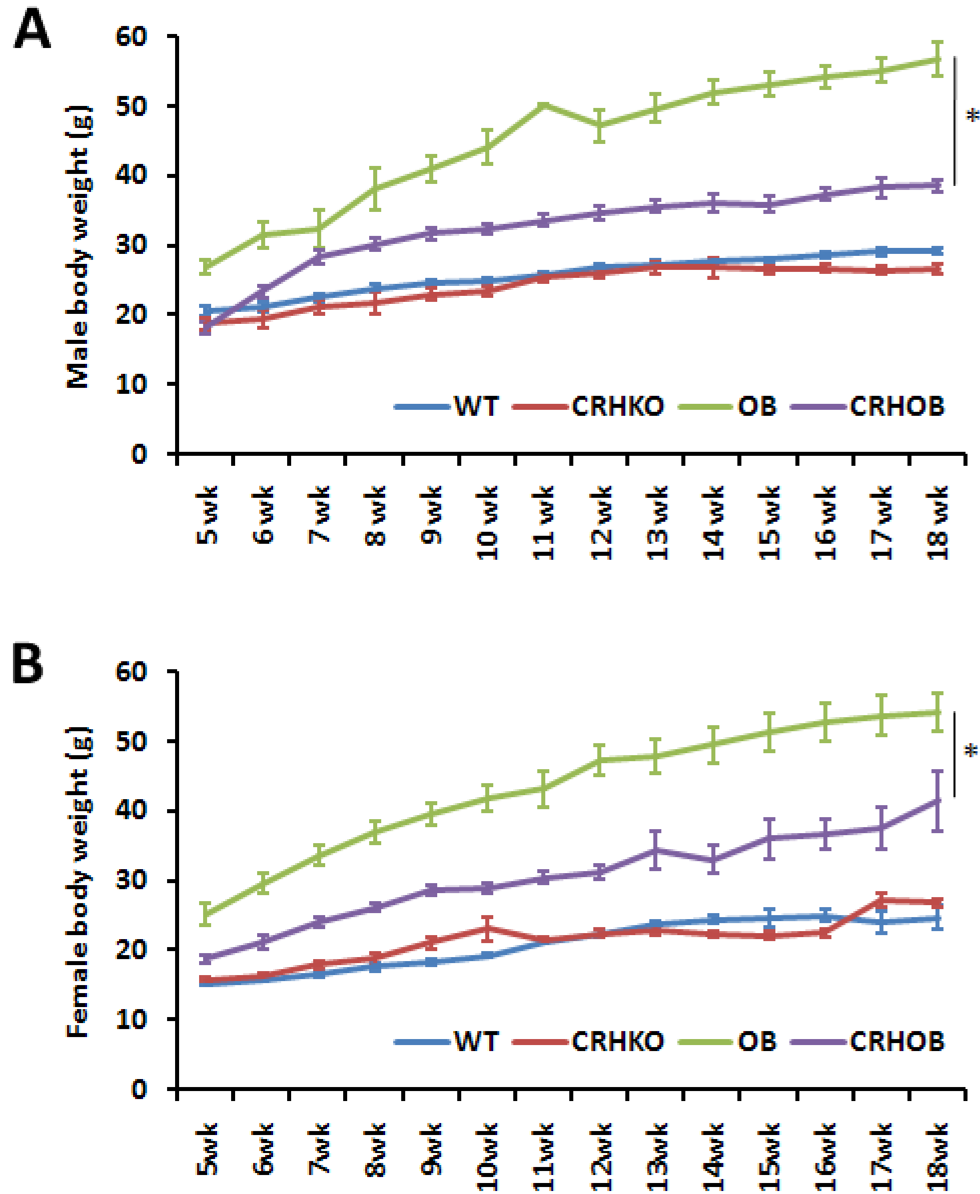


Figure 3.4
(A) Body weight was significantly decreased in CRHOB compared to OB males ($p=1.4x10^{-5}$, linear mixed model $F_{13, 94}=4.3$). **(B)** Body weight was significantly decreased in CRHOB compared to OB females ($p=4.8x10^{-4}$, linear mixed model $F_{5, 80}=5.0$).

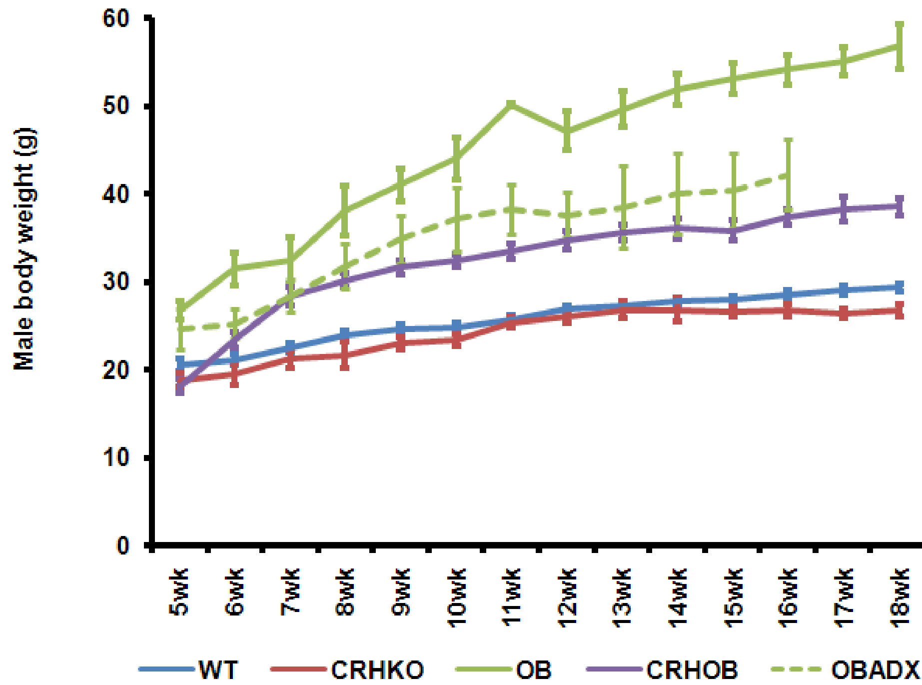


Figure 3.5
In pilot studies, we performed bilateral adrenalectomy on leptin-deficient (OBADX) at 4 weeks of age. Surgical adrenalectomy was reported to cause weight loss in OB mice. Body weight was decreased in OBADX compared to OB animals ($p<0.05$) and comparable to levels seen in CRHOB animals ($p>0.05$).

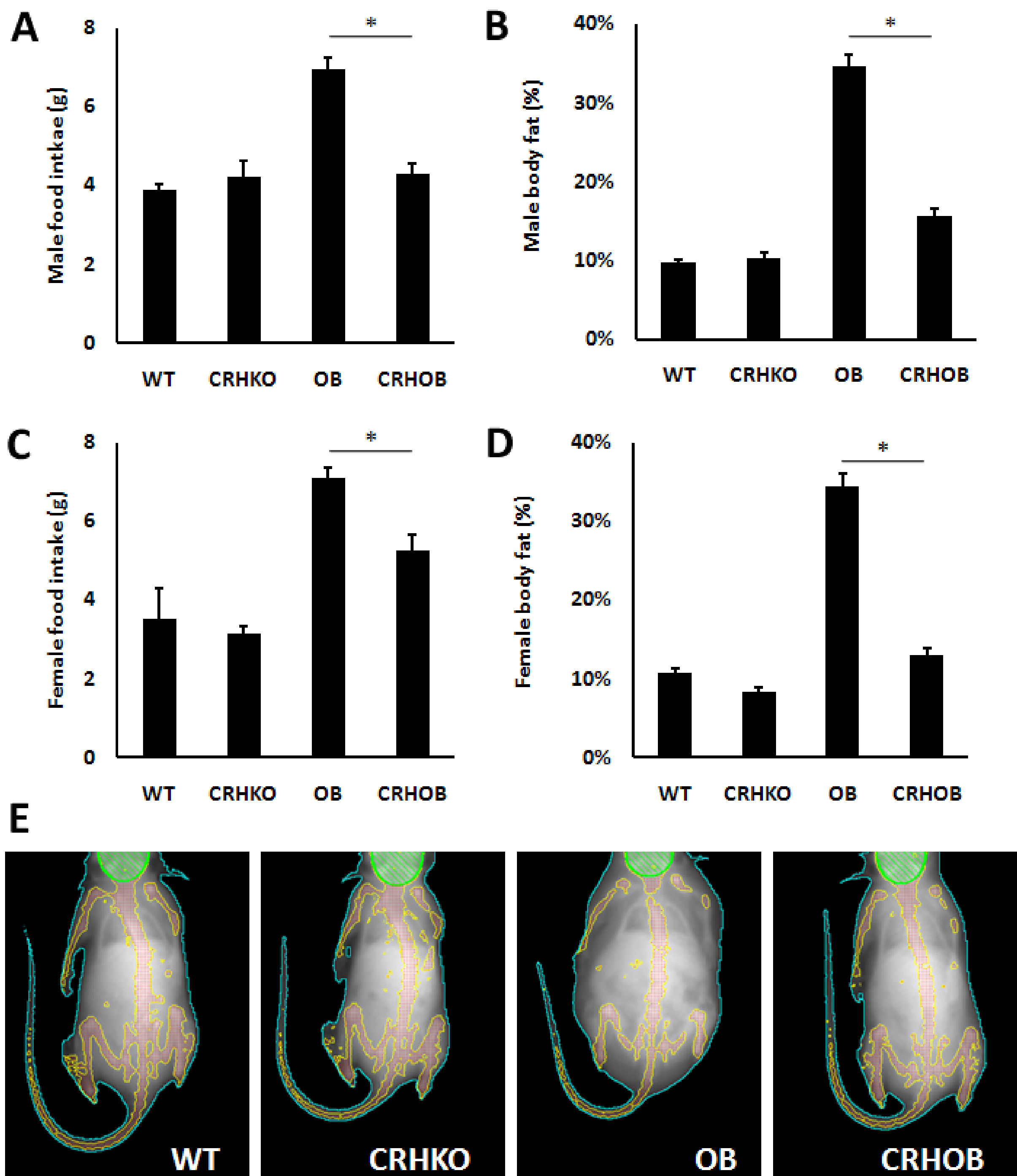


Figure 3.6
(A) Hyperphagia was reduced in CRHOB compared to OB males ($p=4.9x10^{-4}$, LSD *post hoc* test). **(B)** Body fat composition was reduced in CRHOB compared to OB males ($p=1.7x10^{-11}$, LSD *post hoc* test). **(C)** Hyperphagia was reduced in CRHOB compared to OB females (p=0.002, LSD *post hoc* test). **(D)** Body fat composition was reduced in CRHOB compared to OB females ($p=1.7x10^{-13}$, LSD *post hoc* test). **(E)** Representative DEXA body scans.

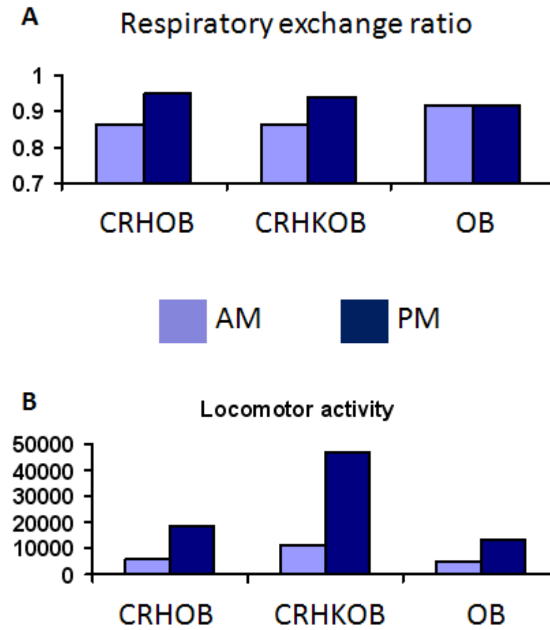


Figure 3.7
In pilot studies, CRHOB mice were monitored in metabolic cages. **(A)** CRHKO control animals oxidized carbohydrates during the night (RER ≈ 1) and fatty acids during the day (RER ≈ 0.7), but OB animals did not exhibit this circadian change in energy utilization. Energy utilization was skewed towards fatty acid oxidation during the nocturnal activity period in OB mice. CRHOB were more similar to CRHKO controls, oxidizing carbohydrates in the activity period and fatty acids in the quiescent period. **(B)** Locomotor activity at night, assayed as the sum of beam breaks in the x-, y-, and z-axes, was significantly lower in OB mice compared to in CRHKO controls. CRHOB animals had slightly elevated locomotor activity compared to OB but not CRHKO animals. Because sample sizes were small (n=2-3) and not all genotypes were represented, we did not perform statistical analyses for these pilot studies.

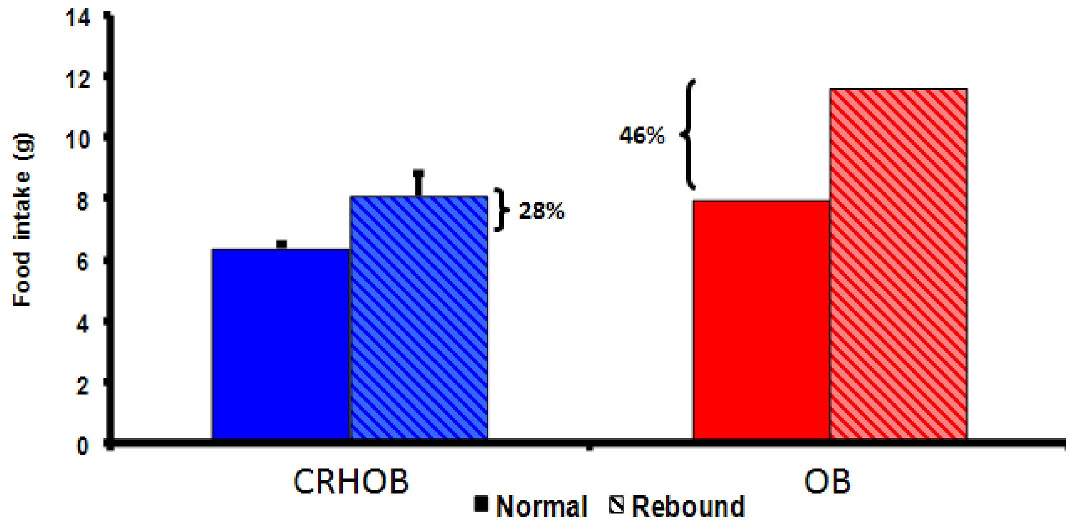


Figure 3.8
In pilot studies, food intake was measured in CRHOB and OB mice for 24 h at baseline ("normal") and after a 24 h fast ("rebound"). It has been shown that in WT mice, 24 h food intake after a 24 h fast is 50% increased compared to baseline. In leptin-deficient mice with intact glucocorticoid secretion, rebound feeding was about 50% higher than baseline food intake. In leptin-deficient, glucocorticoid-deficient mice, rebound feeding was only 28% higher than baseline. Because sample sizes were small (n=1-3) and not all genotypes were represented, we did not perform statistical analyses for these pilot studies.

Glucocorticoid excess also correlates with reproductive axis suppression [125, 129-131, 217], and we hypothesized that increased glucocorticoid secretion in OB mice contributes to infertility. Reproductive organs and hormones were evaluated in glucocorticoid-deficient, leptin-deficient mice. Testis size was significantly increased in CRHOB compared to OB males (**Figure 3.9a, b**). Testosterone trended upward in CRHOB compared to OB males (**Figures 3.9c**). Ovarian and uterine morphologies were normalized in CRHOB compared to OB females (**Figures 3.10, 3.11**). The number of corpora lutea was increased in CRHOB compared to OB females (**Figures 3.10, 3.12a**). Uterine width, plasma luteinizing hormone, and plasma estradiol trended upward in CRHOB compared to OB females (**Figure 3.12b-d**). CRHOB females exhibited normal estrous cycling (**Figure 3.13**). Not only were reproductive organs and hormones normalized in leptin-deficient, glucocorticoid-deficient CRHOB mice, these mice were able to produce offspring. CRHOB males sired litters more frequently than OB males (**Figure 3.14a**), and CRHOB females gave birth to litters more frequently than OB females (**Figure 3.14b**). CRHOB males crossed with CRHOB females were also fertile (n=2).

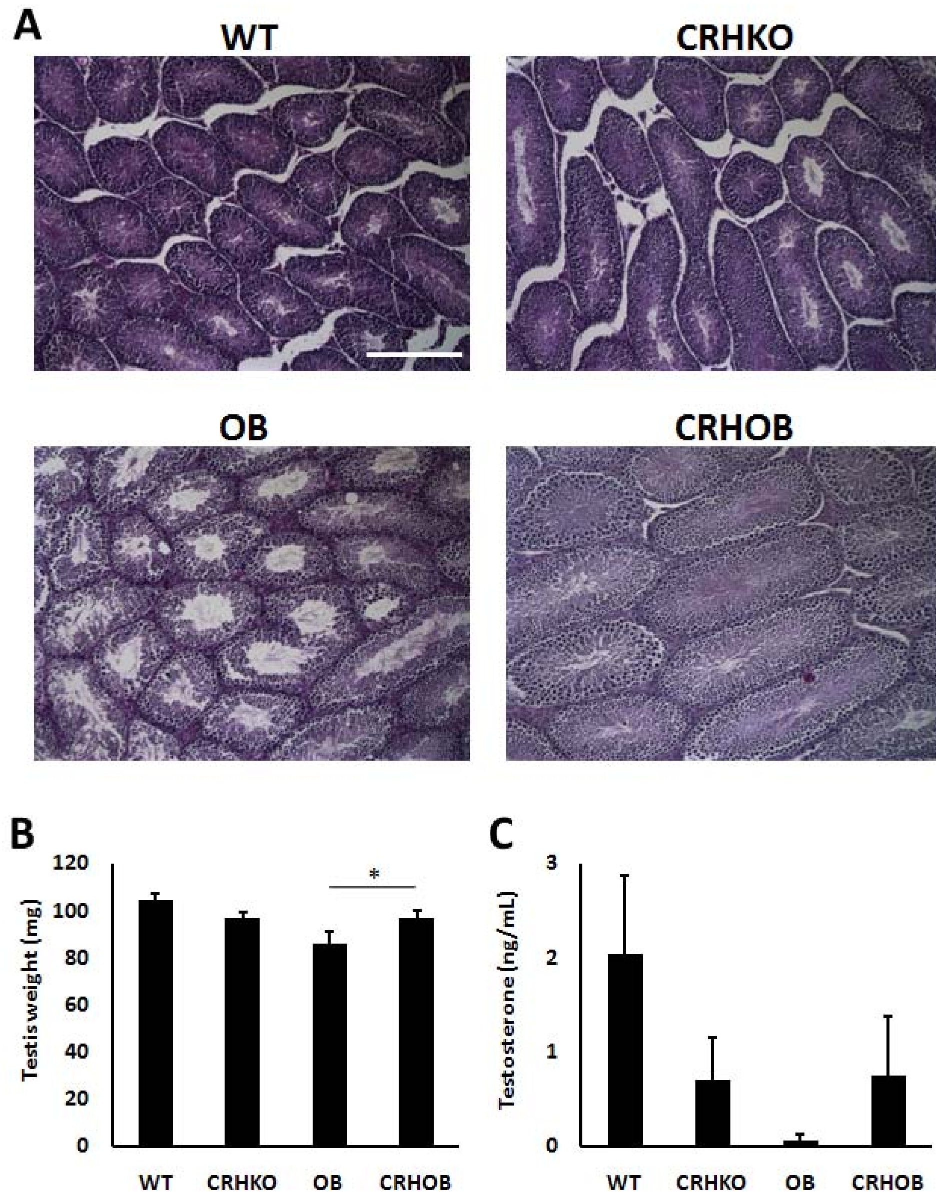


Figure 3.9
(A) Midline sections of the testis were stained by H&E. Scale bar, 250 µm. **(B)** Testis weight was significantly increased in CRHOB compared to OB males (p=0.04, LSD *post hoc* test; p=0.007, one-way ANOVA). **(C)** Testosterone trended upward in CRHOB compared to OB males (p=0.14, one-way ANOVA).

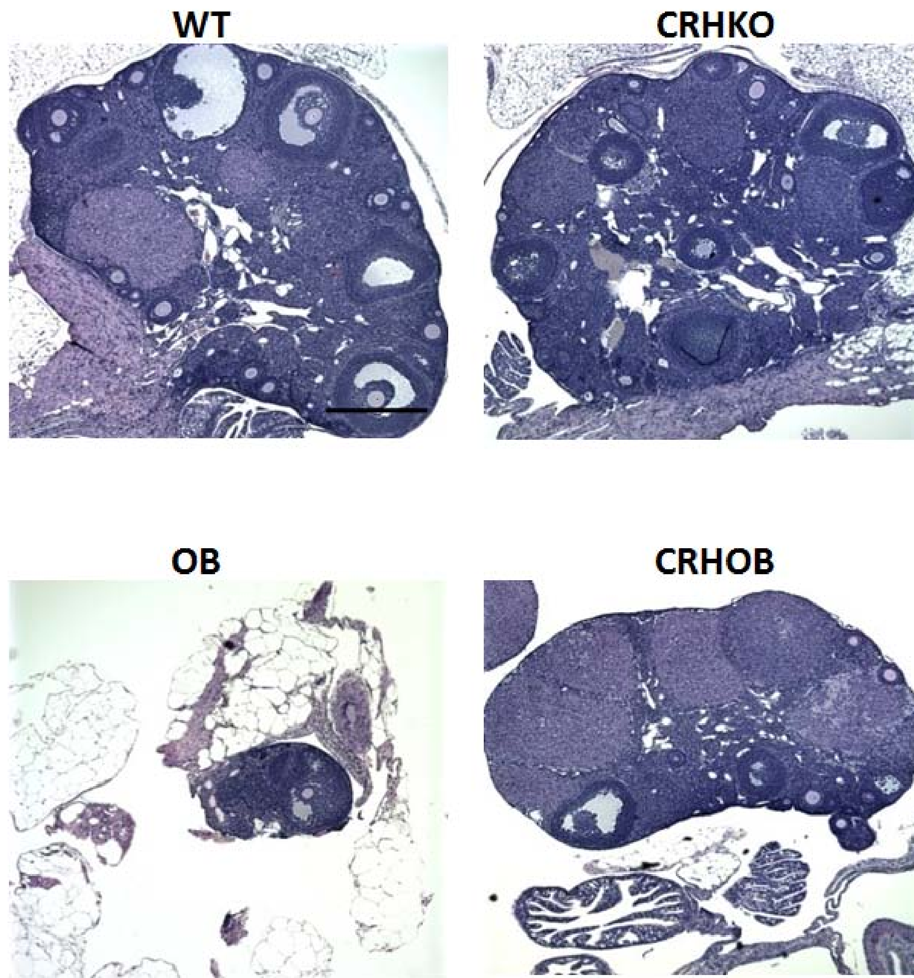


Figure 3.10
Midline sections of the ovary were stained by H&E. Scale bar, 500 μm. Ovary size was increased in CRHOB compared to OB females.

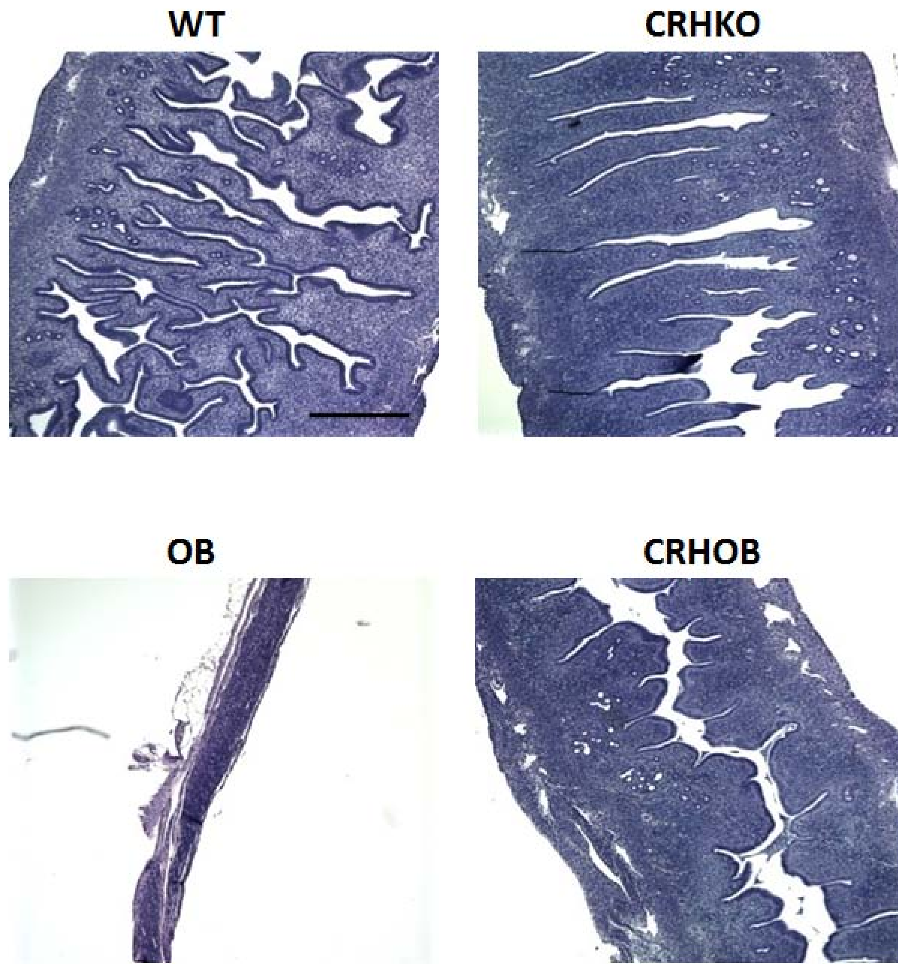


Figure 3.11
Midline sections of the uterus were stained by H&E. Scale bar, 500 µm. Uterus width was increased in CRHOB females compared to the thread-like uterus in OB females.

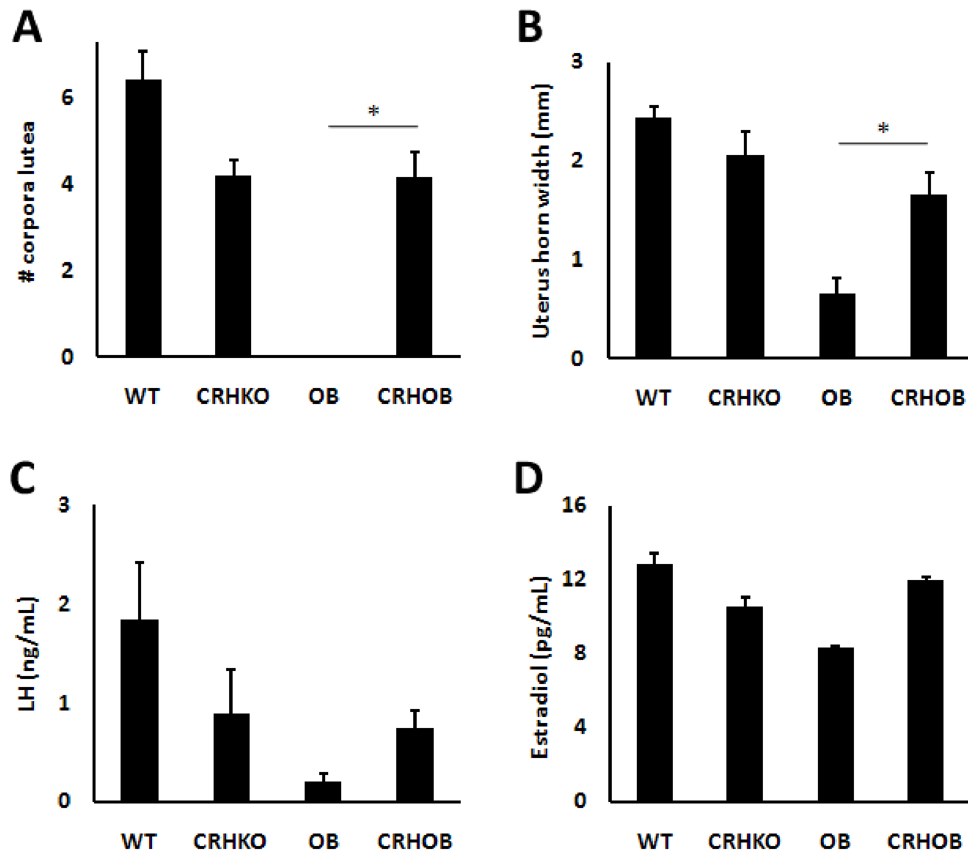
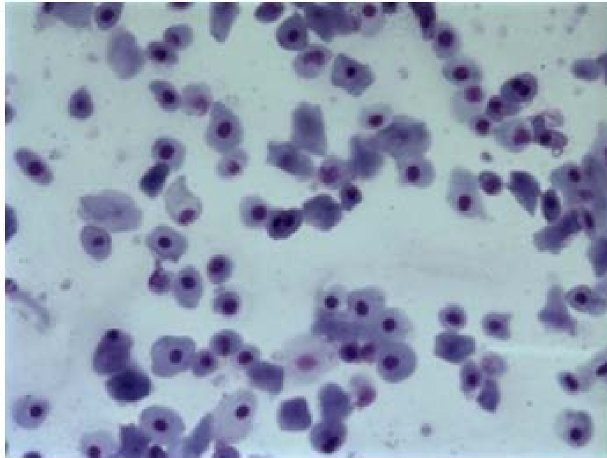
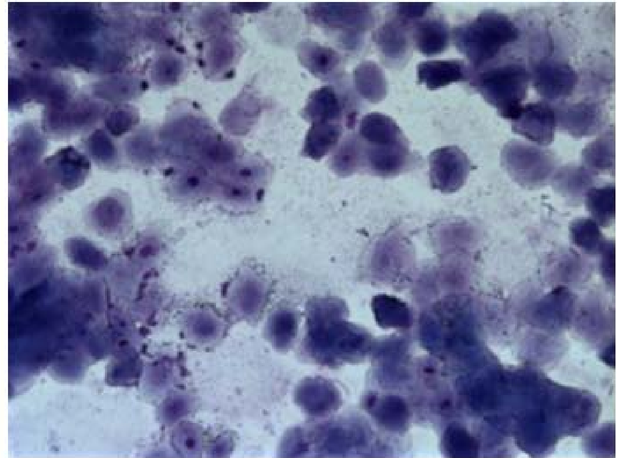


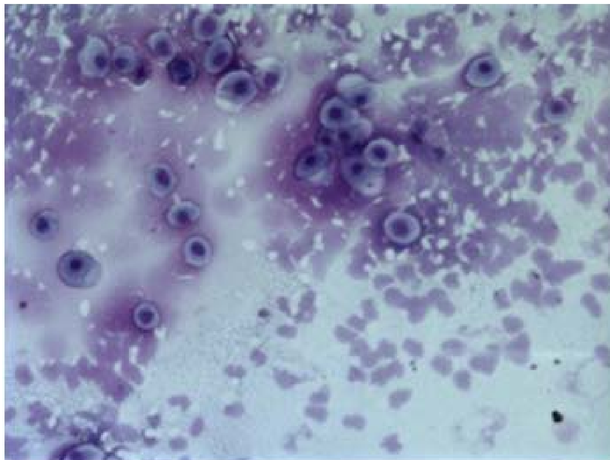
Figure 3.12
Reproductive hormones were evaluated in WT, CRHKO, OB, and CRHOB mice. **(A)** The number of corpora lutea in the ovary was significantly higher in CRHOB than OB females ($p=1.6 \times 10^{-6}$, LSD *post hoc* test; $p=6.1 \times 10^{-8}$, one-way ANOVA). **(B)** Uterine width was increased in CRHOB compared to OB females ($p=0.001$, LSD *post hoc* test; $p=2.7 \times 10^{-4}$, one-way ANOVA). **(C)** Luteinizing hormone trended upward in CRHOB compared to OB females ($p=0.07$, one-way ANOVA). **(D)** Estradiol trended upward in CRHOB compared to OB females ($p=0.10$, one-way ANOVA).



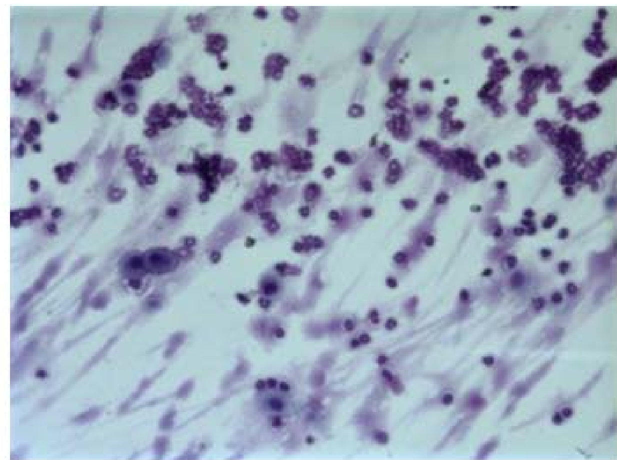
Proestrous



Estrous



Metestrous



Diestrous

Figure 3.13
CRHOB females underwent vaginal opening, a marker of puberty, and exhibited normal estrous cycling as adults. Daily vaginal lavages were obtained, slide mounted, and Geimsa stained.

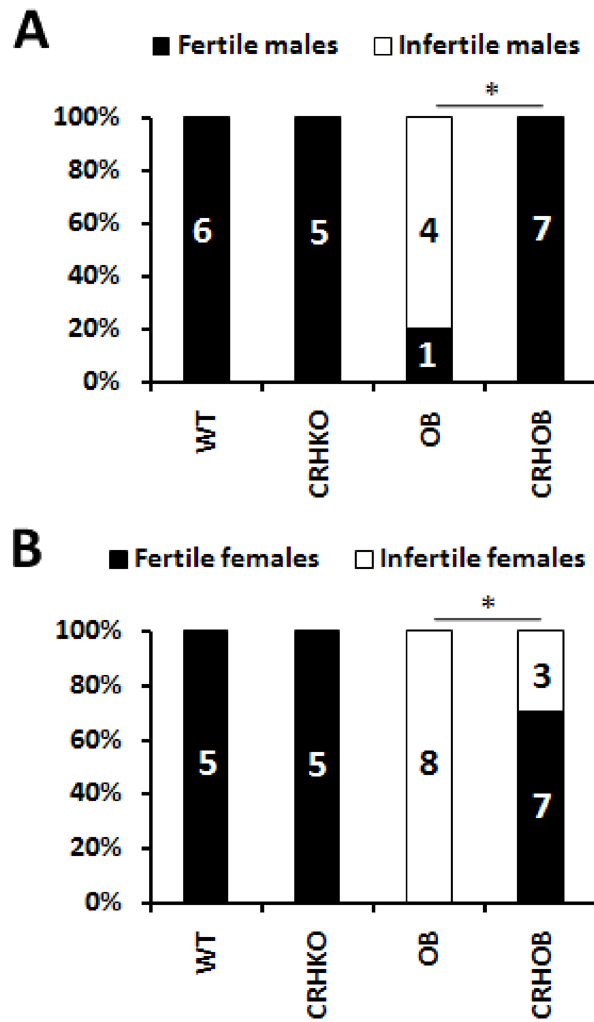


Figure 3.14
(A) CRHOB males sired litters more often than OB males (p=0.01, Fisher's exact test). **(B)** CRHOB females gave birth to litters more often than OB females (p=0.004, Fisher's exact test).

Surgical adrenalectomy, which removes the site of glucocorticoid synthesis, ameliorated obesity in leptin-deficient mice [247]. Adrenalectomy was previously reported to have no effect on LH, FSH, or testosterone [249]. We observed that testosterone was elevated in OBADX males (**Figure 3.15**). However, none of these males were able to sire litters after being paired 1:1 with female mice. By contrast, both WT adrenalectomized males and females successfully produced litters (n=6/6). Upon necropsy 6 weeks after surgery, macroscopic adrenal glands were visible in all OBADX animals. From these pilot studies, it was unclear whether glucocorticoid-mediated reproductive inhibition was rapidly restored following adrenalectomy and suppressed fertility.

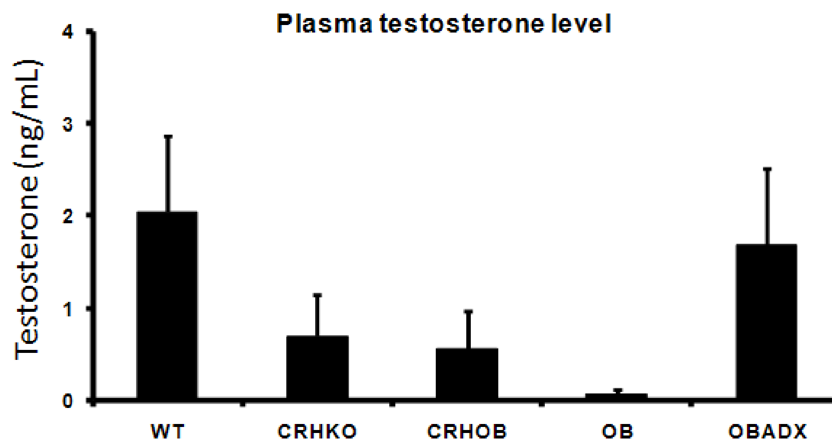


Figure 3.15
In pilot studies, we performed bilateral adrenalectomy on leptin-deficient (OBADX) at 4 weeks of age and obtained plasma at 6 weeks of age. Testosterone trended upward in OBADX compared to OB males.

Because CRHOB mice are CRH- and glucocorticoid-deficient, it is possible that CRH contributes to infertility [132] in leptin deficiency. However, *in situ* hybridization for *Crh* mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus showed no differences in *Crh* expression between WT and OB mice (**Figure 3.16a**). To examine whether changes in fertility were glucocorticoid-dependent, we next treated mice with 30 μg/mL of corticosterone in drinking water, which produced physiologic concentrations of plasma corticosterone in WT, CRHKO, OB, and CRHOB males (**Figure 3.16b**). Compared to untreated controls, plasma corticosterone was elevated only in corticosterone-treated CRHKO and CRHOB mice (**Figure 3.16b**). After 1 week of corticosterone supplementation, CRHOB males were unable to sire litters and became as infertile as OB males (**Figure 3.16c**). Following termination of corticosterone treatment, CRHOB males were fertile (**Figure 3.16d**).

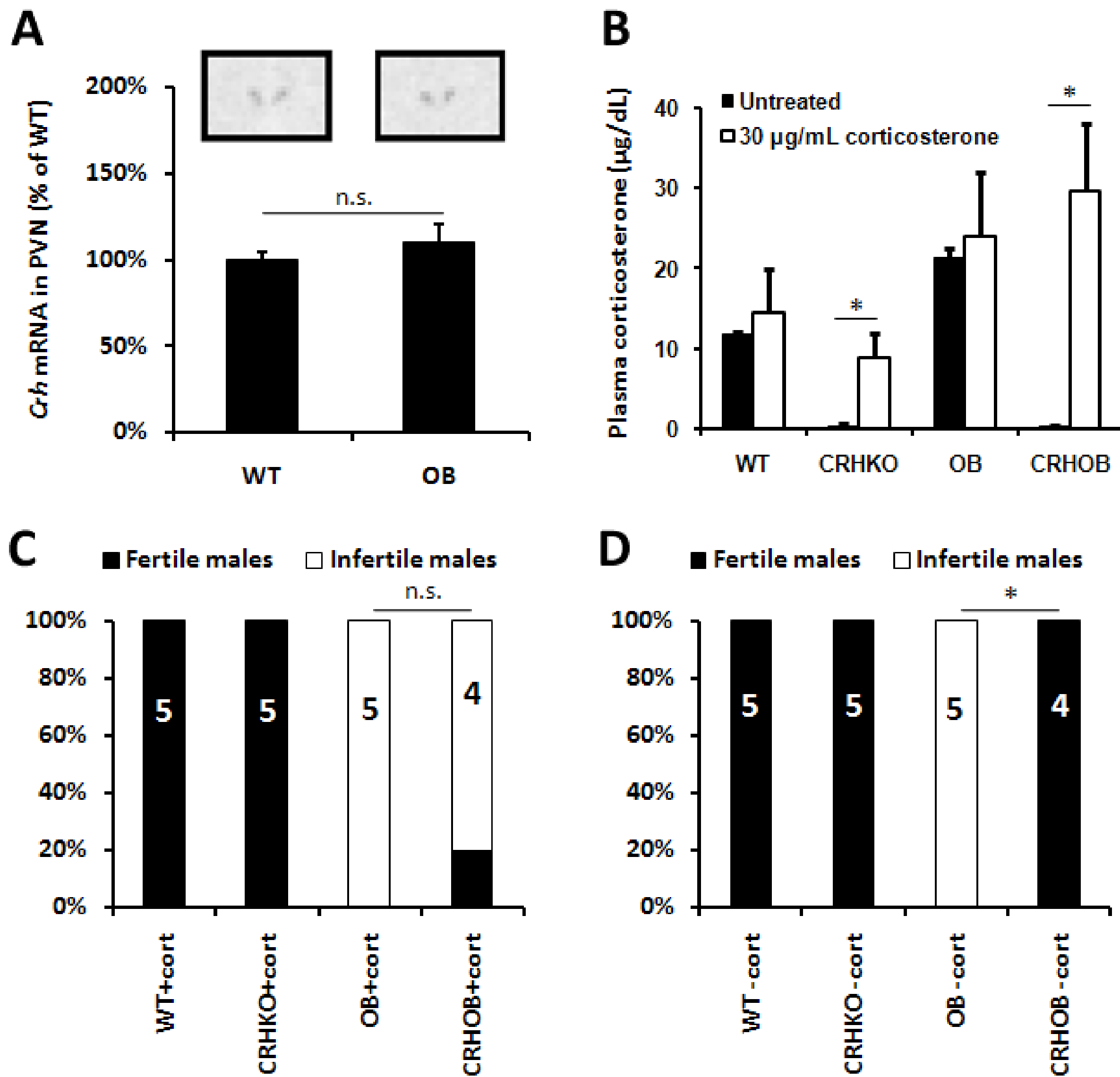


Figure 3.16
(A) *In situ* hybridization for *Crh* mRNA in the paraventricular nucleus (PVN) showed no differences between WT and OB mice ($p>0.05$; data courtesy of Satoru Sakihara). Insets, *in situ* hybridization for *Crh* mRNA in the PVN by autoradiographic film. **(B)** Male mice were supplemented with 30 µg/mL of corticosterone in drinking water. Compared to untreated controls, plasma corticosterone was elevated only in corticosterone-treated CRHKO ($p=0.04$, independent samples t-test) and CRHOB ($p=0.008$, independent samples t-test) mice. **(C)** After 1 week of supplementation with corticosterone, CRHOB males were unable to sire litters. Corticosterone-treated CRHOB males were as infertile as OB males ($p=0.99$, Fisher's exact test). **(D)** Following withdrawal of corticosterone, CRHOB males were once again able to sire litters compared to OB males ($p=0.008$, Fisher's exact test).

Because CRHOB mice are CRH- and glucocorticoid-deficient, it may be possible that CRH contributes to obesity in leptin deficient states. This is unlikely, however, because CRH is a known anorectic [248, 253, 254], and thus, CRH deficiency should promote orexigenic effects. The effect of corticosterone on body weight was evaluated in WT, CRHKO, OB, and CRHOB mice. Male mice were supplemented with 30 μg/mL of corticosterone in drinking water. Body weight was unchanged in corticosterone-treated WT, CRHKO, and OB males compared to untreated controls. Body weight was increased in corticosterone-treated compared to untreated CRHOB males (**Figure 3.17**). This increase in body weight persisted even after termination of corticosterone treatment (**Figure 3.17**). Plasma triglycerides were unchanged in corticosterone-treated WT and OB males compared to untreated controls (**Figure 3.18**). Plasma triglycerides were slightly but significantly elevated in corticosterone-treated CRHKO males (**Figure 3.18**). Plasma triglycerides increased approximately three-fold after corticosterone treatment in CRHOB males (**Figure 3.18**).

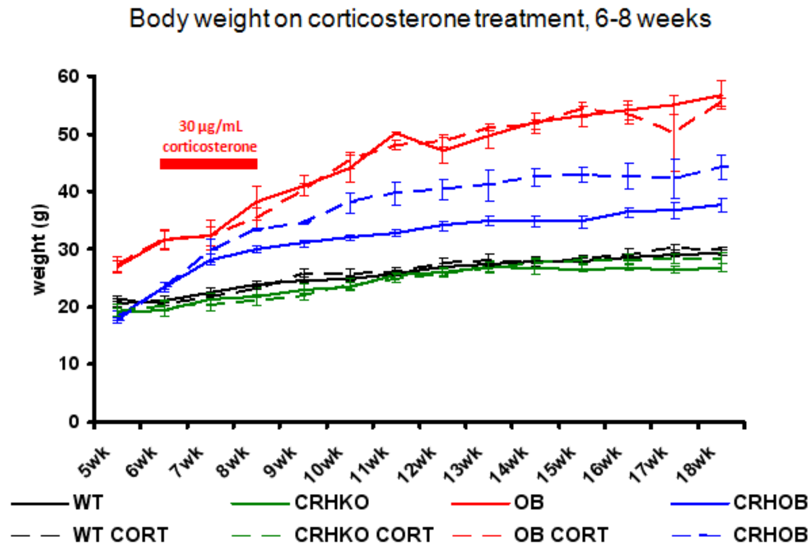


Figure 3.17
In pilot studies, we evaluated the effect of glucocorticoid treatment on body weight in WT, CRHKO, OB, and CRHOB mice. Male mice were supplemented with 30 µg/mL of corticosterone in drinking water. Body weight was unchanged in corticosterone-treated WT, CRHKO, and OB males compared to untreated controls. Body weight was increased in corticosterone-treated compared to untreated CRHOB males. This increase in body weight persisted even after termination of corticosterone treatment.

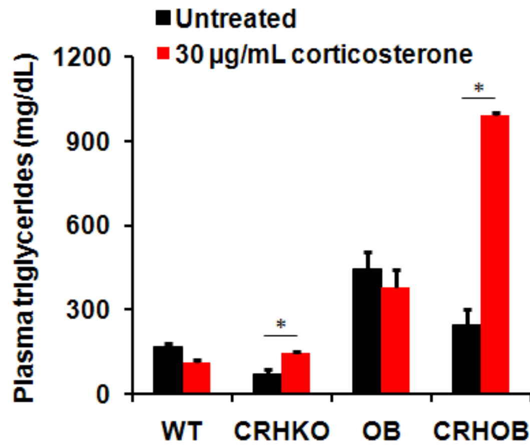


Figure 3.18
The effect of glucocorticoids on plasma triglycerides was evaluated in WT, CRHKO, OB, and CRHOB mice. Male mice were supplemented with 30 µg/mL of corticosterone in drinking water. Plasma triglycerides were unchanged in corticosterone-treated WT and OB males compared to untreated controls. Plasma triglycerides were slightly but significantly elevated in corticosterone-treated CRHKO males. Plasma triglycerides increased approximately three-fold after 1 week of corticosterone treatment in CRHOB males. * $p<0.05$.

To evaluate the role of kisspeptin neurons in the infertile and obese *Lep*$^{Ob/Ob}$ mouse, we first measured *Kiss1* mRNA expression in WT, CRHKO, OB, and CRHOB males. *Kiss1* mRNA expression trended downward in OB and CRHOB males compared to WT and CRHKO controls (**Figure 3.19a**). We generated leptin-deficient mice in which GR signaling was selective deleted from kisspeptin neurons. Thread-like uteri were observed in *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$*::Lep*$^{Ob/Ob}$ (Kiss GR OB) females, and neither males nor females were fertile (**Figure 3.19b**). Body weights (**Figure 3.19c**) and food intake (**Figure 3.19d**) in *Kiss1*^{CreBAC}*::GR*$^{flox/flox}$*::Lep*$^{Ob/Ob}$ mice were comparable to those of *Lep*$^{Ob/Ob}$ controls.

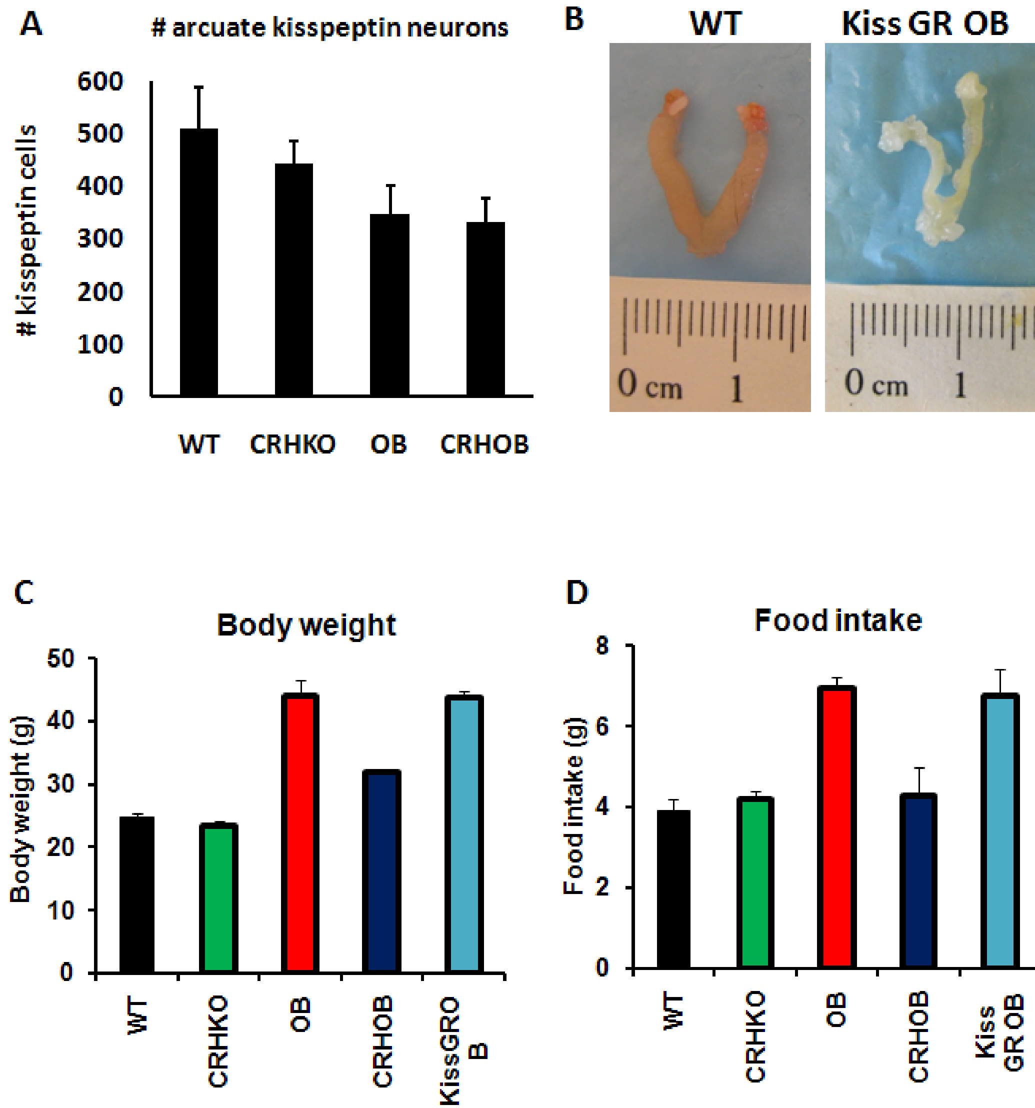


Figure 3.19
In pilot studies, we examined the role of kisspeptin neurons in mediating fertility in CRHOB animals. **(A)** *Kiss1* mRNA expression trended downward in OB and CRHOB males compared to WT and CRHKO controls (n=5-6 per genotype). **(B)** We generated $Kiss1^{CreBAC}$::$GR^{flox/flox}$::$Lep^{Ob/Ob}$ (Kiss GR OB) animals, in which GR was excised from kisspeptin neurons in leptin-deficient animals. Thread-like uteri were observed in Kiss GR OB females (n=2), and neither males nor females were fertile. **(C)** Body weight and **(D)** food intake in $Kiss1^{CreBAC}$::$GR^{flox/flox}$::$Lep^{Ob/Ob}$ male mice (n=6) were comparable to those of $Lep^{Ob/Ob}$ controls (n=5).

In studies of stress-induced reproductive inhibition in WT mice, we demonstrated that suppression of kisspeptin expression was not necessary for reproductive inhibition in cold stress (Chapter 2). Conversely, maintaining *Kiss1* mRNA expression at normal levels during stress in $Kiss1^{CreBAC}::GR^{flox/flox}$ mice did not protect animals from stress-induced suppression of testosterone and copulatory behaviors immediately after acute stress. GR signaling in kisspeptin neurons is unlikely to be the sole inhibitory signal to the HPG axis, as there are multiple glucocorticoid-dependent and -independent stress pathways that inhibit the reproductive axis acutely. For example, catecholamines can mediate direct testicular inhibition [235, 255], which is independent of both glucocorticoid and kisspeptin signaling. Such inhibitory inputs may suppress reproductive function in $Kiss1^{CreBAC}::GR^{flox/flox}::Lep^{Ob/Ob}$ mice despite the lack of GR-mediated inhibition of kisspeptin neurons. It may be interesting to assess whether *Kiss1* mRNA expression is affected in $Kiss1^{CreBAC}::GR^{flox/flox}::Lep^{Ob/Ob}$ mice, as we would predict that *Kiss1* mRNA expression remains unsuppressed in this model compared to $Lep^{Ob/Ob}$ controls.

$Kiss1^{CreBAC}::GR^{flox/flox}::Lep^{Ob/Ob}$ males and females were infertile and obese, suggesting that glucocorticoid receptor signaling in kisspeptin neurons did not regulate fertility or body weight in $Lep^{Ob/Ob}$ mice. Since deletion of GR in kisspeptin neurons did not restore fertility in $Lep^{ob/ob}$ mice, we examined whether neuronal glucocorticoid receptor signaling affected fertility or obesity. We did not use $Nestin^{Cre}$ for Cre-mediated excision of GR in the brain, because $Nestin^{Cre}::GR^{flox/flox}$ animals have decreased body weight [24] and would confound the interpretation of any body weight phenotype in a $Lep^{Ob/Ob}$ background. Instead, we obtained transgenic $Blbp^{CreBAC}$ mice [251], in which the <u>b</u>rain <u>l</u>ipid <u>b</u>inding <u>p</u>rotein promoter drives Cre expression in neural stem cells. We aimed to determine whether $Blbp^{CreBAC}$ would be a suitable

brain-specific driver that did not produce body weight changes in controls. Unlike *Nestin*^{Cre}::*GR*$^{flox/flox}$ mice, neither *Blbp*^{CreBAC} nor *Blbp*^{CreBAC}::*GR*$^{flox/flox}$ animals had altered body weight compared to WT littermates. We proceeded to generate *Blbp*^{CreBAC}::*GR*$^{flox/flox}$::*Lep*$^{Ob/Ob}$ animals, but body weight, food intake, and fertility were not normalized in these animals (**Figures 3.20, 3.21**). Because not all neurons were targeted by *Blbp*^{CreBAC}, however, it is unclear whether this reflects non-central mechanisms of glucocorticoid receptor signaling in fertility and obesity, or insufficient excision of GR in the brain by *Blbp*^{CreBAC}.

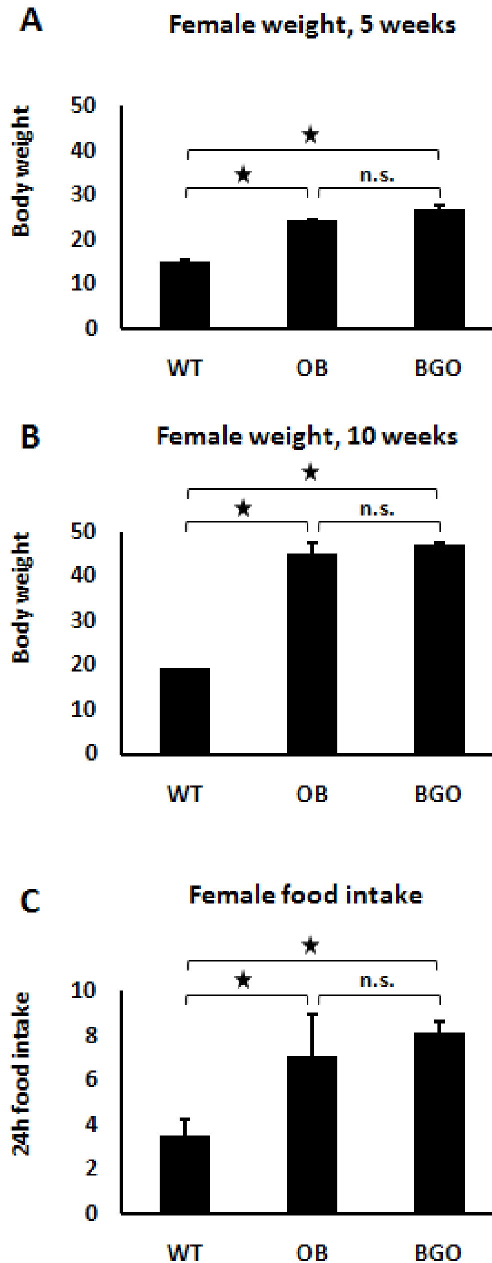


Figure 3.20
In pilot studies, we generated *Blbp*^{CreBAC}*::GR*$^{flox/flox}$*::Lep*$^{Ob/Ob}$ females (n=6), in which the brain lipid binding protein promoter drives Cre expression in neural stem cells and induces Cre-mediated GR excision in a leptin-deficient background. In females, (A) body weight at 5 weeks, (B) body weight at 10 weeks, and (C) food intake were comparable to those of *Lep*$^{Ob/Ob}$ animals. ★$p<0.05$.

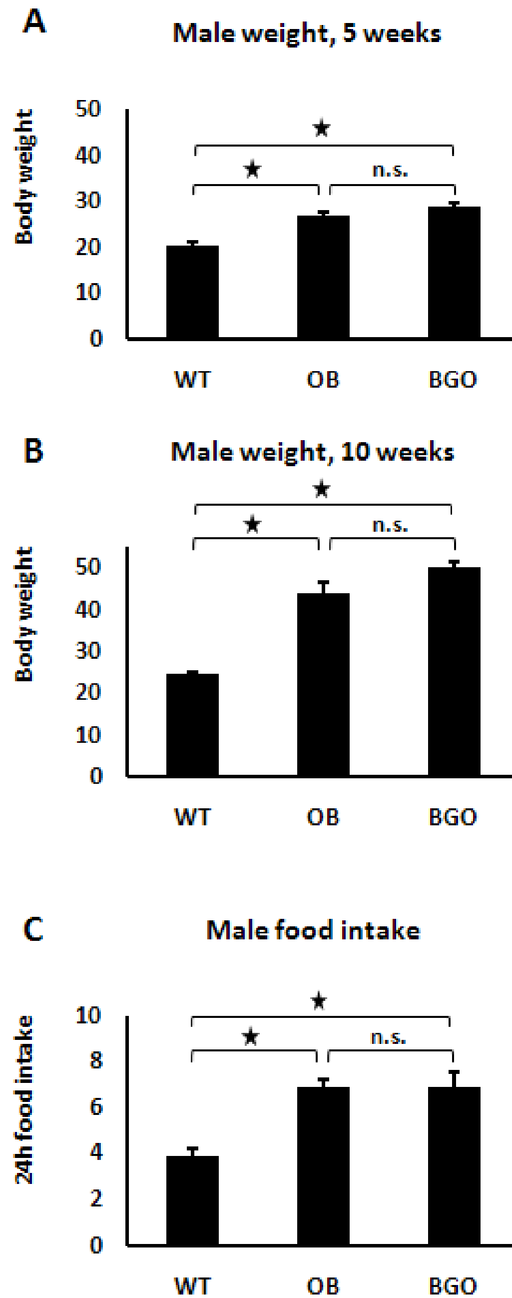


Figure 3.21
In pilot studies, we generated *Blbp*^{CreBAC}*::GR*$^{flox/flox}$*::Lep*$^{Ob/Ob}$ males (n=4), in which the brain lipid binding protein promoter drives Cre expression in neural stem cells and induces Cre-mediated GR excision in a leptin-deficient background. In males, (A) body weight at 5 weeks, (B) body weight at 10 weeks, and (C) food intake were comparable to those of *Lep*$^{Ob/Ob}$ animals. ★$p<0.05$.

3.5 DISCUSSION

Leptin is a potent satiety hormone, and leptin deficiency creates a chronic state of perceived starvation. Starvation causes increased glucocorticoid secretion [246], and glucocorticoids can inhibit reproduction through direct and indirect mechanisms that are leptin-independent [125, 129-131, 256]. Excess glucocorticoids due to glucocorticoid therapy [217] and disease (e.g., Cushing's, anorexia nervosa) correlate with suppressed fertility [132, 189]. Hypercortisolemia in anorexia nervosa and Cushing's disease are both associated with reproductive inhibition, despite dramatic differences in body weight and leptin levels in these disorders.

We hypothesized that elevated glucocorticoid secretion, rather than leptin deficiency per se, caused infertility in leptin-deficient mice. To assess the role of glucocorticoids in fertility, we generated leptin-, CRH-, and glucocorticoid-deficient CRHOB mice. Unlike OB mice, CRHOB mice were fertile and had normalized reproductive organs and hormones. Body weight, fat composition, and food intake were also decreased in CRHOB compared to OB mice. In pilot studies, basal metabolic rate, but not locomotor activity, was normalized in CRHOB animals. In pilot studies, rebound hyperphagia was decreased in CRHOB compared to OB animals. We hypothesized that low leptin and high corticosterone together signal starvation and stimulate rebound hyperphagia. Thus, the OB animal is in a chronic state of perceived starvation and rebound hyperphagia, leading to its morbid obesity. In CRHOB mice that lack excess glucocorticoids, this hyperphagia is ameliorated, and animals are significantly leaner than OB littermates. Body weight in CRHOB animals was comparable to that of adrenalectomized OB animals. In pilot studies, we found that adrenalectomized OB males were unable to sire offspring. Within two weeks of adrenalectomy, macroscopic adrenal glands could be identified

in these animals; it is possible that rapid regeneration of adrenal tissue prevented further activation of the reproductive axis, and the CRHOB model allowed for more complete and permanent removal of glucocorticoid secretion.

CRH is normally inhibited by glucocorticoids and disinhibited by adrenalectomy or glucocorticoid deficiency. In studying the role of glucocorticoids in fertility, we aimed to avoid confounding the decrease in glucocorticoids with the increase in CRH. However, by eliminating both CRH and glucocorticoids in CRHOB animals, it was possible that CRH deficiency, rather than glucocorticoid deficiency, restored fertility in leptin-deficient mice. There is evidence that CRH can inhibit reproductive axis hormones [132, 133]. However, even significantly increased CRH, caused by primary adrenal insufficiency, produces only prolonged estrous cycling but not infertility [257]. Additionally, we demonstrated that *Crh* mRNA expression was not elevated in OB compared to WT animals. We demonstrated that corticosterone supplementation was sufficient to elicit infertility in CRHOB mice that was comparable to that of OB controls. When corticosterone treatment was terminated, CRHOB mice regained reproductive capacity. This indicates that glucocorticoids and not CRH, perhaps in conjunction with low leptin, are responsible for inhibition of fertility in OB mice. Corticosterone supplementation also caused weight gain and increased plasma triglyceride concentrations only in CRHOB mice.

The cellular sites and mechanisms for leptin and glucocorticoid interaction are still unclear. In pilot studies, we deleted glucocorticoid receptors from the brain, using a novel brain-specific Cre targeting strategy, and from kisspeptin neurons in the background of leptin-deficiency. Abrogating GR signaling in kisspeptin neurons did not restore fertility or decrease body weight

in leptin-deficient animals. GR was not sufficiently deleted from all neurons in the brain, and these animals were infertile and obese. It remains unclear whether the interaction between leptin and glucocorticoids occurs in the brain, and if so, which populations of neurons mediate this effect.

Leptin deficiency on a C57BL/6J background produces more severe infertility than leptin deficiency on a BALB/cJ background [184]. Under normal conditions, it is possible that physiologic increases in glucocorticoids and CRH and decreases in leptin together contribute to the suppression of reproductive function. In humans with leptin deficiency, plasma glucocorticoid concentrations are not strikingly elevated, and it is possible that glucocorticoids play a less important role in reproductive inhibition in humans. In our model, mice with global leptin deficiency in conjunction with global glucocorticoid deficiency were fertile. Eliminating hypercortisolism in leptin-deficient mice was sufficient to restore fertility, and leptin was not required for fertility. Thus, glucocorticoids suppress fertility and contribute to obesity in the presence of leptin deficiency. These findings may provide novel molecular targets in the reproductive and metabolic aspects of disorders characterized by glucocorticoid dysregulation, including leptin deficiency, Cushing's disease, anorexia nervosa, and mood disorders.

CHAPTER 4

CONCLUSIONS

Summary

Glucocorticoids are an important output of the hypothalamic-pituitary-adrenal axis, and classical actions of glucocorticoids are exerted through glucocorticoid receptors expressed throughout the body and brain. Stress responses, including increased glucocorticoid secretion, are adaptive and facilitate the return to homeostasis. Adaptations include the suppression of non-essential activities, such as reproduction, growth, and immune reactions.

Chronic or hyperactive stress responses, however, are associated with multiple pathological conditions, including Cushing's disease, post-traumatic stress disorder, anorexia nervosa, major depression, and other affective disorders. Reproductive function is negatively affected in these hypercortisolemic conditions, and glucocorticoid treatment is known to suppress the hypothalamic-pituitary-gonadal axis as well. The cellular substrates and mechanisms responsible for inhibiting and reactivating the reproductive axis during and after stress, respectively, are not well understood.

In Chapter 2, we demonstrated that kisspeptin expression in the arcuate nucleus of the hypothalamus was sensitive to stress-induced changes in glucocorticoids. We examined changes in kisspeptin and the reproductive axis in mice subjected to restraint, food deprivation, cold exposure, and corticosterone administration. In all cases, there was an inverse relationship between plasma corticosterone concentrations and arcuate nucleus kisspeptin expression. Restraint and food deprivation caused increases in plasma corticosterone concentrations and decreases in *Kiss1* mRNA expression. Because these stressors likely elicited stress responses in addition to elevated glucocorticoid secretion, we treated mice with corticosterone to study the

isolated effects of glucocorticoids on kisspeptin neurons. *Kiss1* mRNA expression was decreased in mice treated with corticosterone.

Genetic manipulation of kisspeptin neurons

We next generated mice lacking glucocorticoid receptors specifically in kisspeptin-containing neurons. In these animals, kisspeptin expression was no longer inhibited by restraint stress, and both testosterone and copulatory behaviors recovered faster following restraint. However, male mice lacking glucocorticoid receptors in kisspeptin neurons still exhibited suppression of the reproductive axis immediately after restraint, consistent with the reproductive inhibition observed in male mice following cold exposure despite unsuppressed levels of corticosterone and *Kiss1* mRNA, indicating that other glucocorticoid-dependent and -independent pathways can suppress the reproductive axis. Thus, we conclude that glucocorticoid receptor signaling in kisspeptin neurons regulates the recovery of reproductive function following stress, but not the inhibition of the reproductive axis during stress.

In the studies described above, we analyzed *Kiss1* mRNA expression as a measure of kisspeptin neuron output. Electrical activity, peptide release, and other important synaptic events cannot be captured by measurements of mRNA expression. Changes in gene expression also occur on a slower time scale than changes in electrical activity. Using optogenetic techniques, we could probe the immediate effects of manipulating kisspeptin neurons. Channelrhodopsin (ChR) and halorhodopsin (HR) are microbial ion channels [258]. Channelrhodopsin is activated by blue light and conducts cations, while halorhodopsin is activated by yellow light and conducts chloride anions. Neurons that express ChR are depolarized upon stimulation with blue light,

while neurons that express HR are hyperpolarized and inhibited by yellow light [259]. Delivery of these opsins can be mediated by lentiviral and adeno-associated viral vectors, in which ChR or HR is flanked by loxP sites, allowing for temporal and site-specific control of Cre-mediated recombination and expression [258]. Because these experiments require the implantation of site-specific fiberoptic cables, ventral sites of the brain were relatively inaccessible for many years. Recently, however, optogenic targeting of the ventromedial nucleus of the hypothalamus [260] and orexin neurons of the lateral hypothalamus [261] successfully demonstrated that optogenetic control of ventral brain structures is possible.

Our current findings indicate that kisspeptin neurons govern the time course of recovery from stress-induced reproductive inhibition. We hypothesize that kisspeptin neurons must be reactivated following glucocorticoid-dependent inhibition to turn on the downstream hypothalamic-pituitary-reproductive axis. In one potential experiment, we would induce Cre-mediated expression of HR (e.g., *eNpHR-eYFP*) [262] in kisspeptin neurons in adult *Kiss1*^{Cre} male mice. We would subject these animals to restraint stress, and following release, deliver yellow light to hyperpolarize the kisspeptin neurons. We predict that the inability of kisspeptin neurons to reactivate would prevent the restoration of testosterone and copulatory behaviors. With inhibition of kisspeptin neurons, we would expect copulatory behaviors to remain suppressed for longer than the one week time period described in Chapter 2. Using the same optogenetic model, we could induce hyperpolarization of kisspeptin neurons in the absence of an external stressor to determine whether inhibition of kisspeptin neurons alone is sufficient to suppress reproductive function. Lastly, we would hyperpolarize kisspeptin neurons in prepubertal *Kiss1*^{Cre} mice to determine whether pubertal onset can be delayed, or whether

redundant, kisspeptin-independent mechanisms can elicit puberty. If inhibition of kisspeptin neurons is sufficient to delay puberty, we would then induce acute halorhodopsin-mediated inhibition of these neurons at different prepubertal time points to determine the time course of, and perhaps a critical period for, kisspeptin neuron-dependent regulation of puberty.

Our current findings also suggest that kisspeptin neurons do not wholly mediate the acute suppression of reproductive function during stress. Mice subjected to cold exposure did not exhibit an increase in corticosterone or decrease in *Kiss1* mRNA expression, but testosterone was still suppressed. Male mice in which glucocorticoid receptors were deleted from kisspeptin neurons no longer exhibited a decrease in *Kiss1* mRNA expression after restraint stress, but testosterone and copulatory behaviors were still suppressed immediately after the restraint. We predict that preventing the suppression of kisspeptin neurons during acute stress would not preserve testosterone secretion or copulatory behaviors. Stress levels of glucocorticoids, for example, can exert direct effects on the testis and induce apoptosis of Leydig cells [129, 130]. If one generated an animal in which kisspeptin neurons were constitutively active, for example, we would predict that testosterone and copulatory behaviors would still be suppressed following stress. As a caveat, constitutive activity is not an ideal model, because kisspeptin is likely secreted in a pulsatile manner, much like GnRH, and continuous activation is more likely to suppress than activate the reproductive axis [263]. By using ChR, we could more precisely control the activity of kisspeptin neurons. We hypothesize that 1) kisspeptin activity does not wholly mediate the inhibition of downstream reproductive function during acute stress, and 2) kisspeptin neuron reactivation governs the time course of recovery from stress-induced reproductive inhibition. In potential experiments, we would induce Cre-mediated expression of

ChR (e.g., *ChR2-eYFP*) [262] in kisspeptin neurons in adult $Kiss1^{Cre}$ male mice. This would require preliminary studies to determine the appropriate pulse frequency of blue light in order to mimic physiologic kisspeptin pulsatile release, itself a parameter that has never been characterized. We would subject these animals to restraint and deliver the appropriate frequency of blue light to depolarize the kisspeptin neurons throughout the stressor. We predict that testosterone and copulatory behaviors would still be inhibited following stress, despite the continued activation of kisspeptin neurons, through inhibitory pathways at sites other than the kisspeptin neuron (e.g., direct glucocorticoid-mediated inhibition testosterone biosynthesis [129, 130]). Following restraint, however, we would predict that testosterone and copulatory behaviors would recover faster in mice that express *ChR2-eYFP* and receive blue light stimulation than in *eYFP* controls. The accelerated recovery may be comparable to the one week time period observed in $Kiss1^{CreBAC}$::$GR^{flox/flox}$ males. In $Kiss1^{CreBAC}$::$GR^{flox/flox}$ males, however, we had no control over the time course of kisspeptin neuron reactivation. By continuing to induce optogenetic activation of kisspeptin neurons following restraint, we may be able to restore reproductive function even faster than one week following stress. Using this model, we could also depolarize kisspeptin neurons in peripubertal mice to determine whether early activation of these neurons is sufficient to elicit advanced puberty.

These optogenetic experiments would allow us to isolate the time course of activation and suppression of the reproductive axis, both in juveniles during puberty and in adults during stress-induced reproductive inhibition.

Pharmacologic manipulation of kisspeptin neurons

Our studies may lead to the study of drugs that have not been previously considered for the treatment of stress- and glucocorticoid-induced reproductive inhibition. We would administer mifepristone (RU486) to male mice in order to evaluate the efficacy of possible pharmacological candidates to attenuate the inhibitory reproductive effects of stress on the hypothalamic-pituitary-gonadal (HPG) axis. Mifespristone is a non-specific glucocorticoid and progesterone antagonist that, through it action on progesterone receptors, is commonly known as the "morning-after pill" due to its use as an abortifactant in females [264-269]. Mifespristone may also be an attractive drug candidate to study the reversal of glucocorticoid-induced suppression of the reproductive axis in males. We would predict that preventing glucocorticoid-mediated suppression of kisspeptin neurons would not prevent reproductive axis suppression acutely, but that the reproductive axis would recover relatively quickly following the termination of stress. This may also be achieved by manipulating kisspeptin secretion directly. Kisspeptin-10 is the active decapeptide fragment of endogenous kisspeptin-54 and binds to the kisspeptin receptor with similar affinity but increased bioactivity [270, 271]. We would administer kisspeptin-10 to mice subjected to stress-induced reproductive inhibition and determine whether pre- or post-stress treatment with kisspeptin can prevent prolonged suppression of the reproductive axis. These drugs may demonstrate novel utility in the treatment of the reproductive aspects of post-traumatic stress disorder, mood disorders, and anorexia nervosa.

Kisspeptin neurons contribute to reproductive behaviors

We report that kisspeptin neurons can be protected from stress by the removal of glucocorticoid receptor signaling, and that doing so has the ability to orchestrate a repertoire of complex

behaviors leading to more rapid return of reproductive function, possibly by the downstream regulation of sex steroid secretion. If the kisspeptin neuron-dependent recovery of the reproductive axis in males is mediated by changes in testosterone, we would expect multiple testosterone-dependent behaviors to be affected. Aggression, for example, is a sexually dimorphic behavior widely considered to be mediated by testosterone in mammals [272]. If glucocorticoid receptor signaling in kisspeptin governs the time course of recovery of testosterone, which in turn governs the display of copulatory behaviors, then we would expect aggression to be altered in male mice in which glucocorticoid receptors are selectively deleted from kisspeptin neurons following stress. To test this hypothesis, we would subject control and $Kiss1^{CreBAC}$::$GR^{flox/flox}$ males to restraint stress and assay aggression using the resident-intruder paradigm [273]. The restrained male would serve as the resident in all cases, and age-matched males would serve as intruders. Attack frequency, duration, and latency to first attack would be monitored at baseline, immediately after restraint, and following one week of restraint, consistent with the time course of our studies on copulatory behaviors. We predict that all resident males would fail to attack intruders immediately after restraint, consistent with stress-induced inhibition of testosterone secretion. Following one week of recovery, when $Kiss1^{CreBAC}$::$GR^{flox/flox}$ males exhibited accelerated recovery of testosterone and copulatory behaviors, we predict that these animals would exhibit increased aggression towards intruders compared to control animals with intact glucocorticoid receptor signaling. These experiments may demonstrate that kisspeptin neurons, through the action of testosterone, govern the recovery of multiple complex behaviors following stress.

We do not wish to oversimplify the relationship between glucocorticoids and reproductive axis hormones. Elevated glucocorticoids do not always correlate with suppressed testosterone. In the wild baboon colonies, for example, alpha males can exhibit high levels of testosterone, but also higher levels of glucocorticoids compared to beta males [274]. During times of hierarchy instability, glucocorticoids increase in alpha males, but testosterone is unaffected compared to during times of hierarchy stability [274]. In certain species, the reproductive system of seasonal breeders is also insensitive to glucocorticoids [275]. The mechanisms by which testosterone secretion, and possibly kisspeptin neurons, can become more or less sensitive to glucocorticoids by season or status within a social hierarchy may provide new insights into the glucocorticoid-mediated inhibition of reproduction.

Implications of kisspeptin neuron suppression

Loss-of-function studies have demonstrated that kisspeptin signaling is crucial for pubertal development [83, 84]. Selective deletion of estrogen receptor alpha in kisspeptin neurons elicited advanced puberty [276]. Diphtheria toxin-mediated ablation of kisspeptin neurons in adult mice disrupted estrous cyclicity [216]. Our studies are the first to describe the targeted disruption of a signaling pathway within kisspeptin neurons that alters adult behaviors in mice. We also provide evidence that kisspeptin neurons are directly and potently regulated by an inhibitory input. This insight may lead investigators to consider the role of inhibitory regulation in the characterization of kisspeptin neurons. Much effort is directed towards identifying mechanisms by which kisspeptin neurons are activated during puberty. It is possible that kisspeptin neurons receive tonic inhibitory inputs in prepubertal development, and puberty (like recovery from stress-induced reproductive inhibition) represents a release from inhibition. There

is evidence that disinhibition of GABAergic inputs to LHRH neurons in the median eminence precedes the onset of puberty [277]. Tonic inhibition of kisspeptin neurons may be mediated by GABAergic neurons, the hypothalamic-pituitary-adrenal axis, or other inhibitory inputs. GABAergic networks are known to be plastic during embryonic and postnatal development, and the hypothalamic-pituitary-adrenal axis is especially plastic during the peripubertal period [278-280]. Taking into consideration the mechanisms by which kisspeptin neurons can be disinhibited during puberty may prove to be a valuable complement to studies investigating the circuitry that activate these neurons during puberty.

Model of pathological reproductive inhibition

Our findings may suggest novel therapeutic targets for disorders characterized by dysregulated glucocorticoid secretion, such as mood disorders, anorexia nervosa, and post-traumatic stress disorder. However, the weeklong suppression of reproduction following restraint should not necessarily be interpreted as a model of murine post-traumatic stress disorder. Instead, this suppression could be an adaptive response by which animals, when faced with severely unfavorable conditions, avoid expending energy on reproduction for prolonged periods of time. During a protracted famine, for example, it may not be energetically favorable to reactivate the reproductive axis after finding and consuming just one meal. Naturally slow recovery of kisspeptin neurons may facilitate the continued suppression of downstream reproductive axis signaling or render the axis more sensitive to additional stressors, until such a time when the animal has experienced a number of low-stress days (e.g., plentiful food, lack of predator presence). In the case of starvation, signals such as low leptin and high glucocorticoids may collaborate to turn off the reproductive axis.

Hypercortisolemia in leptin deficiency

Genetic leptin deficiency creates a chronic state of perceived starvation, and leptin-deficient mice exhibit elevated plasma glucocorticoid concentrations, morbid obesity, and infertility. We hypothesized that glucocorticoid excess contributed to obesity and infertility in leptin-deficient mice. In Chapter 3, we demonstrated that leptin-deficient, glucocorticoid-deficient mice exhibited decreased body weight and fat composition, decreased hyperphagia, and normal fertility. When supplemented with glucocorticoids back to the initial levels present in leptin deficiency, these mice gained weight and became infertile. Thus, leptin is not directly required for fertility as previously believed, and glucocorticoids can contribute to obesity and suppress fertility in leptin-deficient mice.

We decreased systemic glucocorticoid secretion and restored fertility in the background of leptin deficiency. The site(s) of glucocorticoid action remain unclear, and the mechanisms by which glucocorticoids and leptin interact are unknown. After the initial discovery of kisspeptin, many investigators believed that kisspeptin neurons were the ideal candidate for the integration of metabolic and reproductive signals. Kisspeptin expression was decreased following food deprivation in mice, rats, and macaques, and in leptin-deficient mice, and kisspeptin expression was restored following leptin replacement [185, 186, 224]. Kisspeptin neurons were hypothesized to be a direct target of leptin action. However, very few kisspeptin neurons expressed leptin receptors, and mice in which leptin receptors were selectively deleted from kisspeptin neurons exhibited neither metabolic nor reproductive phenotypes (unpublished observations, Mayer et al., The Endocrine Society Annual Meeting, 2010).

Our results suggest that decreased *Kiss1* mRNA expression following food deprivation and restraint correlate with, but is not the sole cause of downstream reproductive axis suppression. Mice subjected to cold exposure did not exhibit a decrease in *Kiss1* mRNA expression, but did exhibit a decrease in testosterone. Mice in which glucocorticoid receptors were deleted from kisspeptin neurons no longer exhibited a decrease in *Kiss1* mRNA expression after restraint, but testosterone was still suppressed. Thus, downregulation of kisspeptin expression following food deprivation or in leptin deficiency is not the only inhibitory input to the hypothalamic-pituitary-gonadal reproductive axis. We would predict that if one generated mice in which kisspeptin neurons remained unsuppressed by inducing appropriate pulsatile activation using optogenetic stimulation, the reproductive axis would still be suppressed by food deprivation and leptin deficiency through kisspeptin-independent mechanisms. We generated an animal model in which glucocorticoid receptors were deleted from kisspeptin neurons in the background of leptin deficiency. These mice were as obese and infertile as leptin-deficient controls. It is possible that *Kiss1* mRNA expression is unsuppressed in these animals, as with $Kiss1^{CreBAC}$::$GR^{flox/flox}$ males following restraint stress, but even so, we predict that downstream reproductive axis sites could still be inhibited by other glucocorticoid-dependent and -independent mechanisms. Glucocorticoids exert numerous effects on peripheral tissues [227], and it is possible that the interaction between leptin and glucocorticoids does not occur wholly within the brain. Because animals with brain-specific deletion of glucocorticoid receptors generated by using $Nestin^{Cre}$ were significantly runted compared to littermate controls [24], we do not consider this to be an ideal model in which to study the effects of body weight in the background of leptin deficiency. We attempted to generate animals with brain-specific deletion of glucocorticoid receptors in the background of leptin deficiency by using $Blbp^{Cre}$ transgenic mice, but Cre expression in these

brains was incomplete, and *Blbp*^{CreBAC}*::GR*$^{flox/flox}$*::Lep*$^{Ob/Ob}$ mice were infertile and obese. Generating an animal in which glucocorticoid receptors are selectively deleted from the brain in the background of leptin deficiency may be possible if we were able to identify other brain-specific Cre mice in which Cre expression is widespread, but glucocorticoid receptor deletion in itself does not produce a body weight phenotype.

Sites of glucocorticoid and leptin interaction

As we and others explore sites of glucocorticoid action in regulating fertility and energy metabolism, we bear in mind that the site of glucocorticoid and leptin interaction may remain elusive, because the site of leptin action itself is not clear. Classically, first-order leptin-sensitive neurons are described to be the POMC/CART and AGRP/NPY neurons of the arcuate nucleus of the hypothalamus [281]. However, selective deletion of leptin receptors from POMC/CART and AGRP/NPY neurons produces only mild obesity compared to global leptin receptor knockout mice [282-284]. Selective deletion of leptin receptors from GABAergic neurons, however, produces mice with obesity as severe as that of global leptin receptor knockout mice [285]. In an initial effort to identify the site of interaction between leptin and glucocorticoids, we would generate *Vgat-ires-Cre::GR*$^{flox/flox}$*::Lep*$^{Ob/Ob}$ and *Vglut2-ires-Cre::GR*$^{flox/flox}$*::Lep*$^{Ob/Ob}$ mice in which glucocorticoid receptors are selectively deleted from GABAergic or glutamatergic neurons, respectively, in the background of leptin deficiency. Identifying the site of glucocorticoid action could elucidate an important component of the circuitry of feeding and fertility. Identifying systemic glucocorticoid action as an important contributor to energy metabolism and fertility in leptin-deficient mice introduces a novel therapeutic target in the treatment of obesity and infertility.

Our studies indicate a role for glucocorticoids in the regulation of obesity and reproductive inhibition caused by stress. Together, these findings may provide novel mechanisms and molecular targets in the reproductive and metabolic aspects of disorders characterized by glucocorticoid dysregulation.

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