REGULATION OF ARCUATE NUCLEUS KISSPEPTIN/NEUROKININ B AND GONADOTROPIN-RELEASING HORMONE DURING NEGATIVE ENERGY BALANCE

By

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A DISSERTATION

Presented to the Division of Neuroscience
and Oregon Health & Science University
School of Medicine
in partial fulfillment of
the requirement of the degree of
Doctor of Philosophy

August 2012

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

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List of Acronyms

3V	third ventricle
ACTH	adrenocortiotropic hormone
α-MSH	α-melanocyte-stimulating hormone
AgRP	agouti-related peptide
AHN	anterior hypothalamic nucleus
ARH	arcuate nucleus
AVPV	anteroventral periventricular region
β-END	β-endorphin
BMI	body mass index
BST	bed nucleus of the stria terminalis
CART	cocaine- and amphetamine-regulated transcript
CNS	central nervous system
CR	caloric restriction
CRH	corticotrophin-releasing hormone
DAB	diaminobenzidine
DIO	diet-induced obesity
DMH	dorsomedial hypothalamus
DYN	dynorphin
E	estradiol
ER	estradiol receptor
F	fast
FSH	follicle stimulating hormone
GALP	galanin-like peptide
GC	glucocorticoids
GFP	green fluorescent protein
GnIH	gonadotropin-releasing hormone
GnRH	gonadotropin-releasing hormone
GnRH-R	gonadotropin-releasing hormone receptor
GPR54	G-protein coupled receptor 54
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
icv	intracerebral ventricular
IHC	immunohistochemistry
ip	intraperitoneal
ir	immunoreactivity
ISH	*in situ* hybridization
JACoP	Just Another Colocalization Program
Kiss1	kisspeptin
Kiss1R	kisspeptin receptor

KNDy	kisspeptin, neurokinin B and dynorphin expressing
LH	lutienizing hormone
ME	median Eminence
MPN	medial preoptic nucleus
MPO	medial preoptic area
mRNA	messenger ribonucleic acid
MS	medial septal nucleus
NDB	nucleus of the diagonal band of Broca
NKB	neurokinin B
NK3	neurokinin receptor 3
NiDAB	nickel diaminobenzidine
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
Ob	leptin
ObR	leptin receptor
OC/OCH	optic chiasm
OVX	ovariectomy
OVX+E	ovariectomy and estradiol replacement
P	progesterone
PCOS	polycystic ovarian syndrome
Pe	periventricular region
PMv	ventral premammillary nucleus
POA	preoptic area
POMC	proopiomelanocortin
pSTAT3	phosphorylated signal transduction and transcription 3
PVN	paraventricular nucleus
RFRP3	RF-amide related protein 3
SOCS3	suppressor of cytokine signaling 3
VMH	ventromedial nucleus hypothalamus

ACKNOWLEDGEMENTS

I would like to thank first and foremost my mentor Dr. Susan Smith for the excellent training she has provided me throughout my graduate studies. Not only has she taught me to think critically about scientific methods and experimental design, but she has been a constant source of support through the various challenges of graduate school. My failed experiments were not something to sulk over but learning experiences to guide future studies and this attitude she instilled in me prevented those common feelings of frustration experienced by so many of my peers. Instead these five years have felt informative and stimulating and I credit this solely to my mentor and her unrelenting curiosity for science. Susan has attended every talk I've ever given, made meaningful comments and critiques on every scientific paper I've written in the lab and been a perpetual role model for the type of scientist I hope to become. I am extremely grateful for the support she has provided me as well as the invaluable confidence she has endowed in me as I move on in my scientific career.

I would also like to thank my co-mentor Dr. Kevin Grove, for always challenging me to think critically about my science. I have always valued the stimulating and open discussions that arise from the lab environment Kevin helps stimulate. I would also like to thank my committee members Dr. Judy Cameron, Dr. Henryk Urbanski and Dr. Oline Ronnekliev for their comments and discussions throughout the years and help in guiding this dissertation project forward. I would also like to give special thanks to Melissa Kirigiti, who not only has taught me almost every lab technique I learned in graduate school, but was also a great friend to share the time with. Not many technicians would come in to help you ovariectomize 16 rats on a Saturday, but Melissa did (many times) and made those long days in the lab a fun and happy place to be.

My success in science stems partially from a happy life at home, because I've always believed that hard work should be balanced with some relaxing play. I would like to thank my oldest friends Michael Frederick and Lindsey Maier for always being there to commiserate with me over a drink after a

long experiment gone wrong and also being there to celebrate with me when it all worked out in the end. In addition I am so appreciative of the ever present source of love, support and confidence that my parents both provide me. In particular I would like to thank my mother Kimberly True for being my first role model of women in science. I remember fondly those summer vacations where she would bring me to the lab with her and the projects and techniques she would show me and her obvious enthusiasm and love for her work. She taught me to pipet around the ripe old age of 9 and always kept me coming back to the lab throughout my life (even after I improperly balanced and destroyed her work's centrifuge when I was 14). Lastly I would like to thank my husband David Freas, who knows more about leptin and kisspeptin than he has any right to, and has provided his support day in and day out throughout my post-graduate training.

Chapter 1 - INTRODUCTION

1. Integration of metabolic and reproductive function

Female reproductive function is metabolically-gated, meaning an organism requires sufficient energy intake and stores to maintain fertility. This energy requirement has led to the evolutionary development of seasonal breeding in many species, where breeding is carried out at specific times to coordinate either gestation or lactation with seasons of food availability. However, when these energy requirements are not met and energy expenditure exceeds energy input, this causes an imbalanced metabolic state termed negative energy balance. Negative energy balance results in weight loss leading to the initiation of homeostatic adaptations to increase food intake and decrease energy expenditure in an effort to normalize the metabolic imbalance. One mechanism of energy conservation in female mammals is a suppression of the energy demanding reproductive cycle. By shutting down the ovarian cycle in adulthood, females conserve energy in times of food scarcity. This adaptation also prevents pregnancy and the high metabolic cost associated with gestation and lactation. In humans, this condition is termed functional hypothalamic amenorrhea and can frequently be observed in patients with anorexia nervosa as well as professional athletes (Boyar et al., 1974; Mansfield and Emans, 1989; McArthur et al., 1980; Warren et al., 1999). These two groups of women represent the extreme examples of decreased energy input (anorexics) and increased energy output (athletes), both of which can contribute to an imbalanced and ultimately negative metabolic state.

In addition to regulation of ovarian cyclicity during adulthood, metabolic perturbations can also affect sexual maturation during development. Negative energy balance has been observed to delay sexual maturation and the complex set of physiological changes associated with puberty. One of the most important changes during puberty is an increase in ovarian steroid hormone production, and undernutrition can prevent this increase in steroids resulting in a condition known as hypogonadism in humans. It should be noted that negative energy balance-induced reproductive dysfunction during development may not occur for the sole purpose of energy conservation, as is hypothesized in

adulthood. Previous studies have provided evidence that there may be a growth, or adiposity, threshold that is required for puberty onset. Therefore, it may be that in addition to negative energy balance signals actively inhibiting puberty for energy conservation, undernutrition also prevents the "permissive" signals of body growth required for the initiation of puberty. Regardless of the mechanism, it appears that undernutrition during development delays puberty until certain signals of sufficient growth, which are still not fully understood, have been achieved (Boyar et al., 1974; Ronnekleiv et al., 1978). This role of nutritional status to govern reproduction during development as well as adulthood suggests that pathways of reproductive regulation are likely tightly coupled to those of metabolism throughout an organism's life.

Understanding the metabolic requirements for reproductive function will have far-reaching implications for women's health. By better understanding how reproductive function is inhibited during negative metabolic states, it might be possible to better identify pharmaceutical targets for the treatment of numerous causes of infertility. This could of course benefit patients with metabolic- and stress-induced hypothalamic amenorrhea as well as potentially lead to new intervention therapies for patients with developmental disorders like hypogonadism. Another disorder that may benefit from research into the metabolic regulation of reproduction is polycystic ovarian syndrome (PCOS). This condition of ovarian dysfunction and subfertility is also coupled with an abnormal metabolic phenotype, indicating that the source of this disorder may lie in pathways involved in both reproductive and metabolic regulation. In addition to the treatment of infertility, understanding how inhibitory control is exerted over the reproductive system could also be used for the development of new birth control treatments. The traditional steroidal forms of birth control in use today carry significant health risks including increased prevalence of hypertension, heart attack and blood clotting especially for older women. Identification and development of a non-steroidal birth control may offer a safer alternative to family planning.

In addition to the clinical implications, understanding the metabolic regulation of reproduction on the basic science level would significantly increase our understanding of how multiple signals of these two systems are integrated. For example, understanding the metabolic cues causing reproductive inhibition would provide key insights into how the body senses changes in metabolic state and how these changes are signaled to multiple organs, including the brain. Metabolic regulation of reproduction has been a significant research question for many decades and although our knowledge of how the system works has increased significantly over time, the major causative components of this pathway are still largely unknown. Understanding this basic and highly conserved form of reproductive regulation will increase our understanding of the normal physiology involved in mediating the ovarian cycle and fertility.

2. The hypothalamic-pituitary-gonadal axis

To understand how negative metabolic states result in ovarian acyclicity it is necessary to first understand how the cycle is normally regulated. Ovulation is primarily governed by the hypothalamic-pituitary-gonadal (HPG) axis, which consists of a set of hormonal players from these three organs that tightly regulate ovarian status. Gonadotropin-releasing hormone (GnRH) is produced in the hypothalamus and released in a pulsatile manner into the hypophyseal portal blood where it then circulates to the anterior pituitary. Once in the pituitary, GnRH activates its receptor (GnRH-R) on gonadotrophs to stimulate release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These pituitary hormones circulate and activate their respective receptors in the ovary. LH and FSH are required for follicular growth in the ovary and subsequent steroid hormone production, namely estradiol (E) and progesterone (P). Estradiol and progesterone work in a classical negative feedback loop to dampen hypothalamic GnRH and pituitary LH and FSH release. This negative feedback of estradiol on GnRH release is the dominant form of feedback regulation throughout most of the ovarian cycle. However, as the predominant ovarian follicle grows due to LH and FSH stimulation,

estradiol production increases dramatically. Paradoxically, these higher levels of estradiol act to stimulate GnRH/LH release, and this regulatory action is known as positive steroid feedback. Positive steroid feedback causes a large non-episodic period of GnRH release, although interspersed periods of increased pulsatile GnRH release have also been noted, referred to as the GnRH surge (Clarke, 1993; Clarke and Cummins, 1985; Evans et al., 1995; Moenter et al., 1992; Xia et al., 1992). This striking increase in GnRH release results in a downstream surge in LH, and it is this surge in LH that is critical for evagination of the follicle and ovulation to occur.

Since the identification of GnRH as the decapeptide responsible for pituitary LH and FSH release in the early 1970s (Burgus et al., 1972; Schally et al., 1971), it has been continuously on the forefront of studies investigating reproductive regulation. Given the extreme importance of GnRH for the ovarian cycle, both in responding to varying levels of steroids and stimulating the critical LH surge for ovulation, it is not surprising that it is at this level of the HPG axis where disruption occurs during negative energy balance in adult female mammals. Negative energy balance is characterized by a decrease in both GnRH pulsatile amplitude and frequency and in extreme cases there is a complete absence of pulses (Aloi et al., 1997; Cameron and Nosbisch, 1991; Fox and Smith, 1984; Nagatani et al., 1998). Although it is the surge of GnRH that is crucial for ovulation to occur, without basal pulsatile GnRH release the ovarian cycle cannot proceed normally. Without basal pulsatile GnRH release there is insufficient follicular growth and steroid hormone release, which occludes the maturation of a dominant follicle and high estradiol levels required for positive feedback and the GnRH/LH surge.

Evidence for disruption at the hypothalamus during negative energy balance comes primarily from work demonstrating that exogenous pulsatile GnRH treatment can restore LH and ovarian function, suggesting that the pituitary and ovaries are capable of responding normally (Aloi et al., 1997; Cameron and Nosbisch, 1991; Fox and Smith, 1984; Santoro et al., 1986). However, despite the ability of exogenous GnRH to normalize LH, there have been some indications of abnormal pituitary

responsiveness during negative energy balance. Lowered levels of GnRH release during negative energy balance result in lowered levels of GnRH-R in the pituitary, which is consistent with previous reports of GnRH-R being positively regulated by intermittent, or pulsatile, exposure to its ligand (Bergendahl et al., 1989; Duncan et al., 1986; Katt et al., 1985; Smith and Reinhart, 1993; Yasin et al., 1995). This pituitary hyposensitivity can be frequently missed if exogenously administered GnRH is given at large doses. High enough levels of GnRH likely result in maximal activation of the remaining GnRH-Rs causing observable LH release; however, lower doses of GnRH, similar to those observed under normal physiological conditions, are insufficient to acutely stimulate pulsatile LH release during negative energy balance (Lee et al., 1989). In fact it can take up to 24 hours for low levels of pulsatile GnRH administration to upregulate GnRH-R expression in the pituitary and result in normal LH release (Lee et al., 1989). Although the pituitary is not the focus of the current study, this data indicates that interventions aimed at restoring GnRH function should be carried out for at least 24 hours to allow for upregulation of GnRH-Rs and restoration of pituitary responsiveness. After this time-span, administration of physiological levels of GnRH pulses does normalize changes in the pituitary and ovarian function suggesting that while the pituitary is affected, these changes are likely secondary to the decrease in GnRH release (Lee et al., 1989).

Although it appears clear that GnRH release is disrupted by negative energy balance it is unknown whether this inhibition occurs directly at GnRH neurons or in upstream circuits of the hypothalamus. Changes in upstream cell populations during negative energy balance seem highly likely due to 1) widespread changes in the CNS following negative energy balance and 2) the highly integrated nature of GnRH regulation. For the former, food intake and the availability of energy stores are tightly monitoried by the brain, particularly the hypothalamus. Changes in metabolic state generally lead to an increased drive for food intake and decreased energy expenditure and these adaptations are mediated, at least in part, by neural signals. These homeostatic adjustments in food intake and energy expenditure

are relayed not through single cell populations, but instead through a diverse network of neural circuits. Therefore, neuronal populations sensitive to conditions of negative energy balance that might also regulate GnRH release are numerous. In addition, regulation of GnRH is highly integrated since this neurohormone appears to be the final output signal of reproduction in the brain. Not surprisingly, these cells have been shown to receive input from many populations of cells and GnRH release is regulated not only by metabolic cues but also behavioral, olfactory, and stress stimuli just to name a few. Therefore, the likelihood that upstream populations contribute to negative energy balance-induced GnRH inhibition appears high.

3. Kisspeptin as a critical signal for GnRH regulation

Few upstream GnRH regulators have garnered as much attention as Kisspeptin (Kiss1). Kiss1 was originally characterized for its anti-metastic properties in cancerous cell lines (Lee et al., 1996; Ohtaki et al., 2001) and is expressed in the pancreas, liver, placenta, ovary, testes, small intestine and the brain (Castellano et al., 2006a; Horikoshi et al., 2003; Ohtaki et al., 2001). It wasn't until two groups independently linked mutations in the Kiss1 receptor, GPR54, to hypogonadotropic hypogonadism in humans that Kiss1 was recognized for its importance in reproductive regulation (de Roux et al., 2003; Seminara et al., 2003). Human mutations in GPR54 were quickly compared to transgenic mice homozygous for a knockout in GPR54. Lack of GPR54 in mice recapitulated the major reproductive dysfunction observed in humans, with abnormal pubertal development and lack of normal ovarian cycling and infertility in adult mice (Seminara et al., 2003). Together these early results indicate a critical role for Kiss1/GPR54 signaling for regulation of the HPG axis. Given the critical importance of GnRH release for normal pubertal development, and Kiss1 expression in the brain, it was hypothesized that Kiss1 might act within the hypothalamus to regulate GnRH release.

Soon this hypothesis was confirmed in animal studies demonstrating, by electrophysiological recordings, that GnRH cells are stimulated by Kiss1 application (Han et al., 2005). In fact, Kiss1 is now recognized as the most potent stimulator of GnRH release studied to date, with 1 fmol Kiss1 administration into the cerebral ventricles (icv) eliciting increases in LH release. The concentration at which Kiss1 elicits half of its maximum effect (EC50) on GnRH cellular depolarization is around 3 nM (Zhang et al., 2008). Kiss1's ability to stimulate LH release is blocked by pretreatment with the GnRH-R antagonist acyline in multiple species, confirming that Kiss1's major action occurrs in the brain and not the pituitary (Gottsch et al., 2004; Irwig et al., 2005; Matsui et al., 2004; Mikkelsen et al., 2009; Plant et al., 2006). In addition to its potent effects, it was quickly observed that Kiss1 administration results in dramatically prolonged activation of GnRH neurons, with 1-3 minute application of Kiss1 eliciting increases in GnRH firing frequency for as long as cells can be recorded from, reportedly greater than 80 minutes in some cases (Han et al., 2005). Together these studies demonstrated that Kiss1 actions for reproduction function are mediated by GnRH.

Electrophysiological recordings as well as immunohistochemistry (IHC) studies both provide evidence that Kiss1 acts directly at GnRH cells. For instance Kiss1 stimulates GnRH cell firing in the presence of action potential blockers, indicating direct post-synaptic effects upon GnRH cells (Han et al., 2005). IHC studies demonstrate morphological evidence of Kiss1 fibers in close contact with GnRH cell bodies in the medial preoptic nucleus (POA) and near GnRH fibers in the median eminence (ME) in rodents, sheep and monkeys (Burke et al., 2006; Clarkson and Herbison, 2006; Kinoshita et al., 2005; Ramaswamy et al., 2008; Smith et al., 2011). Finally GnRH neurons express GPR54 mRNA, confirming a potential direct effect of Kiss1 on GnRH cells (d'Anglemont de Tassigny et al., 2008; Dungan et al., 2007; Han et al., 2005; Irwig et al., 2005; Messager et al., 2005). GPR54 is a Gq-coupled GPCR which results in an increased firing frequency in GnRH cells by activation of the PLC pathway and increased intracellular calcium release from the endoplasmic reticulum following IP3 cleavage from PIP2 (Liu et al., 2008). This

cascade ultimately leads to inhibition of potassium channels and activation of nonselective cation TRPC channels to increase GnRH firing (Liu et al., 2008; Zhang et al., 2008). Current research is ongoing to understand how Gq-mediated signaling results in the notable prolonged stimulation of GnRH neurons.

In the rodent there are two major populations of Kiss1 cells; one in the arcuate nucleus (ARH) and another in the anteroventral periventricular nucleus (AVPV) (Gottsch et al., 2004). Strikingly both of these nuclei are strongly implicated in the regulation of GnRH release previously. The AVPV shows activation during sexual behavior (Caba et al., 2000) and is highly sexually dimorphic (Bloch et al., 1993; Davis et al., 1996; Orikasa et al., 2002; Simerly, 1991; Simerly et al., 1985). In addition ERα is abundantly expressed in the AVPV and several AVPV cell types appear to be regulated by estradiol, leading to speculation of a possible role in regulating the LH surge (reviewed in Herbison, 2008; Simerly, 1989; 1991; Simerly et al., 1990). The AVPV nucleus also sends direct projections to GnRH neurons, indicating the importance of this nucleus in the regulation of hypothalamic GnRH release (Gu and Simerly, 1997).

The ARH has been strongly implicated as the site of the GnRH "pulse generator" in multiple species. This hypothesis is based in part on recordings of multi-unit activity (MUA) in the ARH nucleus in which pulsatile field depolarizations correspond with pulsatile LH release detected in serum (Cross and Dyer, 1972; Kawakami et al., 1970; Knobil, 1989; Wakabayashi et al., 2010; Williams et al., 1990). Similar to the AVPV, the ARH is an abundant site of ERα and sends direct projects to the area of GnRH neurons, suggesting a potential role in ovarian-cycle dependent regulation of GnRH release (Chronwall, 1985; McShane et al., 1994; Simerly et al., 1990). Gene expression of opioids and other ARH neuropeptides fluctuate with the estrus cycle (Parnet et al., 1990; Tong et al., 1990). Taken together, these findings have led to the hypothesis that the ARH may be the site of pulsatile regulation of GnRH release.

Overall, the distribution of the two predominant Kiss1 populations suggested a potential role in both pulsatile and surge mechanisms of GnRH release. An important remaining question in

understanding the role of Kiss1 for GnRH regulation will be to determine how Kiss1 release from one nucleus might stimulate pulsatile GnRH release, while Kiss1 release from another nucleus results in surge-like GnRH release. In addition, although Kiss1 fibers appear in close contact with GnRH cell bodies in the POA and fibers in the internal zone of the ME, it is unclear from which populations these projections originate. Work presented in Chapter 2 investigated the projections of ARH versus AVPV Kiss1 cell populations to determine whether the distinct physiological roles of these two populations may be transmitted at distinct neuroanatomical sites.

3.1 Arcuate nucleus Kiss1 cells coexpress Neurokinin B and Dynorphin

An early discovery investigating the role of ARH Kiss1 for GnRH regulation, was that this population coexpresses neurokinin B (NKB) and dynorphin (DYN) in many species, and these cells are now referred to as the KNDy cells (Burke et al., 2006; Goodman et al., 2007; Hrabovszky et al., 2010; Navarro et al., 2009; True et al., 2011b). The discovery of this coexpression suggested a significant role for this population in GnRH regulation, given both DYN (Dufourny et al., 2005; Goodman et al., 2004) and particularly NKB had been previously implicated in the regulation of GnRH.

Similar to Kiss1, disruptions in the genes encoding NKB and its receptor NK3 are linked to hypogonadotropic hypogonadism in humans, suggesting a critical role for this neuropeptide in reproductive development (Topaloglu et al., 2009). NKB fibers are present in close contact with GnRH cell bodies in the preoptic area and fibers in the median eminence (ME) providing additional evidence that NKB might directly regulate GnRH release (Goubillon et al., 2000; Krajewski et al., 2005). While there is no evidence to date of GnRH cell bodies expressing NK3 receptors, NK3 is colocalized with GnRH fibers in the ME as determined by IHC (Amstalden et al., 2010; Billings et al., 2010; Krajewski et al., 2005). Studies investigating the physiological effects of NKB, or the NK3 receptor agonist senktide, on LH release have observed quite disparate results. Despite the stimulatory role implicated for NKB by

genetic mutations in humans, NKB or senktide treatment reportedly decreases LH release (Kinsey-Jones et al., 2012; Navarro et al., 2009; Sandoval-Guzman and Rance, 2004). However, contradictory results have also been reported in which NK3 receptor activation increases both LH release (Billings et al., 2010; Ramaswamy et al., 2010) and multi-unit activity frequency in the ARH corresponding to LH pulses (Wakabayashi et al., 2010). Some of the discrepancy between these results is explained by differences in steroid hormone milieu, given recent reports that estrogen is required for a stimulatory effect of NKB on LH release (Navarro et al., 2011a). Importantly, GnRH cells do not respond to senktide application as measured by electrophysiological recordings (Navarro et al., 2011b); however, it remains unknown whether NKB might directly elicit GnRH release at the ME, which could have been missed by recordings at cell bodies.

There is also evidence that NKB indirectly regulates GnRH release by autoregulation of KNDy neurons. The NK3 receptor is expressed by KNDy neurons, along with the κ-opioid DYN receptor (KOR), suggesting an autoregulatory role of these two neuropeptides (Amstalden et al., 2010; Navarro et al., 2009). In addition, NKB directly stimulates KNDy cell firing and can also elicit increased c-Fos expression in KNDy cells (Navarro et al., 2011a; Navarro et al., 2011b). NKB-induced LH stimulation is absent in GPR54 knockout mice, providing further evidence that NKB's stimulatory effect on GnRH may be solely mediated by regulation of Kiss1 (Garcia-Galiano et al., 2012). Given this hypothesized role of NKB, and its crucial role in the development of the GnRH system in humans, it has been hypothesized that NKB acts to tightly regulate Kiss1 release to elicit corresponding discrete GnRH pulses. More research is needed to better understand how NKB contributes to the *in vivo* regulation of both Kiss1 and GnRH release.

3.2 Regulation of the Kiss1 populations by estradiol

As discussed earlier, estradiol plays an extremely important role in governing the ovarian cycle. It exerts both positive and negative steroid feedback regulation of GnRH release which are both required for normal ovarian function and ovulation. The contradictory negative and positive feedback effects of estrogen were confounding to the research community for many years. The mechanism for estradiol's regulation of GnRH was also controversial, given differing evidence on the expression of the classical nuclear estrogen receptors (ER) in GnRH cells depending on the method of detection (Herbison et al., 1995; Herbison et al., 1993; Herbison and Theodosis, 1992; Rance et al., 1990; Shivers et al., 1983; Skynner et al., 1999; Sullivan et al., 1995; Watson et al., 1992). It should be noted that there is additional evidence that estradiol can exert a direct membrane effect on GnRH neurons through a yet-unidentified membrane receptor (reviewed in Kelly and Levin, 2001). However, strong evidence exists that ERα mediates the LH surge (Wintermantel et al., 2006) and the majority of studies seem to demonstrate a lack of ERα expression in GnRH neurons. Based on this evidence, it is speculated that estradiol acts not directly on GnRH neurons but on populations upstream of GnRH to generate the surge. This hypothesis led to speculation that perhaps two different upstream populations were responsible for negative and positive feedback.

Given the strong role Kiss1 plays in GnRH regulation, a next logical step was to determine whether Kiss1 contributes to steroid feedback regulation of GnRH release. Initial work investigated whether Kiss1 is regulated by estradiol by comparing Kiss1 mRNA expression in OVX and OVX+E animals. Strikingly, while estradiol inhibits ARH Kiss1 expression, it increases AVPV Kiss1 expression (Adachi et al., 2007; Smith et al., 2005). These results immediately led to speculation that Kiss1 populations are the missing link in understanding steroid feedback of GnRH cells. Consistent with this hypothesis, both AVPV and ARH Kiss1 abundantly express ERα, indicating these cells likely respond directly to changes in

circulating estradiol (Adachi et al., 2007; Clarkson et al., 2008; Smith et al., 2005). However, an important remaining question is how the same estradiol receptor, ERα, exerts opposite effects on Kiss1 mRNA production in the two cell populations. Recent evidence indicates the differential effect of estradiol on ARH and AVPV Kiss1 expression may be due in part to differences in ERα signaling within these cells (Gottsch et al., 2009).

The stimulatory effect of estradiol on AVPV Kiss1 production led to work investigating whether this population is involved in positive steroid feedback and generation of the GnRH/LH surge. AVPV Kiss1 cells express c-Fos on the afternoon of proestrus, suggesting activation of these cells during the time of the mounting GnRH/LH surge (Adachi et al., 2007; Clarkson et al., 2008). Proceeding studies provided evidence that exogneous Kiss1 administration is most potent at eliciting LH release during the preovulatory phase of the cycle in women (Dhillo et al., 2007) and an anti-kisspeptin antibody can prevent the LH surge (Adachi et al., 2007; Smith et al., 2011). Importantly, work in transgenic mice revealed that a steroid treatment known to induce a LH surge was incapable of doing so in both GPR54 and Kiss1 knockout mice (Clarkson et al., 2008); however, contradicting evidence for normal LH surges in GPR54 knockout mice also exists (Dungan et al., 2007). Overall, the majority of data continues to point toward an important role for AVPV Kiss1 cells in contributing to the GnRH surge and positive estradiol feedback.

The inhibitory affect of estradiol on ARH Kiss1 mRNA production led to the hypothesis that this population is involved in negative steroid feedback and the regulation of pulsatile GnRH release. Importantly, the original finding of an inhibitory effect of estradiol on ARH Kiss1 mRNA expression has been recapitulated in numerous species (Adachi et al., 2007; Rometo et al., 2007; Smith et al., 2007). Interestingly, ARH NKB is also negatively regulated by estradiol via ERα, indicating that both ARH Kiss1 and NKB could be contributing to negative steroid feedback (Dellovade and Merchenthaler, 2004;

Navarro et al., 2011a; Pillon et al., 2003; Rance and Bruce, 1994). In agreement with evidence of negative regulation of ARH Kiss1/NKB by estradiol, Kiss1/NKB cells are hypertrophied in postmenopausal women (Rance, 2009; Rance and Young, 1991; Rometo et al., 2007). During menopause the depletion of ovarian follicles causes decreased steroid hormone levels and increased GnRH release (reviewed in Rance, 2009). Therefore, increased Kiss1/NKB in post menopausal women could reflect the decreased steroid levels and also be the source of increased GnRH stimulation. This hypothesis is supported by work in non-human primates demonstarting long-term ovariectomy recapitulates menopausal increases in Kiss1/NKB and that this effect is blocked with estradiol administration (Eghlidi et al., 2011). Interestingly, characteristic post-castration rise in LH levels does not occur in GPR54 knockout mice (Dungan et al., 2007), providing additional evidence that Kiss1 is the predominant signal for negative steroid feedback. Studies employing a GPR54 antagonist demonstrated a lack a castration-induced rise in LH as well as an inhibition of basal pulsatile LH, suggesting a role for tonic Kiss1 in GnRH pulsatile release and negative feedback (Roseweir et al., 2009). Furthermore, a Kiss1 antagonist was capable of inhibiting LH pulses when given in the ARH, but not in the POA, highlighting the ARH as the site where Kiss1 likely regulates tonic GnRH pulsatility (Li et al., 2009). While these findings strongly suggest Kiss1 is involved in tonic GnRH regulation, it remains unclear whether Kiss1 release itself is pulsatile or if constant Kiss1 input is intracellularly converted into a pulsatile release pattern within GnRH neurons.

It is clear that the initial finding of differential regulation of the two Kiss1 populations by estradiol has significantly molded our thinking of the role this neuropeptide plays in regulating GnRH release and there is significant evidence to support a role for AVPV Kiss1 in positive steroid feedback and ARH Kiss1 in negative steroid feedback. Questions remain in our understanding of how ARH Kiss1 contributes to the pulsatile release of GnRH release. In addition, it is unclear if AVPV Kiss1 cells are inactive throughout most of the cycle until the time of the surge, or if these cells also play a role in tonic GnRH release. The explosion of research investigating Kiss1 since its discovered link to reproduction in

2003 has significantly advanced our understanding of GnRH regulation and future work will undoubtedly continue to uncover the details that remain in this field.

3.2.1 Species differences in Kiss1 populations and regulation by estradiol

While estradiol regulation of the two populations of Kiss1 cells is well described in mice and rats, the role of the Kiss1 in steroid feedback in other species is less clear. Common features across species are 1) Kiss1 potently stimulates LH release, 2) Kiss1 fibers make direct contacts onto GnRH cells or fibers expressing GPR54, 3) there is an ARH-equivalent population of Kiss1 with subpopulations coexpressing NKB and DYN, and 4) subpopulations of ARH KNDy cells are inhibited by estradiol. One of the major differences between the Kiss1 system in rodents and other mammals is the lack of a large Kiss1 population in the AVPV-equivalent nucleus in the monkey, human, sheep or guinea pig (Bosch et al., 2012; Hrabovszky et al., 2010; Smith et al., 2007). In these species the predominant population of Kiss1 cells is located in the ARH, or its equivalent, the mediobasal hypothalamus (MBH), with only a few scattered Kiss1 cells found rostrally in the hypothalamus. These findings raised the question of whether Kiss1 played a role in positive steroid feedback in these species.

Similar to the rodent there is significant evidence in the non-human primate that estradiol positive feedback at the level of the hypothalamus is critical for an increase in GnRH release driving the LH surge (Levine et al., 1985; Pau et al., 1993). Rostral scattered preoptic area Kiss1 neurons are positively regulated by estradiol in guinea pigs and sheep, but the significance and role of these few dispersed cells for positive feedback is not well understood (Bosch et al., 2012; Smith et al., 2010a). There is also some evidence that a caudal subset of the ARH Kiss1 population is involved in positive steroid feedback in the sheep and guinea pig (Bosch et al., 2012; Estrada et al., 2006; Smith et al., 2009), confounding the simplistic model of two distal populations of Kiss1 being involved in negative and positive steroid feedback. Further research is required to better understand the role Kiss1 plays in

positive steroid feedback and generation of the LH surge in species other than rodents. Interestingly, recent work has also hypothesized that the highly-conserved GnRH II molecule drives positive steroid feedback in the primate and that perhaps positive and negative steroid feedback is relayed by two GnRH populations as opposed to two Kiss1 populations (Urbanski, 2012).

3.3 Kiss1 in negative energy balance

Existing data clearly demonstrates an important role for Kiss1 in GnRH regulation; therefore, it was investigated whether Kiss1 might be involved in the long-studied paradigm of negative energy balance-induced GnRH inhibition. It was hypothesized that during conditions of low GnRH release, upstream Kiss1 levels might also be decreased and thus contribute to a decreased stimulatory drive at GnRH cells. Early studies observed that undernutrition during development leads to a significant decrease in hypothalamic Kiss1 expression that is coincident with lowered GnRH release (Castellano et al., 2005). Consistent with a strong role for Kiss1 in initiating puberty, exogenous treatment of undernourished animals with Kiss1 can restore LH levels and the time at which puberty occurs (Castellano et al., 2005). Interestingly, a rare genetic mutation prevents desensitization of GPR54 and is associated with precocious puberty, indicating Kiss1 is likely a critical signal for the initiation of puberty (Teles et al., 2008). Importantly, these findings also suggest that Kiss1 is a key component in signaling sufficient metabolic growth for puberty.

Previous studies have also investigated whether there are changes in Kiss1 expression in adult models of negative energy balance. Studies employing an experimentally derived diabetes model observed Kiss1 plays a role in negative energy balance-induced GnRH inhibition. Streptozotocin (STZ) administration results in death of insulin-secreting beta cells in the pancreas and an inability to properly utilize glucose intake resulting in perceived negative energy balance. Whole hypothalamic Kiss1 mRNA levels decrease after STZ treatment, and exogenous application of Kiss1 restores LH levels in these

animals (Castellano et al., 2006b). In an alternative model of negative energy balance, a short term fast, Kiss1 mRNA levels are significantly decreased in the AVPV but not the ARH (Kalamatianos et al., 2008). On the other hand, caloric restriction in the sheep is coincidenct with decreases in ARH Kiss1 mRNA expression (Backholer et al., 2010). Therefore, there is still no significant consensus on whether both Kiss1 populations are responsive to negative energy balance. However, given negative energy balance results primarily in an inhibition of pulsatile GnRH release, it is conceivable that the ARH population is the primary Kiss1 population affected. Consistent with this hypothesis is the strong role the ARH is already known to play in metabolic regulation and food intake. Work presented here in Chapters 2 and 3 examines how both Kiss1 protein and mRNA are differentially regulated in three different models of negative energy balance.

One of the major questions remaining in our understanding of Kiss1's role in negative energy balance, and to be discussed in the next section, is what metabolic factors might signal negative energy balance to Kiss1 populations and how do these signals elicit inhibition of Kiss1 expression. There are many potential metabolic regulators of Kiss1 populations that will be discussed, with special attention given to the adipocyte hormone leptin. If inhibition of upstream Kiss1 is crucial for decreased GnRH release during negative energy balance, the inputs and regulators of these Kiss1 cells are of great interest for our understanding of metabolically-gated reproductive function.

4. Metabolic signals of negative energy balance

Negative energy balance results in a myriad of physiological changes. For the regulation of food intake there is an increase in orexigenic signals and decrease in anorexigenic signals during negative energy balance as well as a decrease in energy expenditure (reviewed in Myers and Simerly, 2010; Sanchez-Lasheras et al., 2010). These changes are signaled by numerous peripheral sites, including adipose tissue, muscles, liver, stomach and pancreas. Peripheral changes in negative energy balance

frequently result in changes in hormone secretion that then signal the hypothalamus to drive increased food intake and decreased metabolic rate. Several of these hormonal and neural cues important for regulating food intake during negative energy balance have also been implicated in GnRH regulation (Smith and Grove, 2002; True et al., 2011a). Hormonal and neuronal signals linked to both metabolic and reproductive regulation are key candidates for the integration of these two systems and potentially contribute to GnRH inhibition during negative energy balance.

There are two profiles frequently observed when investigating metabolic signals involved in reproduction. The first is that negative energy balance results in inhibition of anorectic signals, many of which are also stimulatory for GnRH release. Lowering of these satiety signals during negative energy balance is hypothesized to have the dual purpose of increasing food intake and conserving energy through GnRH inhibition. Conversely, negative metabolic states are also associated with activation of orexigenic signals, many of which also play an inhibitory role in GnRH regulation. These signals stimulate feeding and inhibit GnRH in a complimentary manner to achieve the same net compensatory mechanisms of increased food intake and decreased energy expenditure. To look at a single metabolic/GnRH regulator during negative energy balance is undoubtedly an oversimplified view of the system, since increases in orexigenic signals occur simultaneously with decreases in anorexigenic signals. Whether this overlap represents redundancy or adaptability for precise regulation of metabolic and reproduction function is not well understood. However, work isolating individual components to understand their function within the circuit is critical for building a foundation for future studies to investigate integration of several of these signals for metabolically-driven GnRH inhibition.

4.1 Insulin

Perhaps the most obvious signal of energy intake is the pancreatic hormone insulin. Insulin is produced by β-cells, located in the endocrine tissue of the pancrease known as the islets of langerhans,

in response to rises in glucose levels after food consumption. Increases in insulin with nutrient intake acts in a complex negative feedback loop as a satiety factor to decrease food intake and increase energy expenditure (reviewed in Morton et al., 2006; Plum et al., 2006; Woods et al., 1979). Insulin appears to cross the blood brain barrier by a receptor-mediated transport mechanism (reviewed in Woods et al., 2003) where it elicits satiety effects through regulation of hypothalamic neuropeptides involved in regulating energy homeostasis (Bruning et al., 2000). In fact, the arcuate nucleus melanocortin neurons, which are critical for hypothalamic control of energy homeostasis, express the insulin receptor (IR), a member of the tyrosine kinase receptor family. Consistent with this finding insulin is capable of directly regulating the neuronal activity of these neurons (Benoit et al., 2002; Choudhury et al., 2005; Niswender et al., 2003; Rees-Jones et al., 1984; Williams et al., 2010). Consistent with insulin's anorexigenic effects, insulin can inhibit orexigenic neuropeptides and increase anorexigenic neuropeptides (Benoit et al., 2002; Sato et al., 2005; Schwartz et al., 1992; Wang and Leibowitz, 1997).

Not surprisingly based on insulin's role as a satiety factor, conditions of negative energy balance, and particularly undernutrition result in decreased insulin levels. The relationship between insulin and GnRH release has also been investigated to determine if negative energy balance-induced hypoinsulinemia is a factor in GnRH inhibition. Early studies provided evidence that gonadotrophs in the pituitary are sensitive to insulin, and that insulin may enhance GnRH-stimulated LH release (Adashi et al., 1981; Xia et al., 2001). Importantly, transgenic mice with a brain-specific IR KO have disrupted spermatogenesis and ovarian development and a 90% reduction in serum LH levels is observed in females (Bruning et al., 2000). The same study also observed that females lacking IR in the brain are hypersensitive to a GnRH-R agonist, suggesting that the reproductive dysfunction is due to decreased GnRH release from the hypothalamus leading to overexpression of GnRH-R and hypersensitivity at the level of the pituitary. These results support the hypothesis that insulin is important both at the level of the hypothalamus and pituitary for governing GnRH regulation.

Previous work from our lab investigated the role of hypoinsulinemia in the differential regulation of hypothalamic reproductive and feeding neuropeptides during negative energy balance, such as increased levels of orexigenic signals like neuropeptide Y (NPY) and agouti-related peptide (AgRP) and decreases in anorexigenic populations like proopiomelanocortin (POMC)-expressing cells. The lactating rat, to be described later in detail, was used to investigate this hypothesis given the energy drain from milk production results in a perceived negative energy balance. Lowered insulin levels are particularly interesting in this model given that lactating animals are extremely hyperphagic, eating approximately 3 times as much as non-lactating controls in an attempt to compensate for the energy expenditure of milk production. When insulin levels are restored during mid-lactation to physiological levels, many of the changes in feeding neuropeptides are reversed (Xu et al., 2009b). However, restoration of insulin does not attenuate changes in ARH Kiss/NKB or serum LH. These results suggest that while insulin is a major metabolic regulator of neural circuits, hypoinsulinemia during lactation is not required for Kiss1 and GnRH inhibition. However, despite insulin restoration alone not being sufficient for restoration of reproductive function, it remains to be investigated whether insulin might be one of many players that act in concert to elicit changes in Kiss1/GnRH release.

4.2 Leptin

Leptin is a hormone produced in adipocytes and circulating leptin levels are positively correlated to fat mass (Considine et al., 1996; Frederich et al., 1995; Maffei et al., 1995). Leptin is hypothesized to acts as a satiety signal to drive decreases in food intake. Leptin levels also increase transiently with food intake prior to major changes in fat mass (Saladin et al., 1995). In 1950, mutations in a then unknown gene were observed to cause profound obesity, and the gene was termed “obese” and abbreviated *ob* (Ingalls et al., 1950). Cloning later identified the obese gene product as the 16 kDa hormone leptin (Zhang et al., 1994). The severely obese phenotype of mice homozygous for the leptin mutation, ob/ob, appeared to be caused by hyperphagia, reduced activity, reduced core body temperature and decreased

metabolic rate, all of which can be reversed with exogenous leptin administration (Halaas et al., 1995; Pelleymounter et al., 1995). A similar phenotype was observed in rats with a missense mutation in the leptin receptor gene, named the Zucker Fatty rat (fa/fa) (Phillips et al., 1996). Humans with mutations in the leptin gene, *Ob*, or in the leptin receptor gene, *ObR,* also have a severe early-onset obese phenotype (Clement et al., 1998; Montague et al., 1997; Niv-Spector et al., 2010; Strobel et al., 1998). It is clear that disruption of leptin signaling prevents normal satiety responses leading to extreme hyperphagia and obesity.

Leptin receptors are expressed in several nuclei in the brain, including many hypothalamic nuclei involved in food intake such as the ARH and dorsomedial hypothalamus (DMH) (Elias et al., 2000; Shioda et al., 1998). Indeed, many of the orexigenic and anorexigenic neuropeptides involved in food intake (to be discussed in detail later) express the leptin receptor (Baskin et al., 1999a; Elias et al., 2000; Hakansson et al., 1998). Generally, given the anorexigenic role of leptin as a satiety signal, it is frequently thought to stimulate anorexigenic neuropeptides in the brain while also inhibiting orexigenic neuropeptides. One important caveat to leptin's anorectic actions is the observation that leptin appears to have no effect to decrease food intake during obesity. Obese individuals have increased fat mass and elevated leptin levels, which would be hypothesized to cause a reduction in food intake (Considine et al., 1996; Frederich et al., 1995; Maffei et al., 1995). However, most data suggests that obese individuals either have no change or elevated food intake (Frederich et al., 1995; Lin et al., 2000; Togo et al., 2001). In fact, even exogenous administration of leptin, which causes severe hypophagia in lean animals, has an attenuated effect in obese rodents, suggesting impairment in leptin's ability to decrease food intake during obesity (Seeley et al., 1996; Widdowson et al., 1997). This is widely refferred to as leptin resistance, although the mechanism of how this resistance is achieved is still an area of intense investigative research (Levin and Dunn-Meynell, 2002; reviewed in Munzberg and Myers, 2005).

Leptin's role in food intake is undoubtedly complex, with many potential sites of actions in the brain as well as discrepancies in leptin sensitivity depending on an obese or lean phenotype. In addition to leptin's role in food intake, it also clearly plays a role in other aspects of energy homeostasis in a diverse range of target tissues. In fact leptin receptors are expressed in the majority of peripheral tissues. It is important to keep in mind that while this Introduction focuses on leptin's anorectic effects in the brain, leptin also elicits a wide range of effects and regulatory actions in other areas of the body (reviewed in Margetic et al., 2002), including stimulation of glucose homeostasis in the pancreas (reviewed in Marroqui et al., 2012), inhibition of lipid accumulation and stimulation of fibrogensis of the liver (reviewed in Tsochatzis et al., 2006), cytokine actions to regulate immune function (reviewed in Lago et al., 2008), and stimulation of thermogenesis in brown adipose tissue (Commins et al., 1999).

4.2.1 Leptin's role in reproduction

Importantly, mutations resulting in disruption of leptin production cause infertility as well as obesity in both rodents and humans (Fischer-Posovszky et al., 2010; Ingalls et al., 1950; Strobel et al., 1998). Restoring leptin in these animals and humans restores reproductive function (Chehab et al., 1996; von Schnurbein et al., 2012). Based on these findings it is hypothesized that leptin indirectly contributes to the regulation of reproductive function. Leptin also has a role in the development of sexual maturation. Serum leptin levels begin rising prior to puberty and continue to rise during the time of pubertal maturation in humans; however it remains unclear whether this increase in leptin acts a permissive signal for puberty to commence or a trigger for puberty onset (Garcia-Mayor et al., 1997; Mann et al., 2003; Mantzoros et al., 1997). Interestingly, this pubertal rise in leptin does not occur in the non-human primate, suggesting other signals are responsible for conveying sufficient growth for the initiation of puberty in this species (Plant and Durrant, 1997).

Delayed puberty caused by negative energy balance, as discussed earlier, is also theorized to be to hypoleptinemia. Restoring leptin can advance the timing of puberty in undernourished rodents, although whether this results in full normalization of delayed puberty is unclear (Cheung et al., 1997; Gruaz et al., 1998). Further evidence in ad libitum fed mice has demonstrated that puberty can be accelerated when exogenous leptin is administered during early development (Ahima et al., 1997; Chehab et al., 1997); however this does not appear to occur in rats (Cheung et al., 1997). These findings point to an important role for leptin in signaling sufficient energy stores required for the initiation of puberty. The current consensus is that leptin is likely a permissive signal for puberty; thus, while leptin does not act as the trigger for puberty onset, sufficient levels of leptin are hypothesized to be required before the initiation of puberty can begin (Cheung et al., 2001).

Additional evidence exists for a role of leptin in negative energy balance-induced GnRH inhibition during adulthood. Fasting causes a significant decrease in serum leptin levels as well as mean LH levels, and exogenous leptin can partially prevent this inhibition of LH (Ahima et al., 1996; Nagatani et al., 1998). Undernutrishment in sheep also leads to lowered levels of leptin, and exogenous leptin can restore LH in this model as well (Henry et al., 2001a). This evidence, as well as developmental studies, indicate that leptin is likely an important metabolic regulator of GnRH release. However, for many years it was unclear how leptin regulated GnRH, because although leptin crosses the blood brain barrier via endothelial receptor-mediated transport, GnRH cells do not express the leptin receptor (Finn et al., 1998a; Quennell et al., 2009). It is hypothesized that leptin acts indirectly to regulate GnRH release through an intermediate cell population. Many hypothalamic neuropeptides, to be discussed more in the following section, that contribute to GnRH regulation are also sensitive to leptin. Thus there are many candidates that are likely important intermediary populations relaying changes in circulating leptin levels to GnRH cells. The next section will focus on Kiss1 and its potential regulation by leptin.

4.1.2 Leptin and Kiss1

The presence of Kiss1 in the ARH, where there is a high abundance of leptin receptors (Elias et al., 2000), led to the hypothesis that Kiss1 cells may be sensitive to circulating leptin providing a pathway by which negative metabolic conditions are conveyed to GnRH cells. Indeed *in situ* hybridization studies observed coexpression of Kiss1 and ObR in the ARH of sheep and mice, suggesting that at least a subpopulation of these neurons are directly sensitive to leptin (Backholer et al., 2010; Smith et al., 2006a). Leptin-deficient ob/ob mice and calorically-restricted ewes both have decreased levels of ARH Kiss1 mRNA and this can be partially restored with leptin treatment (Backholer et al., 2010; Quennell et al., 2011; Smith et al., 2006a). In the STZ model of experimentally-induced diabetes hypothalamic Kiss1 mRNA is inhibited and exogenous leptin can restore both Kiss1 mRNA and LH levels (Castellano et al., 2006b). Interestingly, not only are leptin-deficient states linked to lowered levels of Kiss1, but diet-induced obesity (DIO) and leptin resistance also result in lowered levels of ARH Kiss1 (Quennell et al., 2011). Finally, electrophysiological recording from guinea pig brain slices demonstrates a leptin-stimulatory effect on ARH Kiss1 cell firing (Qiu et al., 2011). These data overwhelmingly support the hypothesis that Kiss1 cells as important relayors of leptin levels to GnRH cells.

4.2 Hypothalamic neuropeptides involved in regulating energy homeostasis and reproduction

The hypothalamus contains numerous neuropeptides involved in regulating energy homeostasis. These include already discussed anorexigenic and orexigenic neuropeptides that span numerous nuclei and have projections throughout the brain. In addition to having effects on food intake, many of these neuropeptides regulate other aspects of metabolic homeostasis such as energy expenditure, thermogenesis and activity. These neuropeptides are differentially regulated during negative energy balance, with increases in orexigenic signals and decreases in anorexigenic signals.

Effects on GnRH and reproductive regulation have also been highlighted for many hypothalamic neuropeptides involved in feeding, indicating significant overlap in metabolic and reproductive circuits that could very likely contribute to negative energy balance-induced GnRH inhibition.

One of the most protent orexigenic neuropeptides in the brain is Neuropeptide Y (NPY). NPY is one of the most abundantly expressed neuropeptides in the brain, and significant hypothalamic populations are present in the ARH and DMH, as well as several other nuclei (Allen et al., 1983). Hypothalamic NPY mRNA levels are directly inhibited by leptin (Stephens et al., 1995), and many NPY cell populations express leptin receptors (Baskin et al., 1999a; Baskin et al., 1999b; Hakansson et al., 1998; Mercer et al., 1996; Williams et al., 1999). This is consistent with NPY's orexigenic role and elucidates one mechanism by which NPY cells respond to changes in energy balance.

There is also significant evidence that NPY acts directly on GnRH cells to regulate release. NPY fibers are in close contact with GnRH cells and GnRH cells express the NPY Y5 receptor as demonstrated by immunohistochemical studies (Campbell et al., 2001; Li et al., 1999b). NPY's effects on GnRH release are regulated by steroid hormones, with NPY stimulating GnRH in the presence of estradiol and inhibiting GnRH in the absence of estradiol in the rat (Kalra and Crowley, 1984). Given that estradiol levels are low during negative energy balance, it is hypothesized NPY inhibits GnRH secretion in this condition. Consistent with this hypothesis, and NPY's orexigenic role, it is significantly increased in the ARH in conditions of negative energy balance, including fasting, caloric restriction (CR) and lactation (Chen et al., 1999; McShane et al., 1992; Xu et al., 2009a; Xu et al., 2009b). Electrophysiological studies have observed a larger percentage of GnRH cells are quiescent during lactation, and this appears to be due in part to increased NPY tone, since antagonism of the Y5R restores the ratio of firing to quiescent cells to control levels (Xu et al., 2009a). Restoration of insulin during lactation normalizes ARH NPY levels back down to control values (Xu et al., 2009b); however, this normalization of NPY has no effect to

restore LH levels. These results support the hypothesis that while increased NPY signaling contributes to GnRH inhibition during negative energy balance, it is not required.

Orexin is another orexigenic neuropeptide, also known as hypocretin, implicated in both metabolic and reproductive regulation. Orexin is widely expressed in many regions of the brain, including a large population in the lateral hypothalamus in rodents (Broberger et al., 1998; Mondal et al., 1999; Sakurai et al., 1998). Orexin cells express leptin receptors and are directly and indirectly inhibited by leptin, which may be another method by which leptin induces satiety (Hakansson et al., 1999; Iqbal et al., 2001; Leinninger et al., 2011). There are two forms of orexin, A and B, and two orexin receptors, OX1R and OX2R, all of which are hypothesized to contribute to orexin's effects on food intake, as well as other known effects on spontaneous activity and energy expenditure (reviewed in Perez-Leighton et al., 2012). Orexin A may play a role in reproductive regulation since GnRH neurons express OX1R, which has the strongest affinity for orexin A, and are contacted by orexin fibers (Campbell et al., 2003; Iqbal et al., 2001). Consistent with elevated orexin levels observed during negative energy balance, previous work has demonstrated that central administration of orexin significantly decreases LH pulse frequency (Tamura et al., 1999). However, orexin's role in driving energy conservation in the form of LH inhibition is in opposition to previous work demonstrating orexin's profound effect to stimulate activity and energy expenditure (Hara et al., 2001). In fact, orexin is known to play an important role in stimulating wakefulness, since ablation of orexin neurons results in a narcoleptic-like condition in rodents (Hara et al., 2001). However, orexin levels in the lateral hypothalamus are elevated during negative energy balance consistent with orexin's role in stimulating food intake (Mondal et al., 1999; Sakurai et al., 1998), suggesting the orexingenic role of this neuropeptide is dominant in negative metabolic states. A recent study employing electrophysiological recordings form GnRH cells observed orexin-induced hyperpolarization of these cells indicating a predmoninantly inhibitory effect for reproductive function (Gaskins and Moenter, 2012). More work is needed to understand if increased

orexin input to GnRH cells is in fact a significant contributor to negative energy balance-induced reproductive dysfunction.

Cocaine- and amphetamine-regulated transcript (CART) is an anorectic neuropeptide that is abundantly expressed in the hypothalamus. Initial characterization of CART was provided by experiments in which icv administration resulted in a significant decrease in food intake (Kristensen et al., 1998). Consistent with this anorectic role of CART, administration of a CART antibody potently increases food intake when injected into the brain (Kristensen et al., 1998). However, the role of CART in food intake is complicated by the discovery that injection of CART into discrete hypothalamic feeding nuclei such as the ARH, PVN or DMH results in a significant increase in food intake (Abbott et al., 2001). Overexpression of CART in the ARH by adenovirus transfection also results in increased food intake and weight gain, and similar although less profound effects are also found when CART is overexpressed in the PVN (Kong et al., 2003; Smith et al., 2008b). The discrepancy between these results has not been fully elucidated in the field, although there is evidence of a motor abnormality when CART is administered icv (Abbott et al., 2001). It has been hypothesized that these motor abnormalities may decrease movement enough to prevent food intake, potentially indicating the anorectic effect is secondary to decreased activity.

However, consistent with an anorectic role for CART, *CART* expression appears generally inhibited in negative metabolic states. Both fasting and CR result in inhibition of CART mRNA levels, particularly in the ARH population (Adam et al., 2002; Henry et al., 2001b; Kristensen et al., 1998). Overexpression of ARH CART also increases thermogenesis, presumably to promote energy expenditure after food intake (Kong et al., 2003). Many different CART populations in the hypothalamus express leptin receptors and *CART* expression is stimulated by leptin. These data have led to the hypothesis that low levels of CART during negative energy balance are due to hypoleptinemia. Anorexigenic signals that

are also involved in reproductive regulation are typically stimulatory for GnRH release; therefore, experiments examining CART's role for GnRH regulation were carried out. Studies by Bourginounon and colleages observed a CART-induced increase in GnRH pulse frequency from a hypothalamic explant preparation (Lebrethon et al., 2000; Parent et al., 2000). However, work from this group has not been replicated by others to date and this preparation did not clarify whether CART acts directly or indirectly to stimulate GnRH release. Therefore, the mechanism by which CART regulates GnRH release and the contribution of this neuropeptide for negative energy balance-induced GnRH inhibition are both important future research questions. Work presented in Chapter 4 investigated whether hypothalamic CART populations are differentially regulated in both CR and lactation and whether changes in CART relay negative metabolic conditions to reproductive neuroendocrine circuits.

It is clear that many changes in the metabolic system may contribute to negative energy balance-induced GnRH inhibition. Both peripheral hormones, like insulin and leptin, as well as the metabolic neuropeptides discussed in this section likely affect GnRH release. These hormones may also indirectly regulate GnRH through regulation of the metabolic neuropeptides known to contribute to GnRH regulation. There is also evidence that both metabolic hormones and neuropeptides may also affect upstream Kiss1 cells as well as other aspects of the reproductive neuroendocrine pathways. In conclusion, there is a high degree of interconnectivity between circuits regulating metabolism and reproduction, making careful study of these systems critical for the advancement of our understanding of the most essential connections and signals in this integrative process.

5. Animal models of negative energy balance

In order to study metabolic regulation of GnRH and the HPG axis, animal studies are of critical importance for a better understanding of how changes in the CNS occur at the time of inhibited GnRH release. Many species have traditionally been used in reproductive biology including rats, mice, sheep

and monkeys, each with their own benefits and disadvantages. Historically the predominant species studied in reproductive neuroendocrinology is the rat. Similar to humans, rats have pulsatile GnRH release, which is frequently indirectly measured by assay of serum LH levels, and inhibition of this pulsatile GnRH release appears to be the cause of negative energy balance-induced GnRH inhibition (Fox and Smith, 1984; 1985; Gruenewald and Matsumoto, 1993). While the rat offers the benefit of being the past animal model of choice, and therefore has been extensively characterized in the past literature, it differs significantly from the primate and human in several key areas of the ovarian cycle. Particularly rats have a very short 4-5 day ovarian cycle, compared to the 28-day cycle in primates and other mammals. In addition rats lack a true luteal phase of the ovarian cycle and have a complete lack of menstruation, highlighting large differences in reproductive regulation between humans and rodents. Despite these disadvantages, the rat model has contributed significantly to our general understanding of GnRH regulation and the underlying changes in hormones, neuropeptides and neurotransmitters that regulate GnRH release.

While the current work is focused on negative energy balance-induced inhibition of the ovarian cycle, it should be noted that the HPG axis can also be affected by metabolism in males. Indeed negative energy balance reduces pulsatile GnRH and LH release in males and result in lowered testosterone release from the testis (Cameron and Nosbisch, 1991; Castellano et al., 2006b; Gruenewald and Matsumoto, 1993; Howland et al., 1974; Slob et al., 1979). The male offers the advantage of a simpler system, with an absence of the cyclic release of ovarian steroids and changes in hypothalamic and pituitary responsiveness to those steroids. However, the male also has the disadvantage of decreased physiological relevance since males experience less severe reproductive dysfunction during negative energy balance compared to females. While GnRH, LH and testosterone might all be low, it is unclear how, if at all, this affects male reproductive capacity. While spermatogenesis has been found to be modestly decreased during negative energy balance, for the most part males remain fertile and thus

appear less severely affected than females (Glass et al., 1986; Jean-Faucher et al., 1982; Lucia et al., 1996; Slob et al., 1979). It could be that the drive to suppress reproductive function in the female is higher due to greater degree of energy conservation achieved by inhibition of ovarian cycling compared to spermatozoa maturation. In addition, shutting down fertility is likely more important to survival in females given the higher energy expenditure required in females compared to males following successful reproduction.

5.1 Lactation as a model of negative energy balance

Lactation is associated with decreased fertility in many species, including both humans and rodents (Britt et al., 1985; Chao, 1987; Fox and Smith, 1984; Macmillan et al., 1996). This infertility is associated with the suckling stimulus, to be discussed later, in both species and with negative energy balance in rodents (Fox and Smith, 1984; McNeilly, 1979; Sirinathsinghji and Martini, 1984). The negative energy balance associated with lactation in rats is well established, since lactating animals have significantly increased food intake, presumably to attempt to adjust for the high amount of energy output that is associated with milk production (Asakuma et al., 2004; Munday and Williamson, 1983; Ota and Yokoyama, 1967a; b). Despite the large increase in food intake, the rate of weight gain in lactating animals is not significantly different from age-matched non-lactating female controls (Ota and Yokoyama, 1967a; b). Additional evidence of negative energy balance is decreased insulin levels (Xu et al., 2009b), decreased thermogenesis (Trayhurn, 1983; Trayhurn et al., 1982), and numerous changes in hypothalamic feeding neuropeptides indicating the need for increased food intake and energy conservation (Chen et al., 1999; Malabu et al., 1994; Rocha et al., 2003; Smith, 1993; Xu et al., 2009a; Xu et al., 2009b). Leptin levels are also significantly decreased in lactating rats and this is particularly surprising given the increased fat mass lactating animals have compared to non-lactating controls (Brogan et al., 1999; Denis et al., 2003; Pickavance et al., 1998; Steingrimsdottir et al., 1980; Xu et al.,

2009b). This suggests that leptin levels are actively inhibited during lactation, another clear indicator of negative energy balance in these animals.

The reproductive dysfunction during lactation is also well characterized in rats. Most striking is a complete absence of LH pulses during lactation, which can also be measured as a significant decrease in mean serum LH (Fox and Smith, 1984; Xu et al., 2009b). This absence of LH pulses appears to occur upstream in the hypothalamus since exogenous GnRH administration can restore LH pulses in lactating rats (Fox and Smith, 1984). As expected with an absence of LH pulses, there are very low levels of circulating estradiol, indicating a lack of follicular growth in the ovary (Smith and Neill, 1977; Taya and Greenwald, 1982). Ovarian histology also confirms the lack of large mature graffian follicles during lactation (Taya and Greenwald, 1982).

More recent work provides evidence of inhibition of ARH Kiss1 mRNA levels during lactation (Xu et al., 2009b; Yamada et al., 2007). This inhibition of ARH Kiss1 is consistent with the proposed role this population plays in pulsatile GnRH release, and suggests ARH Kiss1 inhibition contributes to inhibition of GnRH release via decreased stimulation. Interestingly, ARH NKB levels are also significantly inhibited with negative energy balance, indicating a role of this population in metabolically-driven GnRH inhibition, while dynorphin levels were unchanged with lactation. It appears that lactation then results in differential regulation of neuropeptides within KNDy neurons, and this may affect both Kiss1 release and the autoregulation of this release by NKB and DYN. Specifically, a decrease in auto-stimulatory NKB in the presence of unaltered auto-inhibitory DYN levels might result in decreased Kiss1 release from KNDy neurons during lactation. Future research is needed to determine whether NKB and DYN do in fact represent stimulatory and inhibitory autofeedback signals, respectively. It should also be noted that the suppression of ARH Kiss1/NKB during lactation was observed in OVX animals when ARH Kiss1/NKB expression is expected to be elevated due to the removal of estradiol and negative steroid feedback.

This indicates that there is likely active suppression of ARH Kiss1/NKB during lactation. In conclusion, it appears clear that reproductive function is altered during lactation in rats and through a primary decrease in GnRH release, potentially mediated by inhibited ARH Kiss1/NKB levels, and a secondary decrease in pituitary function leading to abnormal progression through the ovarian cycle.

Interestingly, it should be noted that GnRH mRNA levels appear largely unaffected during lactation (Marks et al., 1993). This somewhat surprising finding is in contrast to clear data that exogenous pulsatile GnRH administration can restore LH release during lactation in the rat (Fox and Smith, 1984). Instead of changes in GnRH transcript production, it appears that release is inhibited through an increase in the number of quiescent GnRH neurons during lactation as determined by electrophysiological recordings (Xu et al., 2009a). Clearly experimental investigations of neuronal neuropeptides must take into consideration that the complex regulation of electrical excitability in these cells may drive crucial changes in protein release that are otherwise undetectable by examination of protein and mRNA levels alone. Such changes may be due to differences in ion channel expression, changing the basal excitability or responsiveness of the neuron. Another possibility, and what appears to account for GnRH quiescence during lactation, is that electrical suppression of the cells is caused by increased inhibitory tone from presynaptic inputs resulting in hyperpolarization and decreased probabilities of action potential firing and neuropeptide release.

Given this extensive characterization of 1) the complex mechanism of inhibited GnRH inhibition release and 2) metabolic disturbances during lactation, this model clearly offers the opportunity to study a naturally occurring phenomenon of negative energy balance in the rat (Smith and Grove, 2002). However, it should be noted that there are additional complex physiological adaptations that are specific to lactation and not common to all conditions of negative energy balance. Most notable of these lactation-specific physiological changes is the suckling stimulus. Somatosensory stimuli of suckling

are relayed to several brainstem populations, and these populations in turn project throughout the hypothalamus and other brain regions and relay a wide range of physiological effects (Bodnar et al., 2002; Chen and Smith, 2003; Li et al., 1998; 1999a; c; Smith and Neill, 1977). Importantly, the suckling stimulus activates many of the same cell types thought to play a role in the compensatory adaptations associated with negative energy balance. Of particular importance is work suggesting that the suckling stimulus is a powerful repressor of reproductive function. As mentioned above, the suckling stimulus is thought to be the sole contributor of GnRH/LH inhibition in humans, with negative energy balance playing very little role except for extreme cases (reviewed by McNeilly et al., 1994). Similar to the human, the suckling stimulus in the absence of milk release, and subsequent negative energy balance, can result in significant GnRH/LH inhibition during lactation in the rat (Brogan et al., 1999). These results suggest that when studying the negative energy balance of lactation, the potential impact of the suckling stimulus may represent a confounding source of GnRH/LH inhibition.

Previous work from our lab investigated the effect of restoring leptin during lactation (Xu et al., 2009b). Leptin was administered peripherally at a dose of 500 ng/hr for 48 hours between Days 8-10 of lactation, and this resulted in restoration of leptin to levels within the physiological range. Surprisingly, leptin was incapable of restoring ARH Kiss1/NKB mRNA expression or serum LH levels during lactation. This finding stood in opposition to the wealth of previous literature demonstrating leptin's stimulatory effects on LH release, and to a lesser extent on Kiss1 expression, during negative energy balance (Ahima et al., 1996; Henry et al., 2004; Henry et al., 2001a; Smith et al., 2006a). We hypothesized two possible reasons for the discrepancy in this result and those previously described. The first hypothesis was that despite restoration of leptin, inhibitory drive for Kiss1 and LH persisted from the suckling stimulus. If this were true, then any potential stimulatory effect of leptin might have been masked. Since previous work had not looked at leptin's effect during lactation this hypothesis seemed reasonable and work

presented in Chapter 3 sought to examine the effects of exogenous leptin restoration in a model of negative energy balance similar to lactation but lacking the suckling stimulus.

An additional hypothesis was that perhaps leptin's effects were not observed because the dose of 500 ng/hr administred peripherally (intraperitoneal) was significantly lower than doses used in previous studies. The original study by Ahima and colleagues (Ahima et al., 1996) replaced leptin with an intraperitoneal bolus of 1 mg/kg leptin and measurement of acute serum leptin levels observed a 50-fold elevation in serum leptin levels compared to control conditions. Importantly, when animals exit negative energy balance leptin levels increase gradually over time to normal basal levels and do not reach levels as high as those observed with this dose (Szymanski et al., 2007). Additional studies in the field have continued to use high supraphysiological doses of leptin ranging from 1-5 mg/kg for peripheral administration (Nagatani et al., 1998; Quennell et al., 2011; Smith et al., 2006a) and frequently several micrograms for icv administration (Backholer et al., 2010; Henry et al., 2004; Henry et al., 2001a; Quennell et al., 2009). The one exception is work in humans, in which restoration of leptin to levels similar to those observed in normal women increases LH release in women with hypothalamic amenorrhea and during fasting (Chan et al., 2006; Schurgin et al., 2004; Welt et al., 2004). But to our knowledge the study by Xu et al., was the first to use a physiological dose of leptin during negative energy balance in an animal model. Therefore, while it is clear supraphysiological leptin can modestly stimulate LH release it remained unclear whether the much smaller natural increase in leptin experienced upon exit from negative energy balance is critical for normalization of Kiss1 and LH levels in rodents. To test this we proposed to compare our lower dose of leptin to the supraphysiological levels used in previous studies and examine Kiss1 and LH levels. Work presented here, discussed in Chapter 3, investigated whether restoration of leptin to levels in the physiological range is sufficient to restore Kiss1 and LH levels during negative energy balance, and how this response compares to that achieved with supraphysiological doses of leptin.

5.2 Undernutrition models of negative energy balance

5.2.1 Fasting

Perhaps the most well-studied system of negative energy balance is the fasting model. In this model all food, but not water, is removed for a given period of time, usually 48 hours in the rat. This results in an obvious deficit in energy intake and subsequent negative energy balance. Indicative of this negative energy balance are observations of decreased insulin levels and serum leptin levels, the latter of which is surprisingly given only small changes in fat mass (Boden et al., 1996). Fasting also results in decreased LH release, with observations of decreased pulsatile release as well as decreased overall mean levels of LH (Aloi et al., 1997; Bergendahl et al., 1998; Cameron and Nosbisch, 1991; Matsuyama et al., 2004; Nagatani et al., 1998; Veldhuis et al., 1993). As with other models of negative energy balance, decreased pulsatile LH is attributed to changes in GnRH release, given rescue of the phenotype with exogenous GnRH treatment (Bergendahl et al., 1991; Cameron and Nosbisch, 1991).

There is also evidence that fasting results in Kiss1 inhibition. Fasting in prepubertal rats resulted in a suppression of whole hypothalamic Kiss1 levels (Castellano et al., 2005). Two studies to date distinguishing ARH and AVPV Kiss1 mRNA levels have demonstrated that fasting specifically suppresses AVPV Kiss1 levels and not Kiss1 in the ARH (Forbes et al., 2009; Kalamatianos et al., 2008). In the non-human primate exogenous kisspeptin treatment restores LH levels during fasting conditions (Wahab et al., 2008). These findings significantly point to a role of fasting-induced inhibited Kiss1 contributing to GnRH inhibition. Very recent studies have found evidence that ARH NKB mRNA levels are also significantly reduced in a 48 hr fast in pre-pubertal rats (Navarro et al., 2012).

The fasting model offers the primary benefit of its frequent historical use, therefore making for easy comparison to past literature. The physiological relevance of this model is perhaps its main

downfall. For comparison to human disorders of hypothalamic amenorrhea, fasting might offer little practical comparison since it frequently represents a very severe decrease in caloric intake over a relatively short amount of time. This is in contrast to the more gradual transition to a negative metabolic state usually experienced in humans with functional hypothalamic amenorrhea. Fasting may also represent more of a "starvation" state and it is possible that there are differences in the metabolic and reproductive phenotype between starvation and less severe models of negative energy balance. In addition, fasting is known to activate the hypothalamic-pituitary-adrenal axis which can negatively regulate GnRH release, potentially confounding the source of GnRH inhibition in this model (Maeda et al., 1994; Tsukamura et al., 1994; Williams et al., 1990).

5.2.2 Caloric restriction

Caloric restriction (CR) is another model of undernutrition, but unlike the 48 hour fast, CR represents a more modest decrease in caloric input that can be carried out over longer periods of time, usually resulting in more gradual weight loss. Interestingly, a 30% CR model was developed primarily to investigate whether decreased oxidative stress had anti-aging effects in rodents and primates (Sohal and Weindruch, 1996). It should be noted that aging studies of CR frequently seek to replace nutrients, so although overall caloric intake is reduced, necessary vitamins, carbohydrates, and proteins are still present and calories are diminished by a decrease in fat intake. This is in contrast to the caloric restriction used in reproductive neuroendocrinology, previously studied in sheep. In this model total caloric and nutrient input are reduced, usually by 40% or more, resulting in a significantly reduced body weight and an anovulatory state (Henry et al., 2004; Henry et al., 2001a; Tatman et al., 1990). Similar to fasting, CR results in decreased pulsatile LH release thought to be caused by upstream inhibition of GnRH (Adam et al., 1997; I'Anson et al., 2000).

Given that this model has been a well characterized example of negative energy balance-induced GnRH inhibition in the sheep it is surprising how infrequently this model has been used in rodent studies of reproductive regulation. One study has observed that LH desensitization to Kiss1 is delayed in chronically CR animals compared to ad libitum fed animals, indicating a decrease in endogenous Kiss1 and GPR54 during CR (Roa et al., 2008b). It may be that fasting is used instead of CR in adult studies due to the relatively quick and easy experimental design offered by this model. Initial characterization of the CR model in the adult rat would be beneficial for our understanding of how GnRH function is altered with longer-term and more gradual model of negative energy balance, compared to fasting. As such, it may be closer to the condition experienced in women with hypothalamic amenorrhea who usually have gradual weight loss over time due to modestly decreased food intake.

6. The requirement of estradiol for negative energy balance-induced GnRH inhibition

Previous studies investigating metabolically-driven GnRH inhibition have spent considerable time investigating the role estradiol plays in this response. This work has unveiled several key findings that suggest that fasting-induced GnRH suppression is mediated by estradiol signaling. Early indications of an estradiol-component were provided by evidence of a lack of fasting-induced suppression of GnRH in ovariectomized (OVX) animals when compared to intact females and OVX females with estradiol replacement (OVX+E)(Kalamatianos et al., 2008; Matsuyama et al., 2004; Nagatani et al., 1994). This work carried out by Maeda and colleagues provided evidence for a role of estradiol in sensitizing reproductive circuits to the inhibitory influence of catecholamines, leading to increased release of the hypothalamic stress hormone corticotrophin-releasing hormone (CRH)(Cagampang et al., 1992; Maeda et al., 1994; Nagatani et al., 1994; Nagatani et al., 1996; Tsukamura et al., 1994). This increase in CRH was hypothesized to be directly responsible for GnRH inhibition during fasting. Interestingly, estradiol

does not appear required for lactation-induced GnRH inhibition, given evidence that both intact and OVX lactating animals have similarly decreased levels of LH (Fox and Smith, 1984). To date the role of estradiol in CR-mediated GnRH inhibition has not been studied.

Interpretation of studies investigating the requirement of estradiol for negative energy balance-induced-GnRH inhibition is confounded by the known role of estradiol in metabolic regulation. Estradiol is an anorectic hormone, causing decreases in food intake (Butera et al., 1990; Thompson and Cox, 1979; Wurtman and Baum, 1980). OVX results in a characteristic increase in body weight compared to intact animals, and there is evidence that this is due both to increased food intake and decreased metabolic rate (Gao et al., 2007; McElroy and Wade, 1987; Roy and Wade, 1977; Wade and Zucker, 1970). A large part of estradiol effects on energy homeostasis appear to mediated by ERα, since ERα knockout mice have increased adiposity and decreased energy expenditure (Heine et al., 2000), similar to findings in humans with mutations in ER (Smith et al., 1994). The site of estradiol action for reduced food intake and increased energy expenditure remains largely unknown; however, estradiol has been shown to regulate many of the hypothalamic feeding neuropeptides and thus may partially carry out its effects on body weight homeostasis through these cells (Crowley et al., 1985; Messina et al., 2006; Santollo and Eckel, 2008; Tong et al., 1990; Xu et al., 2011). There is also some evidence that estradiol regulates the expression of leptin receptor and may interact with the leptin-receptor signaling pathways to mediate some of its metabolic actions (Clegg et al., 2006; Gao et al., 2007; Rocha et al., 2004). It seems clear that in addition to regulation of stress pathways described by Maeda and colleagues, estradiol also affects how the brain interprets cues of a negative metabolic state to affect GnRH release.

To date these changes in food intake and metabolism have not been accounted for when studying the requirement of estradiol for negative energy balance-induced GnRH inhibition. Therefore, it remains unknown whether estradiol signaling is required somewhere specifically in the circuits

underlying GnRH inhibition or if perhaps OVX animals simply do not experience as severe a state of negative energy balance due to the absence of estradiol. Treatment with high doses of estradiol during negative energy balance also imposes some limitations on interpretations of previous data. Estradiol levels are naturally suppressed during negative metabolic states due to decreased GnRH release, and this can be observed in the form of low uterine weight (Ronnekleiv et al., 1978; Smith and Neill, 1977). Therefore, exogenous replacement of estradiol to the high end of the physiological spectrum could still represent levels several fold higher than those experienced by intact animals during negative energy balance (Butcher et al., 1974). Based on this rationale it is conceivable that negative energy balance is artificially exacerbated with high estradiol replacement resulting in increased metabolic rate. Future studies are needed to address the true requirement of estradiol in mediating negative energy balance-induced GnRH inhibition while controlling for the metabolic effects of this hormone.

Interestingly, the majority of studies investigating estradiol requirements for fasting-mediated LH inhibition were conducted prior to the discovery of Kiss1's role in reproduction and steroid feedback. Observations that Kiss1 is strongly regulated by estradiol levels and is also a potent stimulator of GnRH release have led to speculation that Kiss1 may be an important aspect of the estradiol requirement for GnRH suppression. Indeed, one study investigating this possibility found that AVPV Kiss1 levels are inhibited along with serum LH in fasted OVX+E rats, but AVPV Kiss1 levels remain unaffected in fasted OVX animals (Kalamatianos et al., 2008). However, this study noted that estradiol treated rats had lowered body weights both before and after the 48-hr fast making it difficult to determine if it was the estradiol or added negative metabolic effects of the steroid that were required for Kiss1 and LH inhibition. To gain a better understanding of the role of estradiol in negative energy balance-induced Kiss1 and LH inhibition, experiments presented in Chapter 3 aimed to control for the negative metabolic effect of estradiol by weight-matching OVX animals to OVX+E animals during negative energy balance. By controlling for metabolic effects of estradiol it was the goal to determine what role steroids might

play in the neural circuits and particularly on ARH and AVPV Kiss1 populations during negative energy balance.

7. Statement of purpose

The aim of the current work was to gain a better understanding of both the hormonal cues and neural circuits that underlie GnRH inhibition during negative metabolic states. Given Kiss1's prominent role in GnRH regulation I examined whether either or both Kiss1 populations are affected by various models of negative energy balance, at both the protein and mRNA level. To begin to understand what metabolic cues might regulate Kiss1 and GnRH during negative energy balance I investigated whether restoring leptin could restore reproductive function in two different models of negative energy balance. Finally, the anorexigenic neuropeptide CART was investigated to determine if changes in CART might affect GnRH release during negative energy balance. As a whole this work significantly contributes to our understanding of how the GnRH system is regulated during negative energy balance. In addition detailed neuroanatomical work provides insights into the potential workings of the circuits of both Kiss1 and CART as they pertain to GnRH regulation. Overall this work provides meaningful insights into metabolic regulation of reproduction in the brain and has led to a better understanding of this long-standing basic research question.

AIMS OF THE THESIS AND APPROACH

Aim 1: Characterize projections of arcuate nucleus and periventricular nucleus kisspeptin populations with regard to regulation of GnRH release.

Approach:

Double-label immunohistochemistsry was used to distinguish projections of AVPV (single-labeled Kiss1 fibers) from ARH Kiss1 populations (double-labeled Kiss1/NKB fibers) at the level of GnRH cell bodies and fiber terminals.

Hypothesis. Distinct physiological role of the two rodent Kiss1 populations for GnRH regulation occurs at distinct neuroanatomical locations.

Aim 2: Determine whether kisspeptin is differentially regulated during negative energy balance and the contribution of estradiol to this inhibition.

Approach:

1) In situ hybridization and real-time PCR were used to investigate changes in Kiss1 mRNA levels in the ARH and AVPV during 1) lactation, 2) caloric restriction and 3) fasting.
2) Examine Kiss1 mRNA levels and LH levels by radioimmunoassay in during caloric restriction with and without estradiol present.

Hypothesis. Kiss1 levels are inhibited during negative energy balance, which may result in decreased stimulatory tone for GnRH cells.

Aim 3: Investigate whether normalization of hypoleptinemia during negative energy balance rescues reproductive phenotype.

Approach:

Osmotic minipumps with a low dose of leptin were given during the final 48 hrs of 1) caloric restriction and 2) fasting. Effects of leptin administration were examined at the level of:

1) ARH Kiss1/NKB and AVPV Kiss1 mRNA expression as determined by real-time PCR
2) Serum LH levels as determined by radioimmunoassay

Hypothesis. Restoration of leptin in an undernutrition model of negative energy balance will attenuate inhibition in Kiss1 and LH levels.

Aim 4. Characterize neuroanatomical relationship between CART and GnRH neurons and determine whether hypothalamic CART populations are differentially regulated during negative energy balance.

Approach:

1) Changes in CART protein and mRNA, measured by immunohistochemistry and in situ hybridization respectively, were investigated during 1) lactation and 2) caloric restriction.
2) Immunohistochemistry was used to investigate morphological evidence for CART regulation of GnRH and kisspeptin populations.

Hypothesis. CART cells are sensitive to changes in energy balance and may directly relay these changes to GnRH neurons.

Chapter 2 - Characterisation of Arcuate Nucleus Kisspeptin/Neurokinin B Neuronal Projections and Regulation during Lactation in the Rat

True C, Kirigiti M, Ciofi P, Grove KL, Smith MS. 2011. Characterisation of arcuate nucleus kisspeptin/neurokinin B neuronal projections and regulation during lactation in the rat. J Neuroendocrinol 23(1):52-64.
PMID: 21029216
PMCID: PMC3118985

ABSTRACT

Lactation results in negative energy balance in the rat leading to decreased gonadotropin-releasing hormone (GnRH) release and anestrus. Inhibited GnRH release may be due to decreased stimulatory tone from neuropeptides critical for GnRH neuronal activity, such as kisspeptin (Kiss1) and neurokinin B (NKB). This study aimed to identify neuronal projections from the colocalized population of Kiss1/NKB cells in the arcuate nucleus (ARH) using double-label immunohistochemistry to determine where this population may directly regulate GnRH neuronal activity. Additionally, this study further examined lactation-induced changes in the Kiss1 system that could play a role in decreased GnRH release. The colocalized ARH Kiss1/NKB fibers projected primarily to the internal zone of the ME where they were in close proximity to GnRH fibers; however few Kiss1/NKB fibers from the ARH were seen at the level of GnRH neurons in the preoptic area (POA). Arcuate Kiss1/NKB peptide levels were decreased during lactation consistent with previous mRNA data. Surprisingly, anteroventral periventricular (AVPV) Kiss1 peptide levels were increased, while *Kiss1* mRNA levels were decreased during lactation suggesting active inhibition of peptide release. These findings indicate ARH Kiss1/NKB peptide levels and AVPV kiss1 mRNA are inhibited during lactation which may contribute to decreased GnRH release and subsequent reproductive dysfunction. Furthermore, the absence of a strong ARH Kiss1/NKB projection to the POA suggests regulation of GnRH by this population occurs primarily at the ME level via local projections.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a critical component of the hypothalamic-pituitary-gonadal (HPG) axis governing regulation of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). When metabolic homeostasis is disrupted towards negative energy balance, the HPG axis is inhibited in female mammals, resulting in anovulation. This inhibition of cyclic reproductive function is largely attributed to decreases in GnRH release, but the upstream regulatory events leading to this decrease are not well understood.

Recent research into the regulation of GnRH release has focused largely on the powerful secretagogue kisspeptin (Kiss1) (Gottsch et al., 2006; Popa et al., 2008; Roa et al., 2008a; Smith et al., 2006b). Originally, a mutation in the human gene for the Kiss1 receptor GPR54 was found to result in hypogonadotropic hypogonadism, a disorder characterized by low GnRH release during development (de Roux et al., 2003; Seminara et al., 2003). Subsequent research has demonstrated that Kiss1 administration alone causes large increases in circulating LH and FSH levels through a GnRH dependent mechanism (Matsui et al., 2004; Navarro et al., 2004; Navarro et al., 2005). Importantly, rodent data has demonstrated that Kiss1 appears important for steroid feedback regulation of GnRH since the anteroventral periventricular (AVPV) Kiss1 population is linked to positive feedback and the arcuate nucleus (ARH) population is linked to negative feedback (Smith et al., 2005; Uenoyama et al., 2009). A recent study demonstrated that, like GPR54 mutations, human mutations in the neurokinin B (NKB) receptor, NK3, as well as NKB itself, resulted in hypogonadotropic hypogonadism, indicating NKB may be another critical stimulator of GnRH release (Topaloglu et al., 2009). NKB has also long been known to be inhibited by estradiol and thus NKB may also contribute to negative steroid feedback of GnRH activity (Rance, 2009; Rance and Young, 1991). Interestingly, the Kiss1 population in the arcuate nucleus of the hypothalamus (ARH) is colocalized with NKB in the non-human primate (Ramaswamy et al., 2010), ewe (Goodman et al., 2007), mouse (Navarro et al., 2009) and the rat (present study).

Negative energy balance-induced GnRH inhibition may result from decreased stimulatory tone set by neuropeptides like Kiss1 and NKB. Lactation in the rat is a well-studied model of negative energy balance, in which the high metabolic cost of milk production results in decreased GnRH release. Recent data has demonstrated a decrease in ARH *Kiss1* mRNA and Kiss1 peptide levels during lactation in the rat as well as decreased NKB mRNA levels, but NKB peptide levels are unknown (Xu et al., 2009b; Yamada et al., 2007). The Kiss1 population in the AVPV has also been linked to stimulating GnRH release, particularly in high estrogen conditions during proestrus leading to the LH surge, suggesting inhibition of this population could also contribute to decreased stimulatory tone for GnRH release (Clarkson et al., 2008; Herbison, 2008; Popa et al., 2008; Smith et al., 2006b).

Although ARH Kiss1/NKB neurons have previously been linked to GnRH regulation, the site of regulatory contact between these two neuronal populations is not well understood. The presence of GPR54 mRNA in GnRH cell bodies and NK3 on GnRH fibers suggests direct regulation of GnRH by NKB and Kiss1 (Irwig et al., 2005; Krajewski et al., 2005; Quaynor et al., 2007). Additional evidence for direct regulation is the presence of both Kiss1-immunoreactive (-ir) fibers as well as NKB-ir fibers in the ME, near GnRH fibers, and in the POA, near GnRH cell bodies (Clarkson and Herbison, 2006; Krajewski et al., 2005; Marksteiner et al., 1992; Ramaswamy et al., 2010). Therefore, both Kiss1 and NKB may regulate GnRH release at fibers in the ME as well as cell bodies in the POA, but the nuclei from which these Kiss1 and NKB fibers originate remains unclear.

The regulation of Kiss1 and NKB populations as well as their possible projections to GnRH cells could prove to be critical for our understanding of GnRH inhibition during negative energy balance. The aim of this study was 1) to use double-label immunohistochemistry to characterize projections of ARH Kiss1/NKB fibers and 2) to determine lactation-induced changes in these neuropeptides, which may contribute to decreased GnRH release.

MATERIALS AND METHODS

Animals

Adult female Wistar rats (200-220 g; Simonsen) were used in all studies. Animals were maintained on a 12-hour light (0700 hr) and dark (1900 hr) cycle throughout the experiment and allowed food (Purina Lab Chow 5001) and water ad libitum. All protocols were approved by the Oregon National Primate Research Center Animal Care and Use Committee and conducted in accordance with National Research Council Guide for the Care and Use of Laboratory Animals.

Experiment 1: ME Projections of ARH Kiss1/NKB Neurons

Transgenic rats with GFP expression under the control of the GnRH promoter were used for immunohistochemistry to investigate ARH Kiss1/NKB projections to the ME, where GnRH fibers terminate. Ovariectomy was performed on all animals in order to remove negative steroid feedback and maximize ARH Kiss1 and NKB peptide expression. Adult ovariectomized (OVX) virgins animals (n=4) served as controls for comparison to adult lactating animals (n=4), which were OVX on Day 2 of lactation. Animals were euthanized eight days following OVX, which corresponded to Day 10 of lactation. Day 10 of lactation is mid-lactation, a time when animals are known to be experiencing suppression of GnRH/LH and severe negative energy balance (Xu et al., 2009b). Animals were anaesthetized under pentobarbital anesthesia and then perfused transcardially with ice cold saline followed by ice cold 4% paraformaldehyde in $NaPO_4$ buffer (pH 7.4). Brains were removed and saturated for 24 hours in paraformaldehyde followed by 24 hours in 25% sucrose and then frozen on dry ice and stored at -80 ˚C. Fixed brain tissue was sectioned coronally at 25 µm, and collected serially into 6 groups, so that consecutive sections for a given series are approximately 150 µm apart. Tissue was cut using a sliding microtome and preserved in ethylene glycol cryoprotectant at -20 ˚C.

Immunohistochemistry

A single series of fixed tissue from each animal was used for immunohistochemistry experiments starting with a potassium phosphate buffer rinse followed by blocking in 2% donkey serum and 0.4% Triton X-100 for 30 minutes. Tissue was then incubated for one hour at room temperature followed by 48 hours at 4˚C in a primary antibody solution. All antibodies were titrated to give an optimal signal and minimal background. The Millipore rabbit anti-Kiss1 antibody (Cat . #AB9754, Lot #LV1541898; Millipore, Billerica, MA) used in the current study was initially characterized and distributed by Dr. Alan Caraty (Franceschini et al., 2006). For immunohistochemistry the Kiss1 antibody was used at a dilution of 1:1500 with fluorescence secondary detection. Preadsorption with 10 µM RFRP1 diminished labeling with this Kiss1 antibody suggesting some cross reaction. However, the lack of immunolabeled cells in the DMH where RFRP1 cells are located suggests this cross reactivity is minimal. Preadsorption with 10 µM Kiss1 abolished all staining. The guinea pig anti-NKB antibody (IS-3/61) has been fully characterized as specific for NKB (Ciofi et al., 1994; Marksteiner et al., 1992). This antibody was used at a dilution of 1:4000 for fluorescence labeling and 1:8000 for NiDAB labeling.

Following primary incubation, tissue was rinsed again before hour incubation at room temperature in the secondary antibody cocktail. For fluorescence detection of the Kiss1 antibody a 1:200 dilution of anti-rabbit Cy-5 (Jackson Immunoresearch, 711-175-152) was used while a 1:200 dilution of anti-guinea pig TRITC (Jackson Immunoresearch, 706-026-148) was used for detection of the NKB antibody. After secondary labeling all tissue was immediately rinsed and mounted onto subbed slides and coverslipped for analysis by fluorescence microscopy. For NiDAB secondary labeling of the NKB antibody, tissue was again initially rinsed in buffer then incubated for an hour at room temperature in a biotinylated donkey anti-guinea pig antibody (Jackson Immunoresearch, 706-066-148), followed by an hour incubation in A/B solution (Vectostain Elite ABC Kit, Burlingame, CA; 1:222 dilution of both the A and B solution). Tissue was then incubated in a nickel sulfate DAB solution in sodium acetate until adequate staining was obtained. Mounted tissue was then dried on slides overnight and then

dehydrated through increasing ethanol washes followed by 2 5-minute xylene washes and immediately coverslipped with Permount.

Confocal Microscopy and Photomicrograph Adjustments

The triple-label immunofluorescence was imaged on a Leica TCS SP Confocal microscope (Leica Microsystems, Inc., Bannockburn, IL). 25X images of GnRH-GFP (using a 488nm AR laser), Kiss1-ir (using a 633nm HeNe laser) and NKB-ir (using a 651nm DPSS laser) were taken at 0.5µm increments along the z-axis of the tissue. Each wavelength was imaged sequentially to avoid bleed-through of different fluorophores to the opposing detector. Image stacks were then compiled using Metamorph software. Metamorph was also used to pseudo-color photomicrographs: Millipore Kiss1-ir was converted from infrared to green, and GnRH-GFP was converted from green to blue.

Further color adjustments were carried out with Adobe Photoshop in order to more accurately represent microscope displays. All confocal images were adjusted to 3.5 by 3.5 inch image size and the resolution was adjusted to 150. Photomicrographs were lightly brightened by adjusting input levels to 25, 1.0, and 225. Pseudo-coloring was performed using the "selective color replacement" function to convert NKB staining from red to magenta, magenta staining (overlap of blue GnRH and red NKB labeling) to white, and to lighten blue GnRH fibers for better visibility.

Fiber Quantification and Colocalization Analysis

To determine the extent of Kiss1/NKB cell body colocalization in the ARH, 2 ARH sections were used per animal. Cells were manually counted on one side of the ARH for both sections and the percent of colocalized cells was averaged across the two sections for each animal.

Single Kiss1 and NKB fiber content in the ME was examined from ME photomicrograph stacks using ImageJ software. Photomicrographs of 2 ME sections were used per animal and for all measurements, the values between the two ME were averaged per animal. The ME was traced, with tracing beginning at one corner of the ME and encompassing as much of the ME as was included in the

photomicrograph, usually around half. The "Analyze Particles" measurement was used, excluding particles that were composed of only 2 pixels or less, to determine the area of the region of interest that contains either Kiss1 or NKB staining. Particles were summed through the entire 28 planes (0.5 µm thickness) and then normalized to the total area of the traced portion of the ME, and are expressed as Particles/Area Examined. This analysis was performed for both Kiss1 and NKB labeling to determine whether lactation resulted in decreases in the total area of either Kiss1 or NKB total staining. In addition Just Another Colocalization Program (JACoP) by S. Bolte and F.P. Cordelières (Bolte and Cordelieres, 2006) was used to calculate the Manders' Coefficients, which are the percentages of one staining that has the same location as another staining, to determine the fraction of overlap between Kiss1 and NKB staining in the ME.

Experiment 2: Rostral Projections of ARH Kiss1/NKB Neurons

To determine rostral projections of ARH Kiss1/NKB neurons, specifically in relation to GnRH cell bodies a series of tissue sections were used from the animals described in Experiment 1, with one lactating animal excluded due to a technical error. Immunohistochemistry and confocal microscopy was performed as described above, except no NiDAB secondary labeling was used. For analysis of double-labeled fiber content at the level of GnRH neurons, two confocal images/animal were taken at the level of GnRH neurons for lactating (n=3) and control animals (n=4). No significant differences were observed in colocalized fiber content between the two groups so they were combined for analysis. For each animal, one image was taken at the level of the medial septal nucleus while another image was taken at the level of the Diagonal Band of Broca (NDB). Images containing 28 stacks of 0.5 µm thickness were once again analysed using the ImageJ plugin Just Another Colocalization Program (JACoP) by S. Bolte and F.P. Cordelières (Bolte and Cordelieres, 2006) to determine the fraction of overlap between Kiss1 and NKB staining in the POA.

These two images per animal were also used to analyse close appositions on GnRH cells. Ten neurons across the 2 images were chosen at random and were analyzed for close appositions of either Kiss1 or Kiss1/NKB staining. Close appositions were defined as any double-label with GnRH that was observed within a 1 µm plane. Quantification was carried out similarly as described for the ME, only the entire photomicrograph was analyzed for the POA instead of using a region of interest, as was done for the ME.

Computer Assisted Line Drawing

To generate the computer assisted line drawing of Kiss1 and NKB fiber projections using confocal microscopy, one representative OVX virgin control case was selected and imaged at 10X, taking images at 1µm increments, from the NDB to the level of the ARH. For each section chosen, two adjacent images were taken along one side of the third ventricle. Forebrain levels were identified using the atlas Brain Maps: Structure of the Rat Brain (L.W. Swanson, 1992). Montages of the confocal images were constructed using Adobe Illustrator. Lines color-coded to represent either Kiss1-ir, NKB-ir, or double Kiss1/NKB-ir were drawn over contiguous fiber projections and terminal axonal fields using the pencil tool. At the level of the NDB, GnRH neurons were also traced using the pencil tool and later replaced with triangle symbols.

Experiment 3: Regulation of Kiss1 and NKB Populations during Lactation

All comparisons between lactating and control conditions were performed in intact animals. Rats in the lactation group were received 17-18 days pregnant and the day of pup birth was defined as Day 0 post partum. On Day 2/3 litters were adjusted to 8 pups and tissue was collected Day 10/11 post partum. Tissue was collected from control animals on diestrus after 2 consecutive 4-5 day estrous cycles were observed by vaginal cytology. Immunohistochemistry was performed as described above using the Millipore rabbit Kiss1 antibody (1:1000 dilution) and the guinea pig NKB antibody (1:4000 dilution) and NiDAB secondary labeling.

Cell Counts

For the ARH, cell counts for the total number of Kiss1-ir cells from both sides of the ventricle were summed together for four sections per animal (n=4 animals for lactating and diestrus groups). The same protocol was carried out to determine ARH NKB-ir cell counts (n=4 animals for lactating group and n=3 animals for diestrus group). AVPV Kiss1-ir cell counts were performed in a similar manner except that only 3 sections were counted per animal due to the small size of this nucleus (n=3 animals for lactation group and n=4 animals for diestrus group).

***In situ* hybridization**

Intact virgin and lactating groups were also used for *in situ* hybridization. Animals were sedated with isoflurane before decapitation and rapid removal of the brain, which was subsequently frozen on powdered dry ice and stored at -80 ˚C until sectioning. Fresh frozen brains were cut into a one-in-three series of 20 µm coronal sections by cryostat (MICROM HM500OM, Carl Zeiss IMT Corporation, Maple Grove, MN) from the AVPV through the BST. *In situ* hybridization was performed as previously described (Xu et al., 2009b). One series of fresh frozen tissue per animal was briefly fixed in a phosphate buffered paraformaldehyde solution and subsequently treated with 0.25% acetic anhydride in 0.1 M triethanolamine. Tissue was then taken through 2 washes in sodium saline citrate and dehydrated in a series of ethanol solutions so that delipidation in a choloroform wash could be performed. Tissue was then rehydrated through a reverse series of ethanol solutions and air dried.

The *Kiss1* probe (a gift from the lab of Dr. Robert Steiner), which has been previously characterized (Gottsch et al., 2004), was transcribed using a T7 polymerase in the presence of ^{33}P. The radioactively labeled probe was heat shocked and then diluted in hybridization buffer (50% formamide, 6.25% dextran sulfate, 0.7% Ficoll, and 0.7% polyvinylpyrolidone) and counted for final radioactive concentration. Slides were incubated in this diluted radioactive probe overnight in humidified chambers

at 55 ˚C. After incubation slides were washed in 4X SSC, ribonuclease A at 37 ˚C and in 0.1X SSC at 60 ˚C. Slides were then taken through a series of alcohols for dehydration.

For quantification of mRNA levels, *in situ* hybridization slides were dipped in Kodak NTB emulsion (Eastman Kodak, Rochester, NY) diluted in 600 mM ammonium acetate (Fisher Scientific) and placed in light-tight boxes containing desiccant at 4 ˚C. Slides were left to develop for 9 days. After development, slides were dehydrated in an alcohol series followed by washes in xylene and coverslipping with Permount.

Images of silver grains were taken under dark-field illumination using a CoolSNAP CCD camera (Photometrics, Tucson, AZ) and analyzed using the MetaMorph Imaging system (Universal Imagining Corp., West Chester, PA). Silver grains were analyzed using a size-constant sampling box that encompassed the entire AVPV and measured the integrated intensity. The same sampling box was used for background measurements by taking the integrated intensity in a region just adjacent to AVPV where no *Kiss1* mRNA is present. Background measurements were subtracted from the AVPV measurement to control for variations in emulsion levels. Comparisons of adjusted integrated intensities were compared between lactating and control animals for three sections per animal.

Statistical Analysis

A Student's *t*-test was used to compare lactation and diestrus groups for *in situ* hybridization data and cell counts. Data are represented as mean ± SEM.

RESULTS

Experiment 1: ME Projections of ARH Kiss1/NKB Neurons

Immunohistochemistry confirmed previous findings of nearly complete colocalization of Kiss1-ir and NKB-ir cell bodies within the ARH, with 97% ± 2.6 of Kiss1 cells expressing NKB (Figure 1). Since the ARH is the only known major site of Kiss1 and NKB colocalization, detection of double-labeled fibers by immunhistochemistry can be used to differentiate Kiss1 projections specific to the ARH population.

ARH neurons are known to send projections to the ME where GnRH fibers terminate, and previous work has demonstrated the presence of both Kiss1 and NKB fibers in the ME. To determine whether Kiss1 and NKB fibers in the ME originate in the ARH, double-label immunohistochemistry was performed with fluorescent detection. Extensive Kiss1/NKB-ir fibers were seen in the ME primarily within the sub-ependymal and internal zones near GnRH fibers (Figure 2). Single-labeled Kiss1- and NKB-ir fibers were also observed in the ME, and colocalization analysis demonstrated that in control animals, only 39% ± 6 of Kiss1 staining overlapped with NKB staining, and 43% ± 4.3 of NKB staining overlapped with Kiss1 staining in the ME. Therefore, while double-labeled Kiss1/NKB-ir fibers were common in the ME, they did not represent the majority of either Kiss1 or NKB fiber staining. Consistent with previous findings single-labeled NKB-ir fibers were detected in both the internal and external zone of the ME, (Figure 2, C and G) (Krajewski et al., 2009), while single-labeled Kiss1-ir fibers were found predominantly in the internal zone (Desroziers et al.) (Figure 2, C and G), though occasionally fibers were seen in the external zone as well.

ME fiber distribution was analyzed in both control and lactating animals to determine whether the lactating state resulted in changes in the possible regulation of GnRH by Kiss1/NKB fibers in the ME. No significant differences were observed between total Kiss1 fibers in control (6.41 ±1.31 Kiss1-ir pixels/area) and lactating animals (6.27 ± 1.1 Kiss1-ir pixels/area, *t*-test, p=0.94). Similarly there was no difference in total NKB fibers between control (9.45 ± 2.2 Kiss1-ir pixels/area) and lactating animals

(10.67 ± 1.52 Kiss1-ir pixels/area, *t*-test, p=0.66). There was also no difference in the distribution of Kiss1 or NKB fibers or the percentage of colocalized fibers. Analysis of contact with GnRH fibers by Kiss1, NKB or Kiss1/NKB fibers could not be performed due to the extreme abundance of staining in the ME of both Kiss1 and NKB, as well as the transgenic labeling of GnRH.

Importantly, there was variability in NKB fiber staining within the ME, regardless of group, particularly with the quantity of single-labeled fibers in the external zone varying within individual cases. Two examples from the same animal are shown in Figure 2, with one section containing conspicuous NKB-ir fibers in the external zone (Figure 2, A-D) and another section containing few NKB-ir fibers in the external zone (Figure 2, E-H). None of the variability between sections could be accounted for by rostal-caudal position.

To verify results with fluorescence secondary detection demonstrating section-to-section variability in the quantity of NKB fibers in the external zone of the ME, chromagen (NiDAB) detection was also performed. Although NiDAB detection cannot be used to detect colocalized Kiss1/NKB-ir fibers, it was possible to compare the distribution of NKB-ir fibers within the external zone of the ME to fluorescent detection. Similar to results from fluorescence double-label immunohistochemistry, we observed variability in the quantity of NKB fibers in the external zone of the ME within individual cases by NiDAB labeling (Figure, 3). Once again two examples from one animal are shown, with one section containing conspicuous fibers in the external zone (Figure 3, B-C) and other section containing quite scattered fibers in the external zone (Figure 3, D-E). Once again this variability was not accounted for by rostral-caudal position. Staining of the ARH with NiDAB labeling was also consistent with immunofluorescence labeling (Figure 1, A and Figure 3, A).

Experiment 2: Rostral Projections of ARH Kiss1/NKB Neurons

To characterize ARH Kiss1/NKB rostral fiber projections, as well as single Kiss1 and NKB fiber distributions, immunohistochemistry was performed and used to compile computer assisted line drawings of these fibers. Immunohistochemistry revealed a primarily ventricular projection of ARH Kiss1/NKB-ir fibers (Figure 4), as observed at the level of the anterior periventricular region (Figure 4, D; Figure 5, A), and these double-labeled fibers were diminished in more rostral sections at the level of the AVPV (Figure 4, B; Figure 5, B). Importantly, double-label fibers seemed to diminish significantly prior to the level of GnRH neurons in the NDB and few double-labeled Kiss1/NKB-ir fibers were observed at this level (Figure 4, A; Figure 5, C and D). There were also very few double-labeled Kiss1/NKB-ir fibers near GnRH neurons in other areas of the POA (results not shown). Quantification of colocalization in control animals using Manders' Coefficient, revealed that in fact only 2.6% (± 0.85, n=4) of Kiss1 staining overlapped with NKB staining at the level of GnRH neurons in the medial septal nucleus and NDB (Figure 5, C), while 5.1% (± 0.82, n=4) of NKB staining overlapped with Kiss1 staining. NKB colocalization was likely slightly higher due to the smaller number of NKB fibers in this area. In summary, ARH Kiss1/NKB projections are found medially along the ventricle, and this appears to be the predominant rostral projection path of Kiss1/NKB fibers since very few Kiss1/NKB fibers were observed in lateral nuclei of the hypothalamus (Figure 4, D-F), and they diminished significantly prior to POA regions containing GnRH neurons.

Single-labeled Kiss1-ir and NKB-ir fibers were observed along the ventricle as well as at the level of the GnRH neurons in the NDB and the broader POA (Figure 5, C-D). Particularly, single-labeled Kiss1-ir fibers were abundant near GnRH neurons, which is consistent with the known projections out of the AVPV, where there is an additional Kiss1 population in the rat. Single-labeled Kiss1 and NKB fibers were also found in many lateral hypothalamic areas (Figure 4, D-F).

To determine whether lactation resulted in any significant difference in total Kiss1-ir or NKB-ir staining, sections of the POA at the level of GnRH cell bodies were analyzed for total Kiss-ir and NKB-ir staining, as measured by pixels. Surprisingly, total Kiss1-ir total staining appeared to be significantly increased in the lactating group compared to the control group. The total number of Kiss1-ir pixels in a size constant area of the POA was on average 8150 ± 1182 pixels in the control group (n=4), while the average number of pixels for the lactating group (n=3) was 19988 ± 657 pixels (*t*-test, $p<0.05$). In contrast total NKB-ir pixels did not change in the POA between lactating (5981 pixels ±1006) and control groups (6300 pixels ± 539, *t*-test p=0.81). To determine if increased Kiss1-ir fibers in the POA translated to increased contacts with GnRH cell bodies, analysis of Kiss1-ir close appositions on GnRH cell bodies was performed. Ten cells were analyzed per animal, from the same photomicrographs used to quantify of total Kiss1-ir in the POA. There was no significant difference in the number of Kiss1 single-labeled fiber appositions on GnRH neurons between control and lactating animals (*t*-test, p=0.26). Of the GnRH cells investigated, 32.5% ± 13% had close appositions from single-labeled Kiss1 fibers, while 40% ± 5.8% had close appositions from these fibers in lactating animals. Furthermore, these values of Kiss1 fiber close appositions on GnRH neurons are similar to those reported previously both in the sheep and mouse (Clarkson and Herbison, 2006; Smith et al., 2008a). In contrast to single-labeled Kiss-1-ir close appositions, out of the 70 GnRH neurons studied, only two had close appositions from Kiss1/NKB double-labeled fibers.

Experiment 3: Regulation of Kiss1 and NKB Populations during Lactation

ARH Kiss1/NKB

ARH Kiss1/NKB neurons were found to have projections in the ME near GnRH fibers suggesting a potential regulatory relationship between these ARH peptides and GnRH release. This finding prompted the question of whether changes in ARH Kiss1/NKB during lactation may be responsible for decreased GnRH release. Previous data has demonstrated a decrease in *Kiss1* and *NKB* mRNA within the ARH

during the negative energy balance model of lactation (Xu et al., 2009b; Yamada et al., 2007), and immunohistochemistry was performed to determine whether Kiss1 and NKB peptides are also decreased. In the ARH, both Kiss1 and NKB NiDAB immunoreactive staining was decreased in lactating animals compared to intact diestrus animals (Figure 6, A-B and D-E) consistent with previous findings (Yamada et al., 2007). There was also a significant reduction in cell number in lactating animals versus controls for both neuropeptides (Figure 6, C and F).

AVPV Kiss

The AVPV Kiss1 population has also been implicated as a critical component of GnRH regulation, particularly positive estrogen feedback. Kiss1 mRNA and peptide were measured in the AVPV to determine what, if any, changes occur during lactation in this population. Immunohistochemistry revealed an increase in Kiss1-ir cell number during lactation (Figure 7, A-C). However, *in situ* hybridization revealed a significant decrease in *Kiss1* mRNA in the AVPV during lactation (Figure 7, D-F). These findings could suggest an inhibition of AVPV Kiss1 production as well as peptide release leading to an accumulation of peptide within cell bodies. This accumulation may also account for the apparent increase in total Kiss1-ir in the POA, since peptide likely accumulated in fibers as well.

DISCUSSION

The singularity of GnRH release as a final reproductive output signal from the brain suggests that there is likely an integrated regulation of these cells by many upstream neuronal populations. In the present study, GnRH inhibition during lactation was found to be coincident with the inhibition of ARH Kiss1/NKB peptide levels, consistent with previous findings of inhibited Kiss1 and NKB mRNA (Xu et al., 2009b; Yamada et al., 2007). Double-label immunohistochemistry was used to track projections of colocalized ARH Kiss1/NKB cells in relation to GnRH cell bodies and fibers. This study suggests the first neuroanatomical evidence that the opposing roles of the two Kiss1 populations in regulating steroid feedback of GnRH correlates with different sites of GnRH regulation (Oakley et al., 2009; Uenoyama et

al., 2009). The AVPV Kiss1 population contributes to positive steroid feedback and this regulation takes place at the level of the POA where Kiss1 fibers, likely originating from the AVPV, contact GnRH neurons (Clarkson and Herbison, 2006). The current study demonstrated that most ARH Kiss1/NKB axons do not target GnRH cell bodies but instead project to the ARH-ME area where GnRH fibers terminate. Since ARH Kiss1/NKB cells are linked to negative steroid feedback (Smith et al., 2005), this suggests that while positive feedback occurs at the level of GnRH cell bodies, negative feedback occurs at the level of GnRH fibers in the ME.

Importantly, this study has found that the majority of Kiss1 and NKB staining in the ME does not represent colocalized Kiss1/NKB fibers from the ARH, similar to a recent paper in the monkey reporting both single-labeled Kiss1 and NKB fibers in the ME (Ramaswamy et al., 2010). One explanation is that nuclei other than the ARH send significant Kiss1 and NKB projections to the ME, and the possible sources of such single-labeled Kiss1 and NKB are discussed below. Another explanation is that the beaded nature of Kiss1 and NKB staining in the ME may hamper the detection of double-labeled fibers during analysis. Particularly, anecdotal cases can be observed when scanning through 0.5 µm planes in which a beads-on-a-string type fiber can be seen where some beads appear single-labeled for either Kiss1 or NKB while other beads appear colocalized. Thus, our current immunohistochemistry methods may be flawed and underestimate the percentage of colocalized fibers.

Immunohistochemistry identifying Kiss1/NKB fibers in the ME demonstrated some variability with either fluorescence or NiDAB detection. In particular, there seemed to be section-to-section variability in the quantity of NKB fibers in external zone of the ME within individual cases, this variability did not appear to have a rostral-caudal pattern. Therefore, caution should be used when characterizing NKB fibers in this area, since different distribution patterns could be concluded based on sections taken from the same animal. Overall, labeling in the external ME revealed a moderate number of periportal axons, thus indicating that their modulatory influence is potent, or alternatively that an abundant supply

of axons in the external ME releases the neuropeptides at a high rate preventing their accumulation and subsequent visualization. Interestingly, DAB detection of the Kiss1 antibody also revealed occasional Kiss1 fibers in the external zone (unpublished observation), thus it is possible that external zone fibers contain both Kiss1 and NKB but that this was not detected with fluorescence labeling for an unknown technical reason. Another possibility could be that Kiss1 staining is also labeling RFRP-1, because although we did not detect labeling in the DMH where RFRP-1 cell bodies reside, this does not exclude the possibility that RFRP-1 fibers could be labeled with the Kiss1 antibody. However, this seems unlikely since the distribution of Kiss1 fibers observed here is similar to that seen by Desroziers et al., (2010), including occasional Kiss1 fibers in the external zone of the ME, as well as a recent non-human primate study by Ramaswamy et al. (2010), which found colocalized Kiss1/NKB fibers predominantly in the internal zone. Improved antibodies will be needed to verify whether the few NKB or Kiss1 fibers reported here to project into the external zone are ARH Kiss1/NKB projections.

The lack of significant Kiss1/NKB fibers in the external zone has been a confounding issue in determining whether the ME is truly a site of regulation, since GnRH terminals are in the external zone. One possibility is that Kiss1/NKB fibers do extend into the external zone, but peptide release is so fast that peptide cannot be detected with immunohistochemistry. An alternative hypothesis is that Kiss1/NKB is released into the internal zone and travels by diffusion to the external zone to bind receptors on GnRH terminals. Importantly, the non-human primate does not show significant Kiss1 contacts at GnRH cell bodies but Kiss1 fibers are near GnRH fibers in the internal zone of the ME (Ramaswamy et al., 2008; Ramaswamy et al., 2010). Despite this lack of contact at GnRH cell bodies, Kiss1 administration still causes robust release of GnRH in non-human primates (Ramaswamy et al., 2010; Ramaswamy et al., 2007), suggesting that Kiss1 may regulate GnRH release directly from the ME through non-synaptic regulation.

Surprisingly, total Kiss1-ir and NKB-ir staining in the ME did not decrease in the lactating condition, despite decreases in both Kiss1-ir and NKB-ir in the ARH. This finding using fluorescence labeling was also confirmed qualitatively with DAB staining (results not shown), suggesting that there is likely inhibition of Kiss1 and NKB release in the ME and accumulation of the peptides within fibers. A similar phenomenon seemed to be occurring in the AVPV as well. While AVPV Kiss1 mRNA was decreased during lactation, peptide was increased at the level of cell bodies, and total Kiss1-ir fiber staining was increased at the level of the POA. The increase in Kiss1-ir in the POA during lactation did not translate to a higher percentage of GnRH neurons demonstrating close appositions from single-labeled Kiss1-ir fibers, supporting the hypothesis that higher peptide levels in the POA are associated with inhibition of release and not upregulation of synapses with GnRH neurons. Importantly, the data from the ME and POA suggest that inhibition of peptide release is a common mechanism in both Kiss1 populations, though it may be more severe in the AVPV population since total peptide was actually increased in both cells and fibers.

In addition to the ARH Kiss1/NKB fiber projections to the ME, there were also major rostral projections along the third ventricle. The ARH Kiss1/NKB fibers diminish significantly prior to GnRH neurons, so it is unclear what the function of these rostral projections may be. Many other cell populations rostral to the ARH are also involved in GnRH regulation and thus could be targets for direct regulation by ARH Kiss1/NKB. The orexin and MCH cell populations are prime candidates, since GnRH neurons express the orexin and MCH receptors and both fiber types are known to contact GnRH cell bodies (Campbell et al., 2003; Williamson-Hughes et al., 2005). However, these cells are located in the lateral hypothalamus while the predominant ARH Kiss1/NKB projections were found only medially along the ventricle, thus it is unlikely that these cells are directly regulated by ARH Kiss1/NKB projections. Further research will be needed to determine what, if any, role these rostral ARH Kiss1/NKB play in GnRH regulation. In addition, the lack of Kiss1/NKB direct projections to GnRH cell bodies or terminals

suggests that these neurons may likely be involved in more than just GnRH regulation, but to date little research has focused on this topic.

The AVPV is likely the source of single-labeled Kiss1 fibers since this is the other predominant Kiss1 population in the rat. In agreement with this idea, the AVPV is known to regulate reproductive function via projections to GnRH cell bodies in the POA, and many studies have specifically identified the Kiss1 population of this nucleus as an important contributor to estrogen-mediated positive feedback (Dungan et al., 2006; Popa et al., 2008; Smith et al., 2006c). However, Kiss1 input from the AVPV may also contribute to basal stimulation of GnRH neurons, such that a decrease in AVPV Kiss1 drive onto GnRH cells during lactation may lead to downstream decreases in GnRH release.

The origins of the single-NKB fibers in the POA are less clear. By immunohistochemistry the next most abundant population of NKB cells is in the bed nucleus of the stria terminalis (BST) and this nucleus has been previously implicated in reproductive regulation (Polston et al., 2004; Simerly et al., 1990). Initial studies indicated that during lactation there was an increase in NKB peptide in the BST, but no change in mRNA arguing against inhibition of this nucleus during lactation (results not shown). Although the BST is the next biggest cell population of NKB after the ARH, NKB peptide and mRNA have been localized to many areas of the brain including the cerebral cortex, amygdaloidal complex, and lateral mammillary bodies (Marksteiner et al., 1992). It is possible that single-label NKB fibers in the NDB and POA arrive from one or many of these additional nuclei.

Overall the finding of inhibited ARH Kiss1/NKB and AVPV Kiss1 populations during lactation is consistent with the hypothesis that decreased GnRH release in this model is due to decreases in upstream stimulatory tone. However, the signals responsible for inhibition of Kiss1 during lactation remain largely unknown. Kiss1 has been shown to be inhibited in another model of negative energy balance, fasting, and the predominant hypothesis in this model is that low leptin drives Kiss1 and LH inhibition (Castellano et al., 2005; Kalamatianos et al., 2008; Kalra et al., 1998; Luque et al., 2007;

Nagatani et al., 1998). However, previous work in the lactating model has demonstrated ARH Kiss1/NKB mRNA and serum LH levels remain low even after leptin is restored to physiological levels, suggesting that low leptin is not required for inhibition of ARH Kiss1/NKB in this model (Xu et al., 2009b). This discrepancy between fasting and lactation may be due to obvious differences in the duration and severity of negative energy balance between the two models. It is also possible that leptin was incapable of restoring ARH Kiss1/NKB and LH levels during lactation due to another redundant inhibitory signal in this model: the suckling stimulus. The suckling of pups activates cells in a large number of brainstem nuclei that project to the ARH and removal of this stimulus results in a rapid restoration of GnRH and LH release (Li et al., 1999a; c; Xu et al., 2009b). It is therefore possible that inhibition of ARH Kiss1/NKB is driven in part by brainstem nuclei activated by suckling stimulus in the lactation model.

Regulation of NKB during negative energy balance is less well understood, as is the nature of NKB's effects on GnRH release. Although NKB clearly appears to be stimulatory for GnRH release in humans, there are contradictory reports for NKB's effect on LH release in other species (Corander et al., 2010; Kalra et al., 1992; Navarro et al., 2009; Rance and Young, 1991; Sandoval-Guzman and Rance, 2004; Topaloglu et al., 2009). More recent evidence has demonstrated that while NKB acutely stimulates LH in sheep and non-human primates, intermittent administration cannot maintain pulsatile LH, which could account for contrasting reports on NKB's actions on GnRH release (Billings et al., 2010; Ramaswamy et al., 2010). The data presented here are consistent with a stimulatory effect of NKB for GnRH release, since peptide and mRNA levels are decreased in the ARH during lactation when GnRH release is low (Xu et al., 2009b). The NKB receptor, NK3, has been recently identified on ARH Kiss1/NKB cells, which has lead to the hypothesis that ARH NKB may work in an autoregulatory manner to stimulate ARH Kiss1 release; thus decreases in NKB could lead to decreased Kiss1 release resulting in a reduced stimulatory tone for GnRH release (Navarro et al., 2009; Wakabayashi et al., 2010).

Although ARH Kiss1/NKB neurons likely directly influence GnRH fibers in the ME and these ARH cells are inhibited during lactation, the question of whether inhibition of these nuclei is required for GnRH and LH inhibition in this model remains unanswered. It is also possible that Kiss1/NKB modulation of GnRH release is in part indirect via dopaminergic terminals in the external ME, in line with recent studies (Szawka et al., 2010) showing association of Kiss1 axons with the perikarya of dopaminergic tubero-infundibular neurons of the ARH. In vitro techniques such as hypothalamic explants may allow for a more direct approach to address whether Kiss1 and NKB peptide release is decreased in tissue from animals in negative energy balance and what if any effect restoring these peptides has on GnRH release. While this current study did not directly measure release of Kiss1 and NKB, it has provided evidence that the ARH Kiss1/NKB cells, and likely the AVPV Kiss1 cells as well, are inhibited during lactation when GnRH release is low. Importantly, this study is the first to definitively demonstrate an absence of ARH Kiss1/NKB projections to GnRH neurons in the POA, and suggests that this population likely regulates GnRH activity via projections to the ARH-ME area.

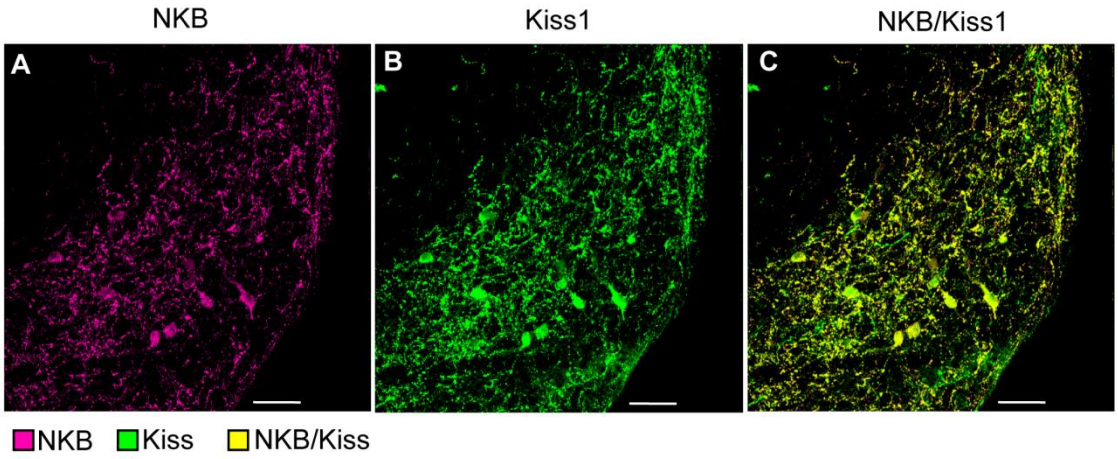


Figure 1: Colocalisation of Kiss1 and NKB in the ARH.
Immunohistochemical results of NKB-ir (A; magenta) and Kiss1-ir (B; green) staining in the ARH revealed almost complete colocalisation (C; yellow) of Kiss1 and NKB cell bodies within OVX virgin rats. Scale bars represent 50 μm; V3, third ventricle.

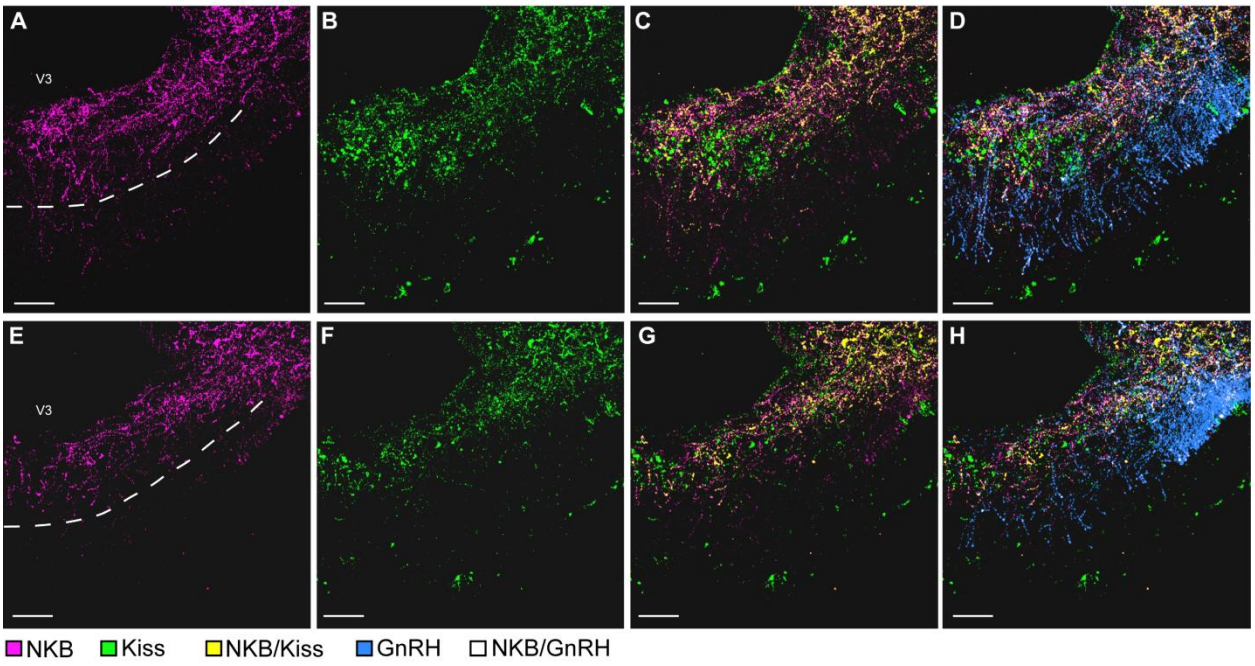


Figure 2. Kiss1 and NKB fiber distribution in the ME.
NKB-ir (A,E; magenta) and Kiss1-ir (B,F; green) fibers were found in the ME and overlay of these images revealed colocalized Kiss1/NKB-ir fibers (C,D; yellow) primarily in the internal zone. Double-labeled Kiss1/NKB-ir fibers were observed in close proximity to GnRH-GFP fibers (D,H; blue). Single-labeled Kiss1-ir fibers were also observed primarily in the internal zone, while single-labeled NKB-ir fibers were observed in both the internal and external zone of the ME. Single-labeled NKB-ir fibers were often found in close proximity to GnRH fibers in the external zone of the ME (white). Two ME examples from the same animal are shown to illustrate variability of staining within the external zone. The dotted lines in panels A and E mark the approximate border of the internal and external zone. Immunohistochemistry was performed in OVX GnRH-GFP virgin rats. Scale bars represent 50 µm; V3, third ventricle.

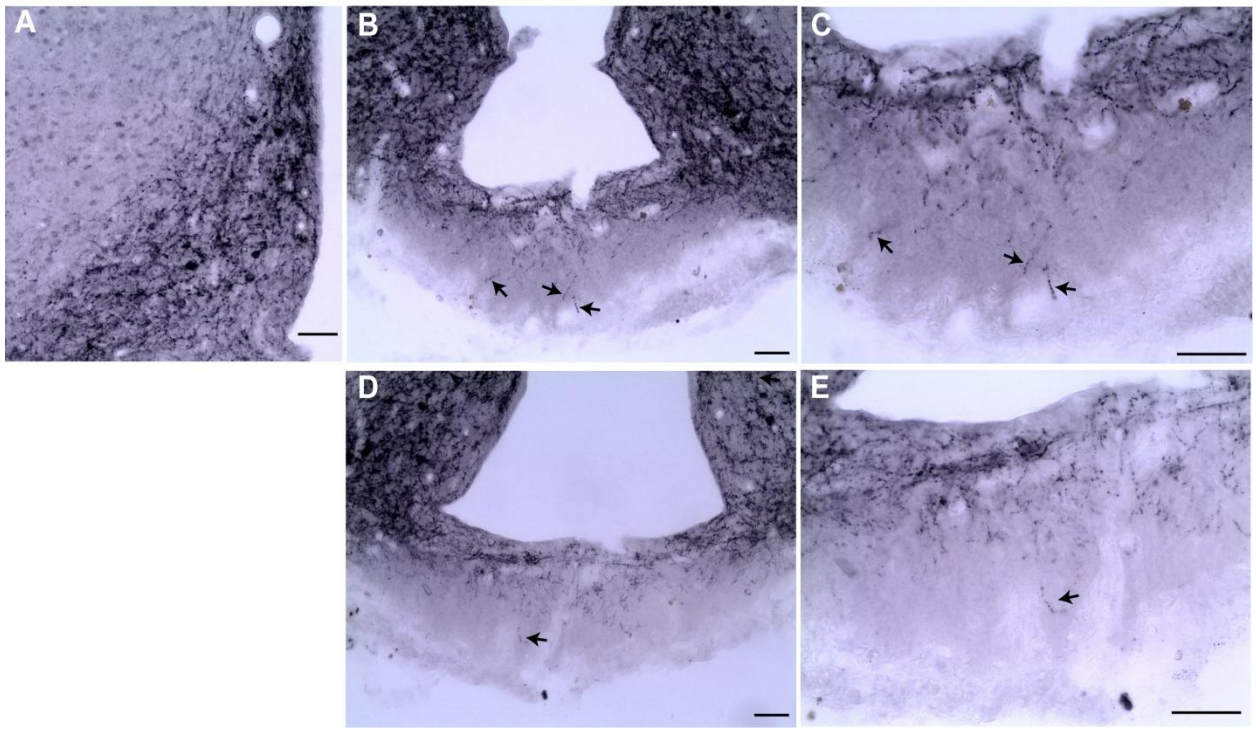


Figure 3. NKB immunoreactivity in the ARH and ME using NiDAB secondary detection.
NiDAB staining was used to verify the variable quantity of NKB fibers in the external zone of the ME as observed by fluorescence labeling. ARH NKB-ir (A) appeared similar to fluorescence immunoreactivity. The quantity of NKB-ir fibers in the external zone of the ME (arrows) varied from moderate (B,C) to light (D,E) in sections taken from the same OVX virgin rat. Panels C and E are magnifications of B and D, respectively. Scale bars represent 50 μm.

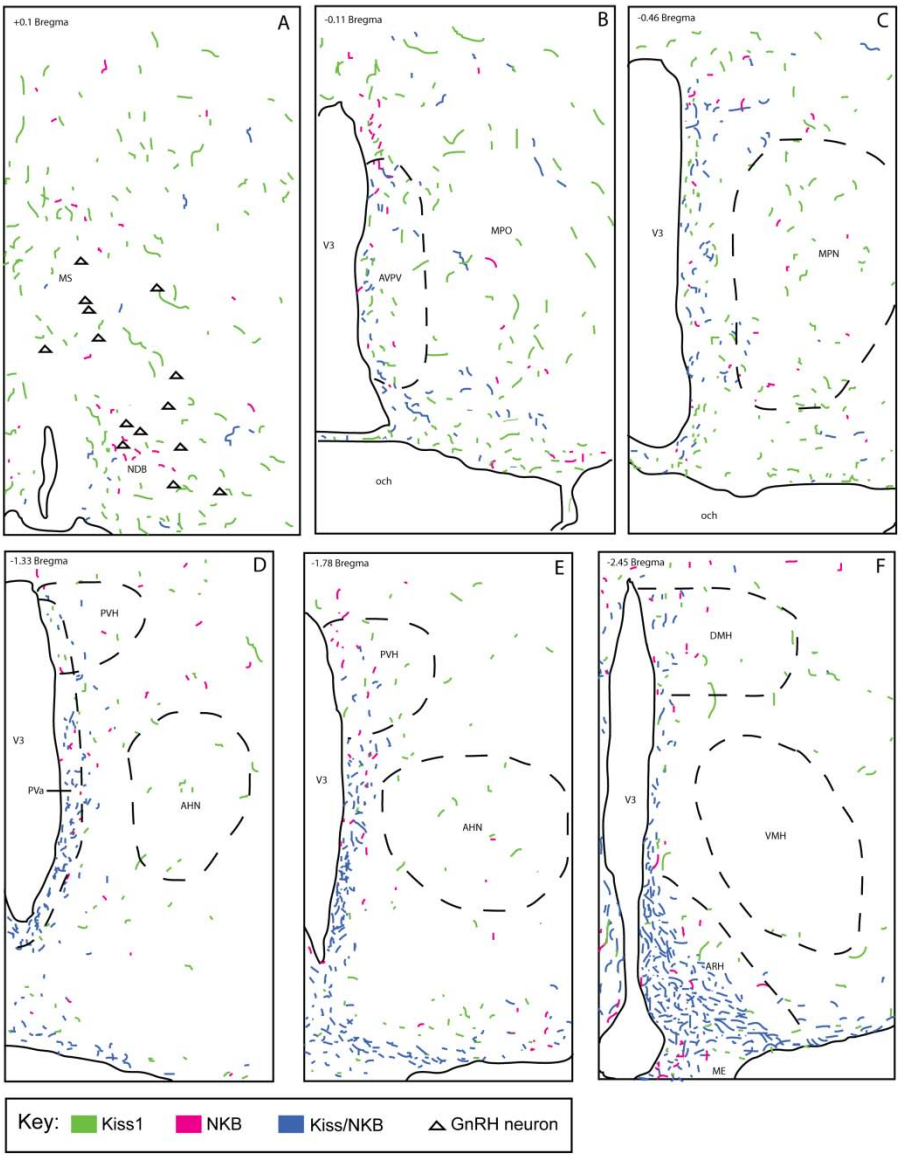


Figure 4. **Computer assisted line drawings of Kiss1/NKB-ir rostral fiber projections from the ARH.** Double-labeled Kiss1/NKB-ir fibers (blue) in OVX virgin control tissue had a rostral projection pattern that closely followed the third ventricle (V3) with fibers diminishing rostrally. Very few double-labeled Kiss1/NKB-ir fibers were observed near GnRH neurons (A; triangles) in the NDB, though single-labeled Kiss1-ir fibers (green) and NKB-ir fibers (magenta) were found in this area. Corresponding coordinates from the Swanson rat brain atlas (1992) are given in upper left corner of each panel. AHN, anterior hypothalamic nucleus; ARH, arcuate nucleus; AVPV, anteroventral periventricular nucleus; DMH, dorsomedial nucleus hypothalamus; ME, median eminence; MPN, medial preoptic nucleus; MPO, medial preoptic area; MS, medial septal nucleus; NDB, nucleus of the diagonal band [Broca]; och, optic chiasm; VMH, ventromedial nucleus hypothalamus.

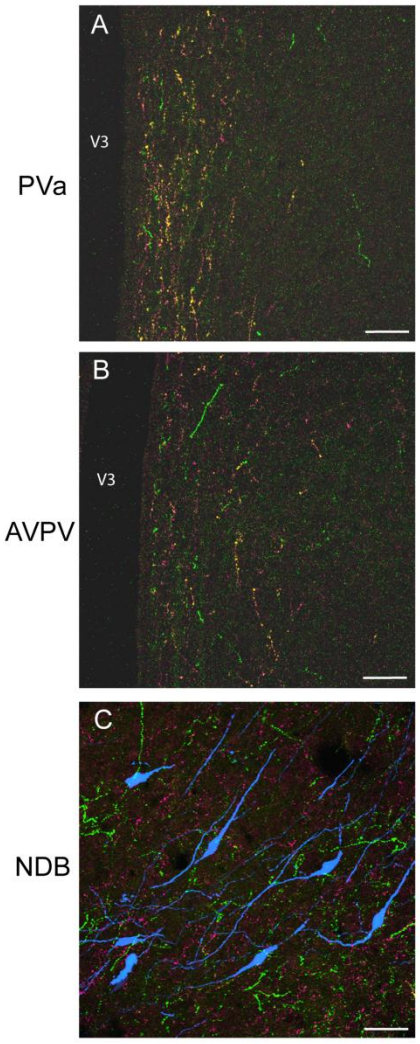


Figure 5. Colocalized Kiss1/NKB-ir fibers at the level of the PVa, AVPV and NDB.
Double label immunohistochemistry in GnRH-GFP OVX virgin control rats revealed a ventricular projection pattern of double-labeled Kiss1/NKB-ir fibers (A; yellow) which diminished rostrally (B), with few double-labeled fibers (C and D; arrow) observed in the NDB near GnRH neurons (blue). Single-labeled Kiss1-ir (green) and NKB-ir fibers (magenta) were observed at all three levels, with single-labeled Kiss1-ir fibers being particularly abundant in the NDB and some close contacts to GnRH neurons were observed (D; light blue, arrowhead). The coordinates of the three micrographs correspond to the following panels in Figure 4:'s panel A to Figure 4D, panel B to Figure 4B and panel C to Figure 4A. Scale bars represent 50 µm.

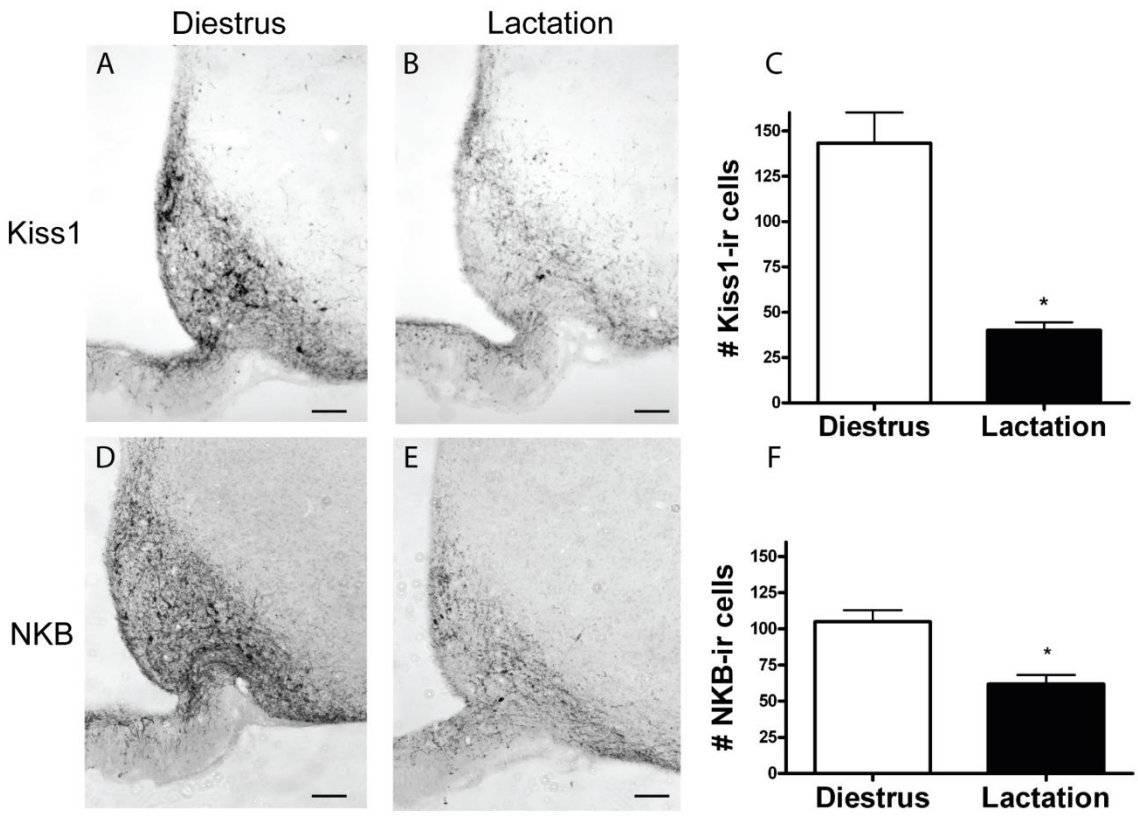


Figure 6. ARH Kiss1 and NKB immunohistochemistry during diestrus and lactation.
Immunohistochemistry for Kiss1 (A,B) and NKB (D,E) revealed decreases in Kiss1- and NKB-ir in the ARH in intact lactating animals compared to diestrous virgin controls, as well as significant decreases in cell numbers (C, F; *, $p<0.05$). Scale bars in photomicrographs represent 50 µm.

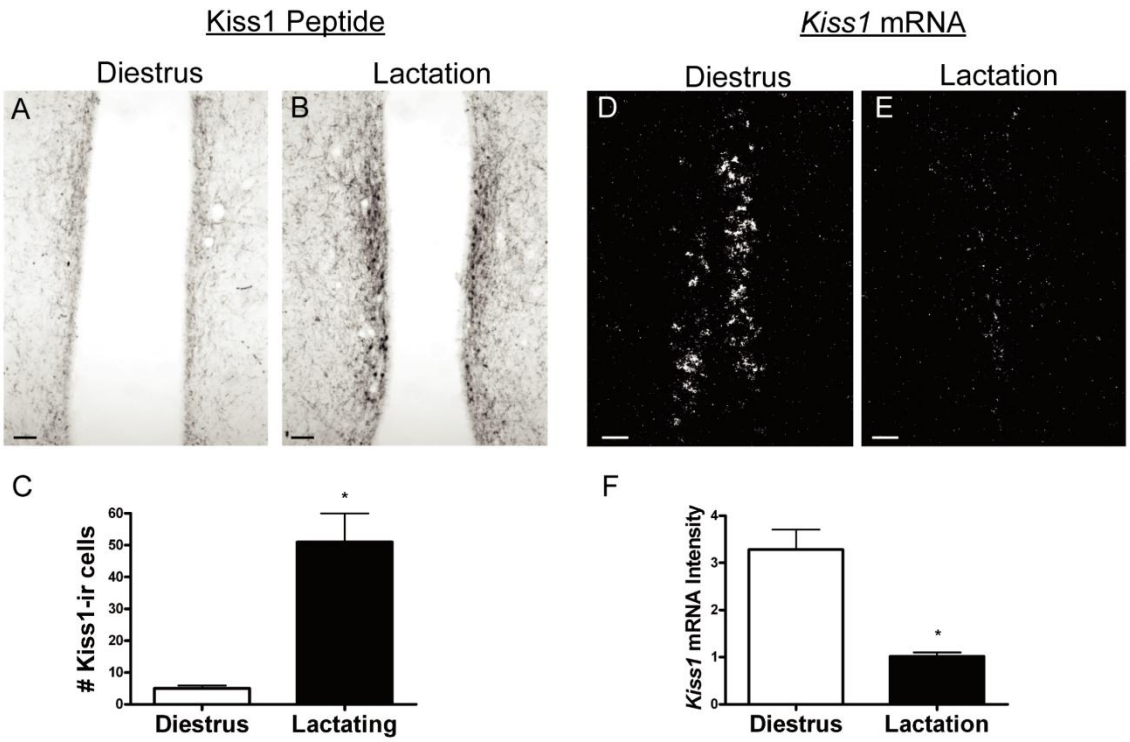


Figure 7. Kiss1 peptide and mRNA in the AVPV during diestrus and lactation.
Kiss1-ir increased in the AVPV in intact lactating animals compared to diestrous virgin controls (A,B), as determined by immunohistochemistry, and there were significantly more Kiss1-ir cells in lactating animals (C;*, $p<0.05$). *Kiss1* mRNA (D,E) significantly decreased in the AVPV in lactating animals as measured by the averaged integrated intensity of silver grains using *in situ* hybridization (F;*, $p<0.05$). Scale bars in photomicrographs represent 50 µm.

CHAPTER 3 -Leptin is not the critical signal for kisspeptin or luteinizing hormone restoration during exit from negative energy balance

True C, Kirigiti MA, Kievit P, Grove KL, Smith MS. 2011. Leptin is not the critical signal for kisspeptin or luteinizing hormone restoration during exit from negative energy balance. J Neuroendocrinol 23(11):1099-1112.
PMID: 21518032

ABSTRACT

Low levels of the adipocyte hormone leptin are thought to be the key signal contributing to inhibited gonadotrophin-releasing hormone (GnRH) release and reproductive acyclicity during negative energy balance. Hypoleptinemia-induced inhibition of GnRH may be initiated with upstream inhibition of the secretagogue kisspeptin (Kiss1), since GnRH neurones do not express leptin receptors. The aim of the current study was to determine whether eliminating the hypoleptinemia associated with caloric restriction (CR), by restoring leptin to normal basal levels, could reverse the suppression of the reproductive neuroendocrine axis. 50% CR resulted in significant suppression of anteroventral periventricular (AVPV) Kiss1 mRNA, arcuate nucleus (ARH) Kiss1 and neurokinin B (NKB) mRNA levels and serum LH. Restoring leptin to normal basal levels did not restore Kiss1 or NKB mRNA or LH levels. Surprisingly, leptin did not activate pSTAT3 expression in ARH Kiss1 neurons indicating these neurons may not relay leptin signaling to GnRH neurons. Previous work in fasting models showing restoration of LH using a pharmacological dose of leptin. Therefore, in a 48-hour fast study, replacement of leptin to pharmacological levels was compared to replacement of leptin to normal basal levels. Maintaining leptin at normal basal levels during the fast did not prevent inhibition of LH. In contrast, pharmacological levels of leptin did maintain LH at control values. These results suggest that although leptin may be a permissive signal for reproductive function, hypoleptinemia is unlikely to be the critical signal responsible for ARH Kiss1 and LH inhibition during negative energy balance.

INTRODUCTION

Negative energy balance is the metabolic state in which energy input is insufficient for energy output. In mammals, negative energy balance results in a halting of cyclic reproductive function that is thought to be triggered by decreased release of gonadotrophin-releasing hormone (GnRH), which then results in lowered levels of luteinizing hormone (LH) (Aloi et al., 1997; Bergendahl et al., 1991; Kile et al., 1991). However, the signal mediating this inhibition of GnRH release is still unclear.

Leptin is believed to be a key metabolic signal conveying energy reserve to the brain (Elmquist and Flier, 2004; Morton et al., 2006). Leptin is produced in adipocytes, and during negative energy balance, decreases in fat mass result in lowered levels of circulating leptin (Maffei et al., 1995). Importantly, leptin has been implicated in stimulating LH release (Ahima et al., 1996; Barash et al., 1996; Chehab et al., 1996; Finn et al., 1998a); therefore, many have hypothesized that low levels of leptin during negative energy balance are critical for lowered GnRH release. Indeed, several studies have found that giving exogenous leptin during a 48-hour fast maintains normal LH release (Ahima et al., 1996; Cunningham et al., 1999; Donato et al., 2009; Nagatani et al., 1998). However, GnRH neurones do not appear to express leptin receptors (Finn et al., 1998a; Quennell et al., 2009) and it has long been hypothesized that an intermediate cell population likely mediates leptin's effects on GnRH.

The GnRH secretagogue kisspeptin (Kiss1) is critical to our understanding of GnRH regulation, since Kiss1 populations in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARH) have been implicated in positive and negative steroid feedback, respectively (for recent review see Popa et al., 2008; Roa et al., 2008a; Smith, 2008; Uenoyama et al., 2009). ARH Kiss1 cells are of particular interest since they also express two other neuropeptides, neurokinin B (NKB) and dynorphin (DYN) across many species (Burke et al., 2006; Goodman et al., 2007; Navarro et al., 2009; Ramaswamy et al., 2010; True et al., 2011b; Wakabayashi et al., 2010), and are referred to as KNDy neurons. NKB is also thought to be stimulatory for GnRH release (Krajewski et al., 2005; Ramaswamy et al., 2010; Topaloglu

et al., 2009), and may act autosynaptically to stimulate Kiss1 release (Navarro et al., 2009; Wakabayashi et al., 2010). ARH KNDy cells were found to express leptin receptors in the mouse, and leptin has been demonstrated to stimulate Kiss1 (Castellano et al., 2006b; Smith et al., 2006a), making them a likely candidate for the intermediate cells involved in leptin's regulation of GnRH (de Roux et al., 2003; Gottsch et al., 2004; Matsui et al., 2004; Navarro et al., 2004; Seminara et al., 2003; Smith et al., 2006a). Both AVPV Kiss1 and ARH Kiss1/NKB mRNA levels appear to be inhibited in some models of negative energy balance (Castellano et al., 2005; Castellano et al., 2006b; Kalamatianos et al., 2008; Luque et al., 2007; True et al., 2011b; Xu et al., 2009b; Yamada et al., 2007), suggesting decreases in Kiss1 or NKB could translate to decreased stimulation of GnRH release. Taken together, the above findings have led to the updated hypothesis that low leptin levels during negative energy balance may drive inhibition of Kiss1, and possibly NKB, which, in turn, leads to less stimulation of GnRH release.

To date, leptin's effects on LH release during negative energy balance have primarily been studied at pharmacological doses. A recent study in our lab using the lactation model of negative energy balance demonstrated that eliminating hypoleptinemia by restoring leptin to normal basal levels did not relieve inhibition of ARH Kiss1, NKB or LH (Xu et al., 2009b). Importantly, when animals exit negative energy balance leptin levels rise, but only to levels observed in conditions of normal energy balance (Xu et al., 2009b). Therefore, while high concentrations of leptin may be capable of stimulating Kiss1 and LH (Ahima et al., 1996; Castellano et al., 2006b; Donato et al., 2009; Nagatani et al., 1998), the natural restoration of leptin levels after negative energy balance may not be a sufficient signal alone to relieve reproductive inhibition.

It is possible that in our lactation study (Xu et al., 2009b), leptin restoration to normal basal levels did not restore LH because its effects were masked by continued inhibitory signals specific to the suckling stimulus (Brogan et al., 1999). The first goal of the current study was to determine whether AVPV Kiss1 and ARH KNDy mRNA levels were reduced with long-term caloric restriction (CR) and

whether eliminating hypoleptinemia was capable of restoring these reproductive neuropeptides or serum LH in this model lacking the suckling stimulus. The second goal was to compare the relative effectiveness of maintaining leptin at normal basal levels versus at pharmacological levels in preventing inhibition of LH secretion in animals fasted for 48 hours. The 48-hr fast was chosen for this comparative study since pharmacological leptin doses have been previously shown to be effective at maintaining LH in this model.

MATERIALS AND METHODS

Animals and Tissue Collection

Adult female Wistar rats (Simonsen, Gilroy, CA), weighing between 200-220 grams, were used in all studies. Animals were singly-housed and maintained on a 12-hour light (0600 hr) and dark (1800 hr) cycle throughout the experiment and allowed water ad libitum. All protocols were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

At tissue collection animals were briefly anaesthetised under isoflurane and decapitated. Trunk blood was collected and the brain was rapidly removed. A 1 mm coronal slice was made at the level of the optic chiasm, and a 2 mm^2 punch was made from this slice corresponding to the AVPV and rapidly frozen. The remainder of the brain was further blocked with a caudal boundary of the mammillary bodies and lateral boundaries of the temporal sulci. This brain block was mounted ventral side up for vibratome sectioning in Kreb's Solution and the bottom 600 µm of the brain pertaining to the ARH was removed and rapidly frozen, as described previously (Xu et al., 2009b). In addition, the uterus was dissected and weighed at the time of tissue collection. The remaining carcasses were then frozen until dual-energy X-ray absorptiometry (DEXA) was performed at a later time.

Experiment 1. 40% Caloric Restriction

All animals were ovariectomised (OVX) under a mixture of isoflurane/oxygen gas anaesthesia and during the same surgery implanted subcutaneously with silastic capsules (10 mm in length/100 grams of body weight) containing either 30 μg/ml 17β-estradiol dissolved in oil or oil alone. This method and dose of estradiol replacement has been previously shown to result in low physiological levels of estradiol corresponding to those observed during diestrus (Goodman, 1978) or negative energy balance (Smith and Neill, 1977). Importantly, this low dose of estradiol does not significantly blunt the OVX-induced LH rise, unless also combined with progesterone treatment (Goodman, 1978); therefore LH levels remain high and differences between groups can be more easily detected. Animals were given 5 mg/kg of the analgesic carprofen subcutaneously for recovery. The animals receiving estradiol were split into 3 groups (Table 1): 1) control animals that were ad libitum fed (OVX+E, n=8), 2) animals on a 40% caloric restriction compared to food intake measured from the OVX+E group, for 14 days (OVX+E CR, n=8), and 3) animals on 40% CR also receiving leptin for the final 48 hours of CR (OVX+E CR+L, n=8). To assess the requirement of low levels of estradiol for LH inhibition during CR, we included OVX control ad libitum fed animals without estradiol replacement (OVX, n=8), as well as OVX animals weight matched (OVX CR*, n=8) to the CR group with estradiol replacement (OVX+E CR) to determine whether a primary action of estradiol is required, beyond its known effects on metabolism and body weight (Table 1). CR began 4 days after OVX and implantation of silastic implants to allow recovery from surgery prior to food intake manipulations.

The 40% CR for the OVX+E CR and OVX+E CR+L groups was calculated each day based on the average food intake of the OVX+E group from the previous day. Food intake for the OVX CR* group was adjusted daily so that animals lost a comparable amount of weight as the OVX+E CR group, thus controlling for the metabolic effects of estradiol. Animals on CR received food at 0700 hours. Leptin treatment for the OVX+E CR+L group was administered continuously through an osmotic minipump (Alzet, Cupertino, CA) at a rate of 500 ng of recombinant rat leptin (400-21, Peprotech, Rocky Hill, NJ)

per hour; this dose restores leptin to normal basal levels, as previously described in our lactation studies (Xu et al., 2009b). The biological efficacy of this leptin dose was demonstrated by its ability to induce phosphorylated signal-transducer and activator of transcription-3 (pSTAT3), a downstream effector of leptin-receptor activation, within 2 hours following minipump implantation during a hypoleptinemic state (48-hr fast; see Supplemental Figure 1 for more details). The four remaining groups received minipumps containing saline. Osomotic minipumps were incubated in a 37^{0}C water bath for 12-24 hours prior to subcutaneous implantation under isoflurane/oxygen anaesthesia at 0700 hours on day 12 of CR. Tissue was collected on day 14 of CR beginning at 0700 hours.

Experiment 2. 50% Caloric Restriction

There were three groups in the 50% CR experiments (Table 1): 1) OVX and estradiol-replaced ad libitum fed controls (CTRL, n=8), 2) OVX and estradiol-replaced animals receiving 50% less calories than control for 14 days (50% CR, n=8), and 3) OVX and estradiol-replaced animals on a 50% CR receiving leptin treatment (50% CR+L, n=8). This experiment was carried out similarly to the 40% CR, with food intake and body weights measured and food allotment given daily at 0700 hours for each animal. Leptin treatment was also similar as described for the 40% CR study except it began on day 11 of CR so that animals were exposed to 72 hours of leptin treatment. Tissue was once again collected at 0700 hours on day 14 of CR.

Experiment 3. 48-Hour Fast

Animals were OVX and estradiol replaced as described for the CR studies and split into 4 groups (Table 1): ad libitum fed controls (CTRL, n=6), 48-hour fast (F, n=6), 48-hour fast combined with minipump infusion of leptin as described for the CR studies (F+Leptin, n=6) and 48-hour fast combined with a pharmacological leptin treatment (F+High Leptin, n=6). Fasting and leptin treatments began on the fourth day following OVX. The administration of leptin to achieve normal basal levels was the same as described for Experiments 1 and 2, and minipumps were implanted at 0700 on the first day of the

fast. The pharmacological leptin treatment was modified from the protocol used by Nagatani et al. (Nagatani et al., 1998). Animals in the F+High Leptin group were given intraperitonial (i.p.) injections of 3 µg of leptin per gram of body weight at 0700 and 1700 hours each day of the fast. Animals in all other groups received i.p. saline injections. These different methods of leptin administration were used to replicate previously published results for both normal (Xu et al., 2009b) and high (Nagatani et al., 1998) levels of leptin. Animals were euthanised and tissues were collected at 0700 following the 48 hour fast.

Experiment 4. Leptin induction of pSTAT3 in ARH Kiss1 neurones

To determine whether leptin acts directly at ARH Kiss1 neurones, acute leptin injections were given followed by immunohistochemistry to investigate potential colocalization of ARH Kiss1 and pSTAT3. Female rats were OVX, but not estradiol replaced to keep ARH Kiss1 staining as high as possible, and four days later given an acute i.p. injection of either leptin (1 µg/g of body weight; n=4) or saline (n=4) at 0900. A high pharmacological dose of leptin was used to ensure maximum stimulation of pSTAT3. Forty-five minutes later animals were anaesthetised with tribromoethanol and perfused transcardially with saline and 4% paraformaldehyde. Brains were removed and kept in 4% paraformaldehyde overnight followed by 24 hours in a 25% sucrose solution. Brains were then rapidly frozen and later cut into a 1-in-6 series of 20 µm sections using a sliding microtome.

RNA Isolation and Quantitative PCR (Experiments 1-3)

RNA was isolated from ARHs and AVPVs using a Qiagen MiniPrep Kit (Qiagen, Valencia, CA), quantified with a Nanodrop Spectrophotomter (ND1000, Thermo Scientific, Wilmington, DE) and treated with DNase (1 µg/µg RNA) prior to reverse transcription using random hexamer primers (Promega Corp., Madison, WI). Quantitative PCR was carried out in 10 µL reactions consisting of 5 µL of Taqman universal PCR master mix, 2 µL of cDNA used at dilutions of 1:50 for ARH samples and 1:20 for AVPV samples, 300 nM of the primer and probe of interest, 80 nM of 18s primers and 250 nM of the 18s probe. Amplification was performed using the ABI/Prism 7700 sequences detector system (Applied

Biosystems, Carlsbad, CA) with 2 minutes at 50^{o} C, 10 minutes at 90^{o} C, and then 40 cycles each at 95^{o} C for 15 seconds followed by 60^{o} C for 60 seconds. The following primer probe sets were all purchased from Applied Biosystems: Kiss1 (Rn00710914_m1), NKB (Rn00569758_m1), PDYN (Rn00571351_m1), AgRP (Rn01431702_g1), NPY (Rn01410146_m1), POMC (Rn00595020_m1) and SOCS3 (Rn00585674_s1).

The threshold for raw CT values for each gene of interest was adjusted to be in the exponential range of amplification. Standard curves on serial dilutions of pooled ARH cDNA were drawn on the basis of the log of the input RNA versus the critical threshold (CT) cycle. The efficiencies of these primers, as determined by the R^2 value from standard curves, were all at or above 0.95. Once CT values were normalised for cDNA content using the line of best fit for the standard curve, CT values for genes of interest were normalised to 18S CT values, which was the house-keeping gene used previously in the lactation study (Xu et al., 2009b). Normalised CT values were then averaged across triplicates. qPCR results were required to meet stringent criteria before they were included for analysis. Samples were excluded if 1) at least two CT values did not fall within one logarithmic degree of each other, 2) 18s CT values were 3 or more logarithmic degrees away from the mean 18s CT value or 3) normalised CT values were more than 2 standard deviations away from the group mean.

Immunohistochemistry for Kiss1 and pSTAT3 (Experiment 4)

One series of tissue sections per animal was used for immunohistochemistry (IHC). Tissue prepared for pSTAT3/Kiss1 IHC was rinsed with potassium phosphate buffer and incubated in 1% $NaOH/H_2O_2$ solution for twenty minutes, followed by ten minute incubations in 3% glycine and 0.03% SDS. Tissue was then blocked in 2% normal donkey serum followed by incubation in the primary mouse anti-pSTAT3 antibody (Cell Signaling, 4113; 1:2000) for one hour at room temperature followed by 24 hours at 4^{0} C. For NiDAB detection, tissue was rinsed and incubated for an hour in either a biotinylated donkey anti-mouse or anti-rabbit antibody (Jackson Immunoresearch, 715-065-150 CAT #, respectively; 1:600), followed by an half hour incubation in A/B solution (Vectostain Elite ABC Kit; 1:222 dilution of

both the A and B solution). Tissue was then incubated in a NiDAB solution (Vector Laboratories, SK 4100) until adequate staining was observed. Tissue was then rinsed and incubated for an hour at room temperature, and 48 hours at 4^0 C in the rabbit anti-Kiss1 antibody (#564, a gift from Alan Caraty). This antibody has been previously characterized and demonstrates highly specific Kiss1 detection in the ARH (Desroziers et al.). Following primary incubation, tissue was processed as above except DAB staining was used instead of NiDAB. All tissue was mounted and dehydrated through a series of increasing concentrations of ethanol, followed by xylene treatment and finally slides were coverslipped with Permount. For cell counts, the total number of Kiss1-ir cells and Kiss/pSTAT3-ir cells were counted in five sections pertaining to the medial ARH for each animal.

Radioimmunoassay

Trunk blood was put on ice immediately after collection until it was spun at 2500 rpm for 25 minutes. Serum was collected from these samples and aliquoted for each RIA prior to storage at -20^0 C. Leptin RIAs were performed by the Oregon National Primate Research Center Endocrine Services Lab using the leptin RIA kit with a lower detection threshold of 0.5 ng/mL (Linco Research, Inc., St. Charles, MO). All LH RIA assays were performed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core and had a reported lower detection threshold of 0.4 ng/mL.

Statistical Analysis

All analyses of group differences for qPCR, immunohistochemistry, RIA, DEXA and uterine weight results were performed by one-way ANOVAs with a Newman-Keul's *post-hoc* test used for pair-wise multiple comparisons. The group differences in the percent change in body weight for the 48-hour fast study was also measured with this test. Repeated measures for daily differences in body weights were determined using a two-way ANOVA and Bonferroni post hoc test. ARH Kiss1/pSTAT3 cell counts were analysed using a two-way ANOVA. All values are presented as mean ± standard error.

RESULTS

Experiment 1. 40% Caloric Restriction

Low levels of estradiol have previously been shown to be required for LH inhibition during a 48-hour fast; therefore, to determine the possible necessity of estradiol for LH inhibition during CR, we included OVX control animals without estradiol replacement, as well as OVX animals weight-matched (OVX CR*) to the CR group with estradiol replacement (OVX+E CR). Ad libitum fed controls with low estradiol (OVX+E) gained significantly less weight than those without estradiol (OVX), despite similar levels of food intake (Figure 1, A; see Supplemental Figure 2 for daily body weight and food intake measurements). Consistent with these observed metabolic effects of estradiol in the control groups, the OVX CR* group did receive slightly less food than the OVX+E CR group to achieve comparable loss in body weight (Supplemental Figure 2); however, the cumulative food intake over the CR period was not significantly different (Figure 1, A). The total percent change in body weight was greater in the OVX CR* group compared to the OVX+E CR group; however, a two-way ANOVA to determine differences between changes in body weight on a daily basis (Supplemental Figure 2) did not reveal significant differences between the two groups ($p>0.05$). The OVX CR* and OVX+E CR groups had similar body compositions as determined by DEXA measurements, with both groups showing significant loss in both total body fat and lean mass (Figure 1, B). This comparable loss in both fat and lean mass as well as similar final loss in body weight suggests that similar levels of negative energy balance were achieved regardless of steroid environment. Final uterine weights were significantly increased in groups receiving estradiol (Figure 1, B), confirming the efficacy of the low estradiol treatment.

Leptin was significantly inhibited in the OVX+E CR and OVX CR* groups (Figure 1, C), with the OVX CR* group having lower leptin values compared to the OVX+E CR group. Leptin replacement resulted in serum levels slightly higher than those seen in controls; however, these leptin levels are still within the physiological range. Consistent with previous work, the low estradiol treatment did not

significantly blunt LH levels (Goodman, 1978), as there was no difference in LH levels between the two control groups (Figure 1, C). LH levels were significantly inhibited in the OVX+E CR group compared to controls; however, LH levels were not significantly inhibited in the OVX CR* group (Figure 1, C), suggesting that estradiol is required for CR-induced LH inhibition. Leptin replacement had a small effect on LH, resulting in levels not significantly different from the OVX+E or OVX+E CR group; however, a large amount of variability was observed in the LH values for the three CR groups (Figure 1, C).

Hypothalamic mRNA levels

ARH Kiss1 mRNA was significantly suppressed with CR, regardless of estradiol replacement, suggesting that while estradiol is required for CR-induced LH inhibition, it is not required for ARH Kiss1 inhibition (Figure 2, A). Restoration of leptin to normal basal levels had no effect on inhibited ARH Kiss1 levels. ARH Kiss1 mRNA levels were not significantly different between the OVX+E and OVX groups. AVPV Kiss1 mRNA was not significantly inhibited in the OVX+E CR group; however levels were significantly lower in the OVX+E CR+L group, suggesting a possible combined effect of CR and leptin treatment (Figure 2, A). AVPV Kiss1 levels were significantly higher in the OVX+E group compared to OVX animals, consistent with the stimulatory effect of estradiol on this nucleus and a previously reported higher level of sensitivity to estradiol compared to the ARH Kiss1 population (Takase et al., 2009).

Despite significant suppression of ARH Kiss1 with CR, NKB mRNA was only partially inhibited in the OVX+E CR group, and leptin appeared to have no effect on NKB mRNA levels (Figure 2, B). Similar to results in the lactation model (Xu et al., 2009b), PDYN mRNA levels were not differentially regulated by negative energy balance or leptin treatment (Figure 2, B). SOCS3, a downstream signaling molecule of the leptin receptor, was significantly inhibited with CR and restoration of leptin to normal basal levels partially attenuated this inhibition (Figure 2, B). The negative energy balance condition was confirmed by the presence of increased ARH NPY and AgRP mRNA levels, although POMC mRNA levels were

unchanged by CR (Supplemental Figure 3, A). Leptin had a small effect to attenuate the rises in AgRP and NPY levels, but had no affect on POMC levels.

Experiment 2. 50% Caloric Restriction

We hypothesized that the highly variable LH levels seen in the 40% CR groups were due to animals being at a threshold of sufficient weight loss required for LH inhibition. To reduce the LH variability observed with 40% CR, and discern true leptin effects on LH, a more severe 50% CR was performed to induce more severe negative energy balance and weight loss. Since estradiol appeared necessary for LH inhibition in 40% CR, only estradiol replaced groups were included in this experiment. 50% CR did result in a greater loss in body weight compared to the 40% CR (Figure 3, A), with animals losing a significant amount of both total body fat and lean mass (Figure 3, B). Leptin treatment was carried out for 72 hours to determine if more significant leptin effects are observed with longer treatment. Despite the longer administration, leptin treatment could not attenuate CR-induced LH inhibition (Figure 3, C). In contrast to the variable LH levels in the 40% CR experiment (Figure 1, C), there was little LH variability in either the 50% CR or 50% CR+L group (Figure 3, C), suggesting there may be a threshold of sufficient weight loss required for LH inhibition during CR.

Hypothalamic mRNA levels

ARH Kiss1 mRNA was significantly suppressed by 50% CR, and leptin had no effect to restore ARH Kiss1 (Figure 4, A). Unlike the 40% CR, AVPV Kiss1 mRNA was also significantly suppressed by 50% CR and restoring leptin had no effect to restore AVPV Kiss1. NKB mRNA was also significantly suppressed by 50% CR, and leptin treatment had no effect to attenuate this inhibition (Figure 4, B). Once again PDYN mRNA levels were similar across all groups suggesting that PDYN does not appear to be regulated by CR or by leptin. Similar to the results with a 40% CR, leptin treatment partially restored the suppressed levels of ARH SOCS3 mRNA (Figure 4, B). Orexigenic AgRP and NPY mRNA levels were

significantly increased by CR, and leptin partially reversed these increases. Similar to Experiment 1, POMC mRNA levels were unaffected by CR and leptin treatment (Supplemental Figure 3, B).

Experiment 3. 48-hour fast

To determine if the lack of leptin effects on LH observed in Experiments 1 and 2 was specific to the CR model, the leptin infusion regimen producing normal basal levels was given during a 48-hour fast and compared to the previously reported effects of pharmacological doses (Nagatani et al., 1998). Control (CTRL) animals had a slight increase in body weight over the 48-hour period, while all three fasted groups lost a comparable percentage of body weight (Figure 5, A). Leptin levels were not significantly inhibited by the 48-hour fast; however many samples in the fasted group (F) were below the detectable range; therefore reported leptin levels for this group are an overestimate of true levels (Figure 5, B). The leptin infusion (F+Leptin) once again resulted in leptin levels slightly higher than controls at the time of tissue collection (Figure 5, A). The pharmacological leptin treatment (F+High Leptin) resulted in significantly higher serum levels of leptin, even when measured 14 hours after the last bolus injection (Figure 5, A).

Serum LH was significantly inhibited by the 48 hr fast (Figure 5, B), and consistent with previous results (Ahima et al., 1996; Nagatani et al., 1998), the pharmacological dose of leptin did prevent LH inhibition. Unlike the pharmacological dose, maintaining leptin at normal basal levels was incapable of preventing the inhibition of LH. ARH Kiss1 mRNA was also significantly suppressed with a 48 hour fast, and surprisingly both doses of leptin seemed to have a small effect to partially restore ARH Kiss1 mRNA levels (Figure 5, B).

Experiment 4. Leptin induction of pSTAT3 in ARH Kiss1 neurones

To determine whether leptin exerts a strong direct effect on ARH Kiss1 neurones, immunohistochemistry for ARH Kiss1 and pSTAT3 was performed in brains taken from animals receiving acute i.p. injections of either saline or leptin (1 μg/g body weight). Abundant pSTAT3-ir was observed in

the hypothalamus ARH of leptin-injected animals (Figure 6, A), but few colocalized Kiss1/pSTAT3-ir cells were observed and there were no differences in the number of colocalized cells between saline or leptin-injected animals (Figure 6, B).

DISCUSSION

The current study investigated whether abolishing hypoleptinemia with a physiologically-relevant dose of leptin was capable of restoring GnRH/LH secretion during negative energy balance. The results demonstrate that restoring leptin to normal basal values does not attenuate LH inhibition in a 50% CR. This dose of leptin was also incapable of maintaining LH levels during a 48-hr fast. Therefore, it seems unlikely that elimination of hypoleptinemia is the critical driver of LH restoration. ARH Kiss1 is also suppressed with CR and fasting, and exogenous leptin could not restore ARH Kiss1 levels in the CR models, though there was a small effect of leptin in the fasting study.

There is a wealth of literature on the importance of leptin for normal reproductive function. Leptin is required for normal reproductive development, since mutations in leptin or leptin receptors results in abnormal reproductive function (Chehab et al., 1996; Swerdloff et al., 1976; Todd et al., 2003), and exogenous leptin administration results in early onset of pubertal development (Ahima et al., 1997). Studies across many species have also shown stimulation of LH by exogenous leptin under both normal and metabolically challenged conditions (Ahima et al., 1996; Barash et al., 1996; Chan et al., 2006; Chehab et al., 1996; Finn et al., 1998a), and critically, human studies suggest leptin may be a viable treatment for women with exercise-induced hypothalamic amenorrhea (Welt et al., 2004).

While the above literature clearly points to an important role for leptin in reproductive function, recent studies have complicated the hypothesized role of leptin for negative energy balance-induced reproductive inhibition. Szymanski et al (2007) found that when food restricted ewes are refed, LH parameters were restored prior to any increases in circulating leptin levels, suggesting elimination of hypoleptinemia is not required for restoration of LH after negative energy balance. A recent study from

our lab found that restoring leptin to normal basal levels had no effect to restore LH levels in the lactation model of negative energy balance (Xu et al., 2009b). This dose of leptin, also used in the current study, resulted in normal basal leptin levels that are at least 50 fold lower than levels reported with a standard pharmacological dose previously shown to attenuate LH inhibition (Ahima et al., 1996). Indeed, the vast majority of studies demonstrating leptin-induced attenuation of LH inhibition have used similarly large pharmacological doses (Ahima et al., 1996; Donato et al., 2009; Finn et al., 1998a; Nagatani et al., 1998). However, it is important to acknowledge that although administration of leptin by minipump in the current study resulted in serum levels in the normal physiological range, this administration cannot itself be termed "physiological" since the diurnal leptin pattern was disrupted with continuous leptin infusion. Therefore, it remains possible that leptin effects in our studies were not observed due to a lack of diurnal rhythm.

Our previous work demonstrating a lack of leptin effects upon negative energy balance-induced LH inhibition was performed in the lactation model (Xu et al., 2009b), and we hypothesized that leptin effects in this model may have been masked by other inhibitory signals specific to lactation, such as the suckling stimulus (Brogan et al., 1999). Therefore, the current study was designed to determine if leptin was ineffective during lactation due to the redundant inhibitory signals specific to lactation or the lower dose of leptin administered. Seventy-two hours of exogenous leptin treatment, which restored leptin to normal basal levels, was incapable of restoring LH during 50% CR, suggesting the previously demonstrated lack of leptin effects during lactation is not a characteristic specific to this model. Additionally, while pharmacological leptin did prevent LH inhibition during a fast, consistent with previous results (Ahima et al., 1996; Nagatani et al., 1998), maintaining leptin at normal basal levels did not. Leptin levels rise modestly when animals exit negative energy balance to return to normal basal levels and do not reach the very high levels observed with pharmacological doses (Ahima et al., 1996; McCowen et al., 1998). Therefore, although leptin appears to be required for normal reproductive

development and can stimulate LH at pharmacological concentrations, hypoleptinemia may not be the critical signal responsible for suppression of LH during negative energy balance.

In addition to LH inhibition, the current study has demonstrated that ARH Kiss1 is inhibited in both fasting and CR models of negative energy balance. Previous data describing fasting effects on ARH Kiss1 have been inconsistent, with data arguing both for and against inhibition of these cells (Castellano et al., 2005; Forbes et al., 2009; Kalamatianos et al., 2008; Luque et al., 2007; Quennell et al., 2011). Given previous results suggesting a role for ARH Kiss1 in negative steroid feedback of GnRH release, the current results of ARH Kiss1 inhibition in this study are consistent with past work demonstrating a loss of pulsatile LH during a 48-hour fast (Nagatani et al., 1998). AVPV Kiss1, which is believed to contribute to positive steroid feedback and the GnRH/LH surge, was only significantly inhibited in the 50% CR study, and not during fasting or the 40% CR experiments. An unexpected finding in the 40% CR study was the significant inhibition of AVPV Kiss1 in the presence of leptin. There are no obvious explanations for this result, as inhibitory effects of leptin on AVPV Kiss1 have not been reported. AVPV Kiss1 is also suppressed during lactation (True et al., 2011b), indicating this population may only be inhibited under severe conditions of negative energy balance. Interestingly, NKB and PDYN, which are coexpressed within the ARH KNDy neurones, were not consistently inhibited with negative energy balance, although NKB levels were inhibited with the more severe 50% CR, similar to lactation (True et al., 2011b). More research is needed to understand how differential regulation of these three reproductive neuropeptides within the same KNDy cells may contribute to reproductive inhibition.

Similar to LH results, restoring leptin to normal basal values was unable to attenuate inhibition of ARH Kiss1 or NKB mRNA or AVPV Kiss1 mRNA inhibition in the 50% CR experiment. Despite previous results showing leptin-receptor expression on ARH KNDy neurones in the mouse (Smith et al., 2006a), the current study found a lack of ARH Kiss1/pSTAT3 colocalization after acute treatment with a pharmacological dose of leptin, arguing against a strong direct regulatory relationship in the rat. Taken

together with recent evidence observing a lack of leptin-receptor signaling in AVPV Kiss1 neurons (Quennell et al., 2011), these findings suggest Kiss1 neurons are unlikely to be the cell population relaying leptin signaling to GnRH neurons. Pharmacological leptin treatment has been shown to stimulate Kiss1 levels (Castellano et al., 2006b; Smith et al., 2006a), and in the current study leptin replacement to normal basal levels seemed to partially attenuate inhibition of ARH Kiss1 in the least severe model of negative energy balance, the 48-hr fast model, suggesting there may be differential regulation of ARH Kiss1 by leptin depending on the model of negative energy balance.

Due to the lack of leptin effects on Kiss1 mRNA and LH levels in the current study, it was important to demonstrate that the physiologically-relevant dose of leptin administered by minipump infusion was biologically active in the brain. To definitively answer this question pSTAT3 staining was measured in fasted animals receiving either saline or the physiologically-relevant dose of leptin via osmotic minipump. Two hours of peripheral leptin administration via the osmotic minipump at the physiological dose significantly increased pSTAT3 staining compared to animals receiving saline, confirming biologically activity of this dose in the brain. Although it is conceivable that leptin may degraded at body temperature with longer minipump treatments like those in Experiments 1-3, this appears unlikely given the abundance of studies showing significant leptin effects after prolonged minipump administration for up to two weeks (Correia et al., 2001; Kievit et al., 2006; Nishiyama et al., 1999; Pal and Sahu, 2003; Pearson et al., 2001; Sindelar et al., 1999; Wetzler et al., 2004). In addition to pSTAT3 staining, ARH SOCS3 mRNA was also increased in the 40% CR and the 50% CR with the low leptin treatment, although this difference only reached statistical significance in the former model. Furthermore, this dose of leptin was used previously in our lab and shown to completely reverse the suppression of POMC in lactating rats (Xu et al., 2009b), confirming that this dose is biologically relevant in the brain.

Surprisingly, in the current study POMC was not significantly inhibited in either CR model. This lack of POMC regulation with CR suggests that decreases in POMC may not be as strongly regulated with negative energy balance in females as has been previously reported for males (Mizuno et al., 1998; Mizuno et al., 1999; Schwartz et al., 1997; Ziotopoulou et al., 2000), and therefore POMC inhibition is only observed in female rats with the severe hyperphagia and negative energy balance of lactation (Xu et al., 2009b). Given the lack of POMC inhibition with CR, it was not surprising that leptin administration had no affect on POMC levels. Leptin infusion was also incapable of completely attenuating the large increases in NPY and AgRP in response to CR, consistent with previous work from our lab finding no effect of restoring leptin to normal basal levels on NPY and AgRP levels in lactating rats (Xu et al., 2009b). While this lack of leptin regulation on NPY and AgRP may seem controversial, it should be noted that previous effects of leptin on NPY and AgRP have been demonstrated with male mice using pharmacological doses (Mizuno et al., 1999; Mizuno and Mobbs, 1999; Ziotopoulou et al., 2000). It appears likely that differences between the current study and previously reported leptin affects on NPY and AgRP are likely due to either gender or doses of leptin.

Consistent with earlier fasting studies, our results found a requirement of estradiol for negative energy balance-induced LH inhibition (Nagatani et al., 1998; Nagatani et al., 1994). As expected, the low dose of estradiol administered alone did not blunt the OVX-induced LH rise (Goodman, 1978); however, the estradiol levels were clearly biologically-active, since significant effects on body weight, uterine weight, and AVPV Kiss1 were observed. Interestingly, the current results demonstrate that while estradiol is required for inhibition of LH it is not required for of the inhibition of ARH Kiss1. In the 40% CR studies, ARH Kiss1 was uniformly suppressed in all CR groups with or without estradiol treatment, whereas the LH values were widely variable, with some in the normal control range. Thus, it appears that suppression of ARH Kiss1 is not always tightly coupled to the suppression of LH secretion, lending support to the notion of Kiss1-independent regulation of LH secretion (Chan et al., 2009). The variable

LH levels with a 14 day 40% CR suggest that there may be a threshold of sufficient weight loss required for LH inhibition. The uniform suppression of LH with 50% CR suggests all animals had achieved sufficient weight loss for LH inhibition. Importantly, it appears that once animals are in a severe enough state of negative energy balance, as demonstrated with the 50% CR, restoring leptin to normal basal levels has no effect to restore LH. These findings highlight the fact that many aspects of LH inhibition remain poorly understood, and further studies are needed to understand the multitude of signals contributing to LH inhibition.

The current findings, coupled with our earlier studies of lactation (Xu et al., 2009b), suggest that metabolic factors other than low leptin likely contribute to inhibition of reproductive pathways in models of negative energy balance. Previously studied candidates include ghrelin, insulin, glucose, and NPY to name a few (for recent review see (Castellano et al., 2009; Tena-Sempere, 2008)). NPY and insulin do not appear be to critical players since insulin replacement during lactation did not restore reproductive function, and attenuation of elevated levels of NPY in both lactation and 50% CR were not accompanied with changes in LH (Xu et al., 2009b). Ghrelin is also an interesting candidate for linking metabolic and reproductive function, since ghrelin has been shown to be inhibitory to LH (Furuta et al., 2001). However, while the elevated levels of ghrelin during fasting are consistent with a potential role in the inhibition of LH (Toshinai et al., 2001), during lactation ghrelin levels are low (Shibata et al., 2004) and exogenous ghrelin has no affect on LH (Fernandez-Fernandez et al., 2004), suggesting it is unlikely to contribute to the suppression of LH. Clearly, much remains to be learned about the metabolic regulation of reproduction, but results from the current study argue against a critical role of hypoleptinemia in the suppression of LH during negative energy balance, since restoration of leptin to normal basal levels does not restore LH. Taken together with previous work clearly demonstrating a strong role for leptin in reproductive regulation, it is becoming clear that this pathway is more complex than previously hypothesized. Therefore, understanding the neurocircuitry involved in the inhibition of

Kiss1 and GnRH release and the potential multitude of metabolic signals that could be involved in this process still remain two critical and unresolved questions in the field.

Table 1. Experimental Group Descriptions

Experiment	Group Name	Steroids	Food Intake	Leptin Treatment
40% CR	*OVX + E*	OVX+E	Ad libitum	None
	OVX+E CR	OVX+E	40% CR for 14 days	None
	OVX+E CR+L	OVX+E	40% CR for 14 days	500 ng/hour for last 48 hours
	OVX	OVX	Ad libitum	None
	*OVX CR**	OVX	CR to weight-match "OVX+E CR" group	None
50% CR	*CTRL*	OVX+E	Ad libitum	None
	50% CR	OVX+E	50% CR for 14 days	None
	50% CR+L	OVX+E	50% CR for 14 days	500 ng/hour for last 72 hours
48-hr Fast	*CTRL*	OVX+E	Ad libitum	None
	F	OVX+E	48 hr fast	None
	F+Leptin	OVX+E	48 hr fast	500 ng/hour, for 48 hours
	F+High Leptin	OVX+E	48 hr fast	3 µg/g twice daily, for 48 hours

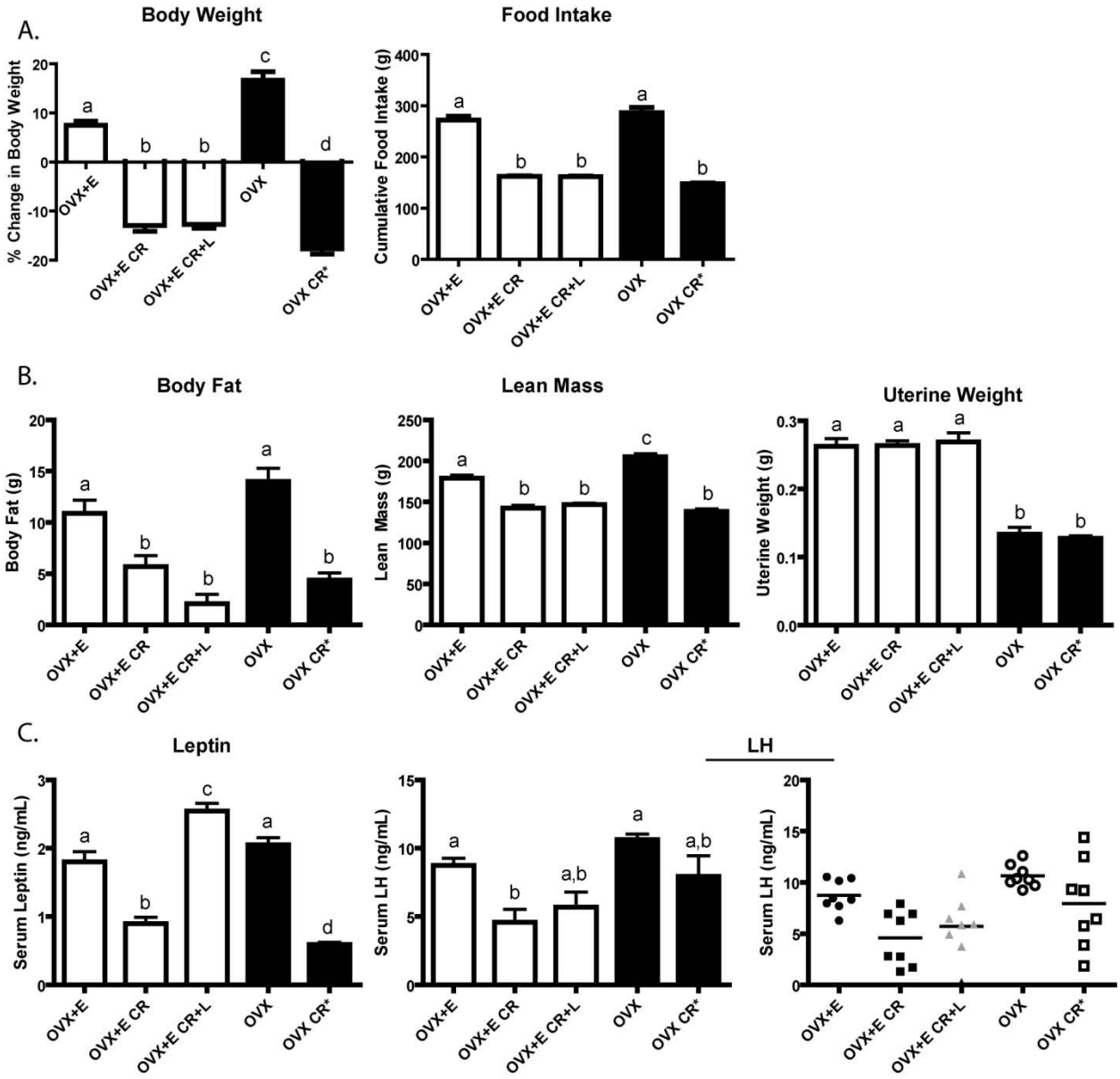


Figure 1. Body composition and serum leptin and LH levels in response to 40% CR.
A) Change in body weight and cumulative food intake. OVX+E, ad libitum fed controls receiving 2.5 cm silastic implant containing 30 μg/ml estradiol; OVX+E CR, estradiol replaced animals on 40% CR (compared to OVX+E group); OVX+E CR+L, estradiol replaced animals on 40% CR implanted with an osmotic minipump delivering 500 ng of leptin/hour for the last 48 hours of CR; OVX, ad libitum fed controls not receiving estradiol replacement; OVX CR*, animals without estradiol that were CR to achieve comparable weight-loss as the OVX+E CR group. B) Both body fat and lean mass were measured by DEXA post mortem. Uteri were dissected and weighed at the time of euthanization to verify effectiveness of estradiol treatment. C) Serum leptin and LH were measured by RIA. LH results are presented both as a bar graph and scatterplot to demonstrate high variability in LH levels in all three CR groups (OVX+E CR, OVX+E CR+L, OVX CR*). Columns with different letters are significantly different, $p<0.05$.

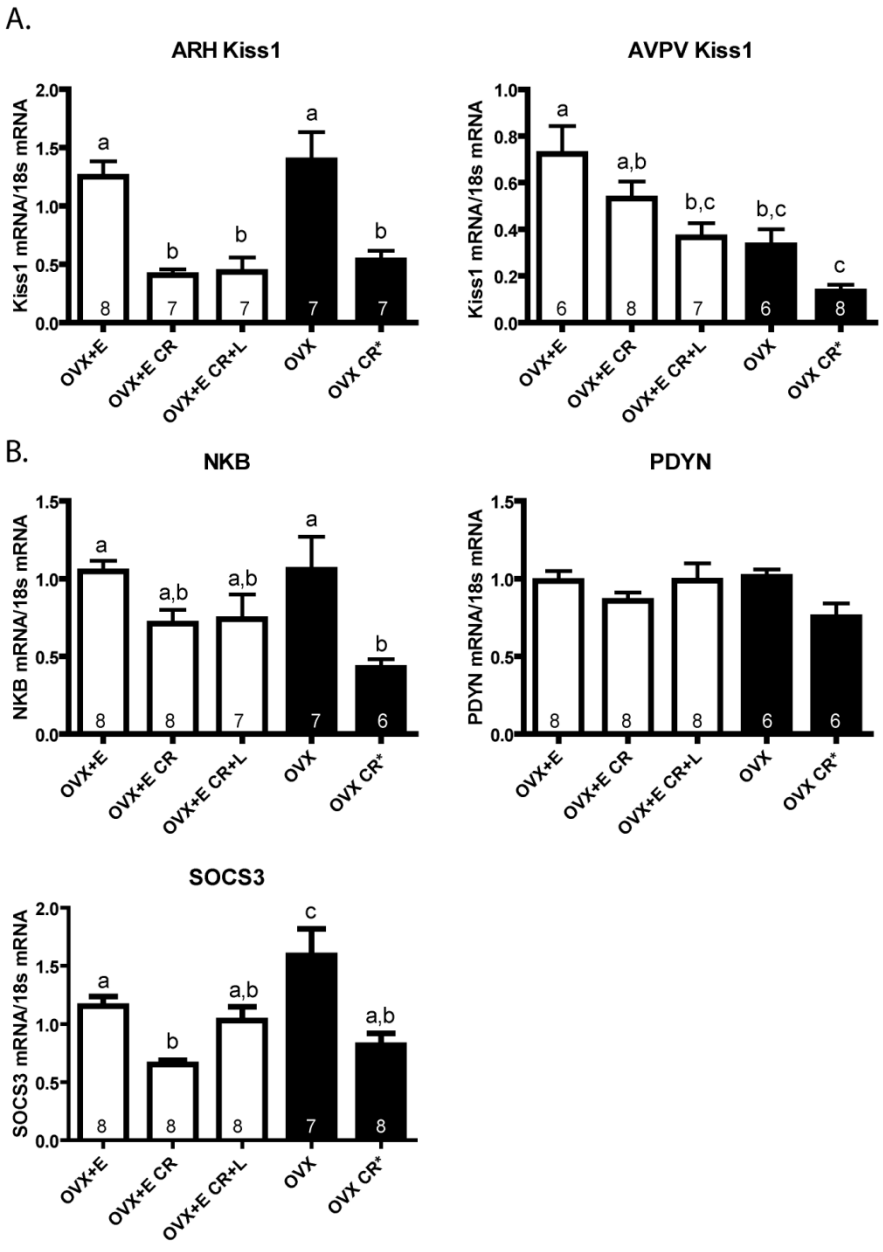


Figure 2. Hypothalamic mRNA expression in response to 40% CR.

A) Kiss1 mRNA was measured by Real-time PCR from microdissected ARH and AVPV samples. B) ARH reproductive mRNAs (NKB, PDYN) and SOCS3 mRNA were also measured from the microdissected ARHs. Columns with different letters are significantly different, $p<0.05$; numbers inside histograms represent group size.

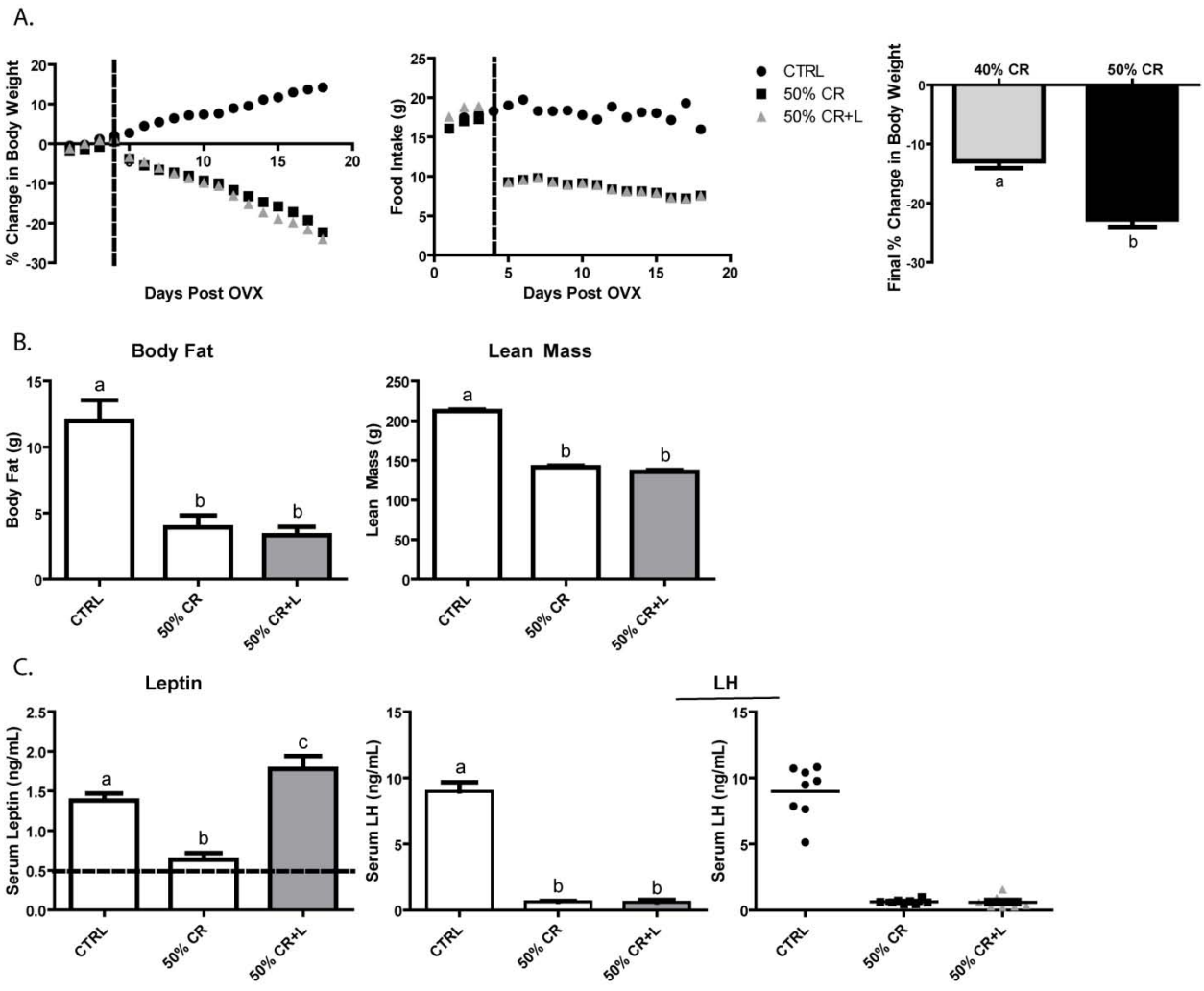


Figure 3. Body composition and serum leptin and LH levels in response to 50% CR.
A) Body weight was measured daily and is presented as the daily accumulative average of % change in body weight. Food intake represents the average amount of food consumed per day. OVX and estradiol silastic implantation were performed on Day 0, and the solid line marks the beginning of CR treatments on Day 4. Leptin treatment had no additional effect on body weight in CR animals and changes in body weight were significantly different in CR groups compared to the CTRL group beginning on Day 5, one day after beginning CR. Right panel: Comparison of total weight loss for experimental groups across CR studies: 40% CR, OVX+E CR group from experiment 1; 50% CR, 50% CR group from experiment 2. B) Body fat and lean mass were determined by DEXA post mortem. C) Serum leptin and LH were measured by RIA. Horizontal line in the leptin bar graph represents the lower threshold of detectability for the leptin RIA. LH data is presented both as bar graph and scatterplot. Columns with different letters are significantly different, $p<0.05$.

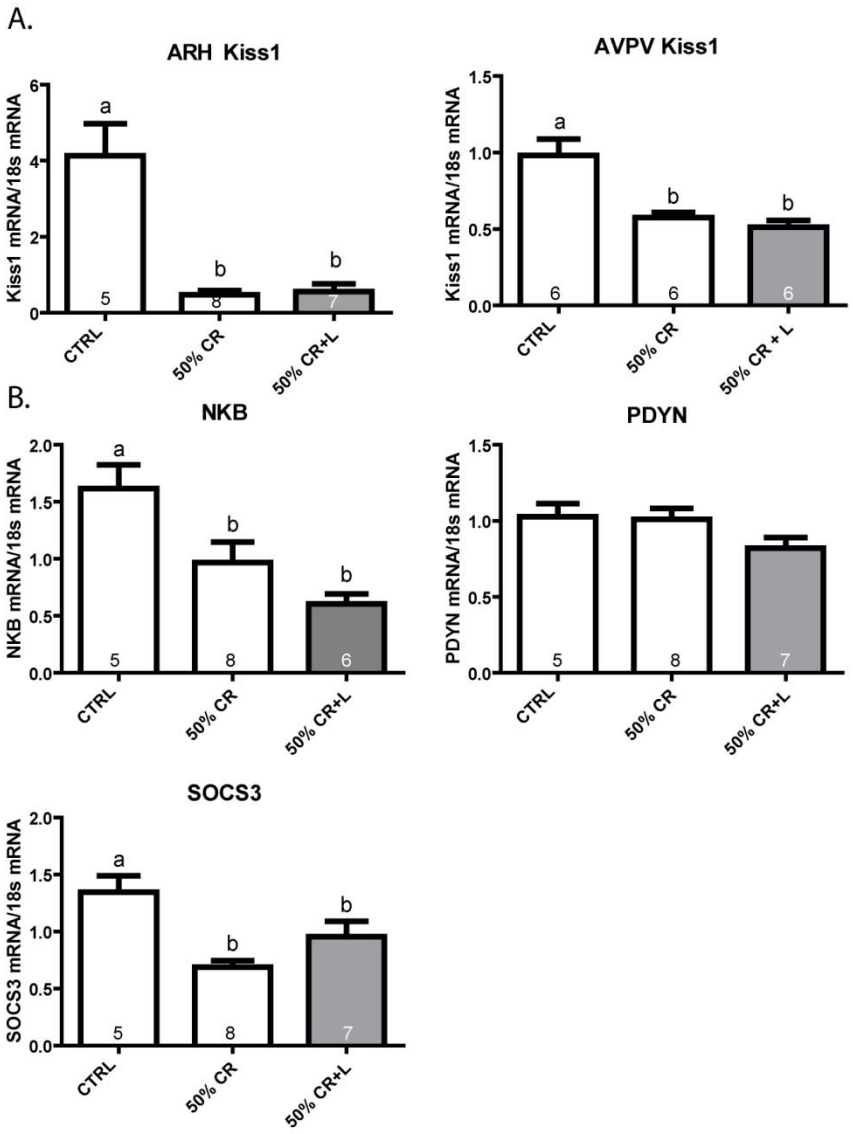


Figure 4. Hypothalamic mRNA expression in response to 50% CR.
A) Kiss1 mRNA was measured by Real-time PCR from microdissected ARH and AVPV samples. B) ARH reproductive mRNAs (NKB, PDYN) and SOCS3 mRNA were also measured from the microdissected ARHs. Columns with different letters are significantly different, $p<0.05$; numbers inside histograms represent group size.

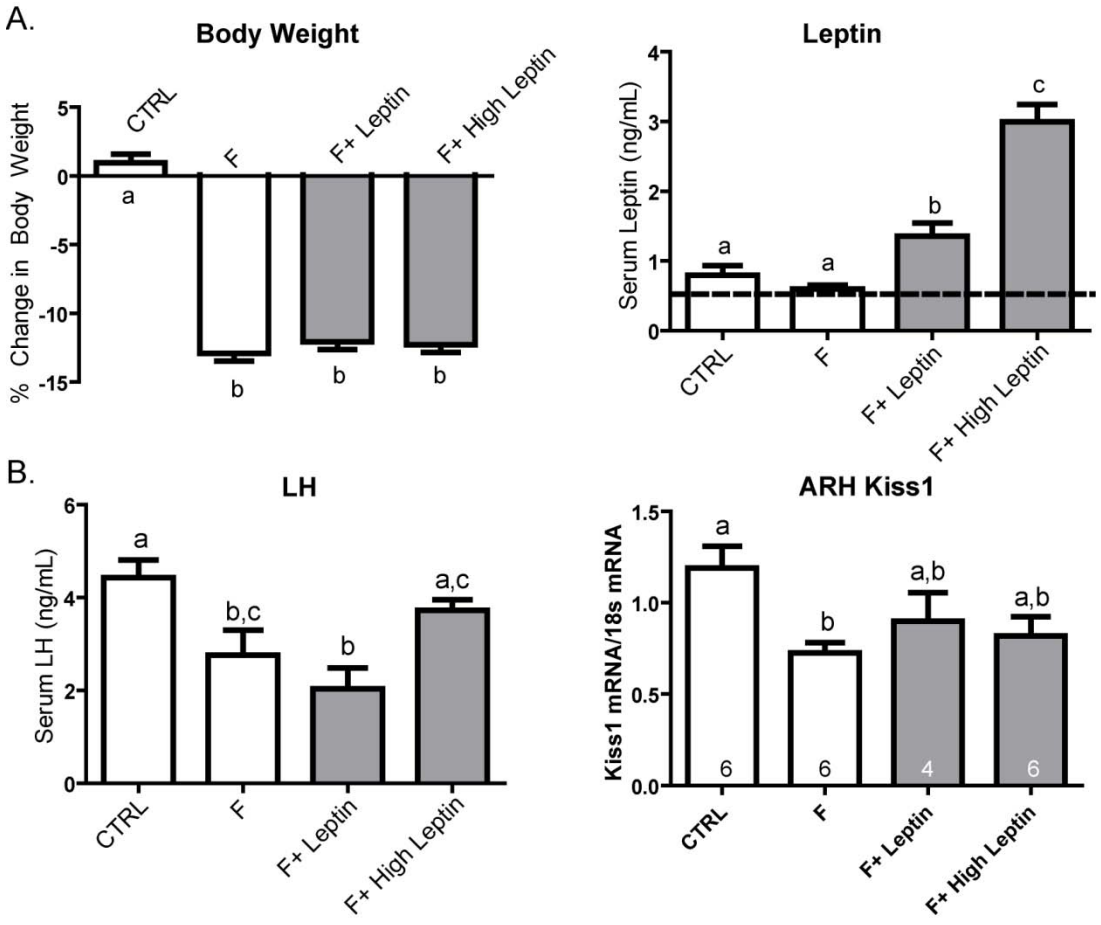


Figure 5. Changes in body weight, serum leptin, serum LH, and ARH Kiss1 mRNA levels in response to the 48-hour fast.

A) Change in body weight was calculated as the total % change in body weight over the 48 hour period; CTRL, control; F, fasted; F+Leptin, fasted with 500 ng/g of body weight leptin treatment via osmotic minipump; F+High Leptin, fasted with twice daily injections of 3 µg/g of body weight leptin. Serum leptin was measured by RIA and the dotted line denotes the lower threshold of detectability for the leptin RIA. B) Serum LH was also measured by RIA. Kiss1 mRNA was measured by real-time PCR from microdissected ARH samples. Columns with different letters are significantly different, $p<0.05$.

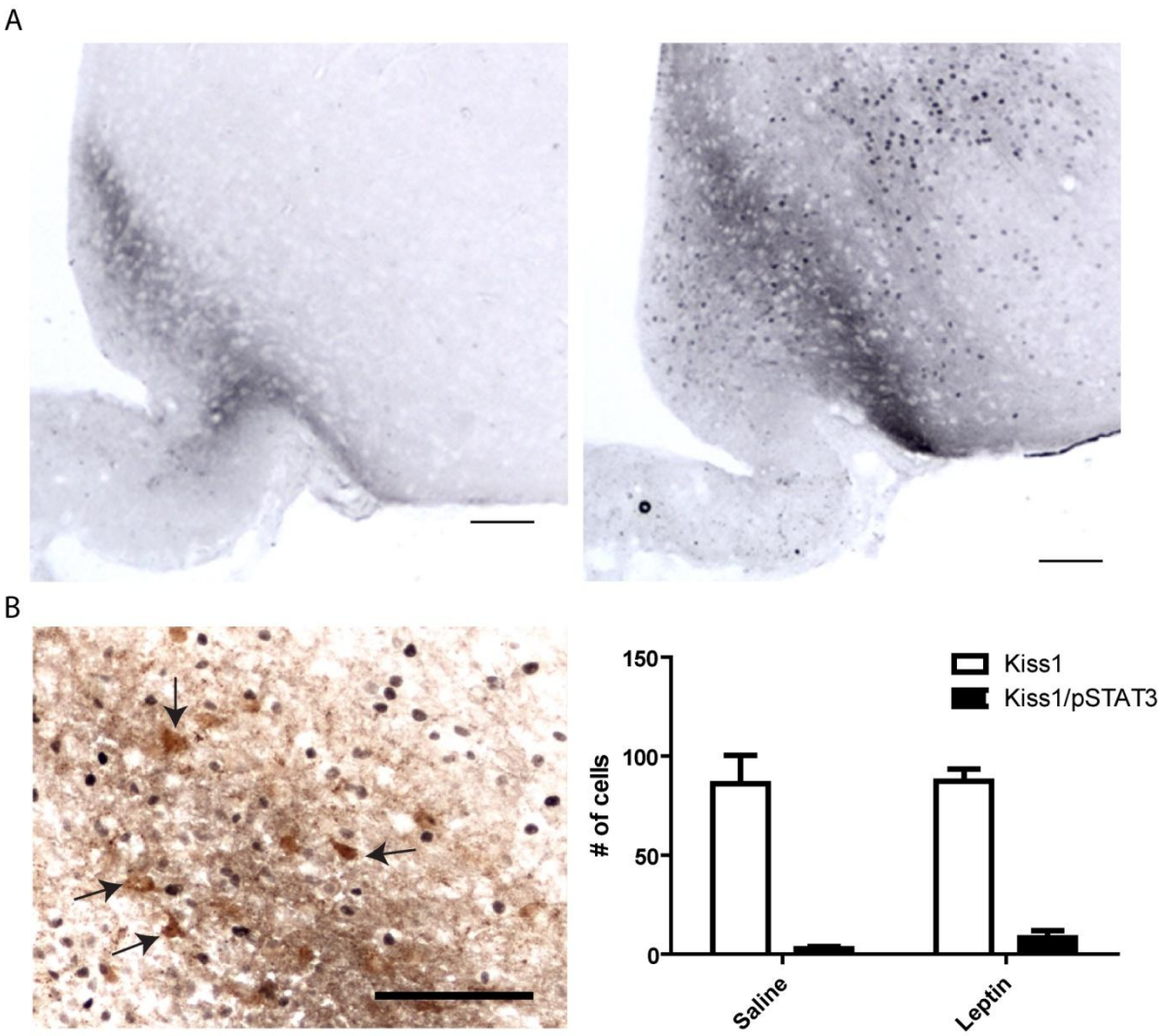
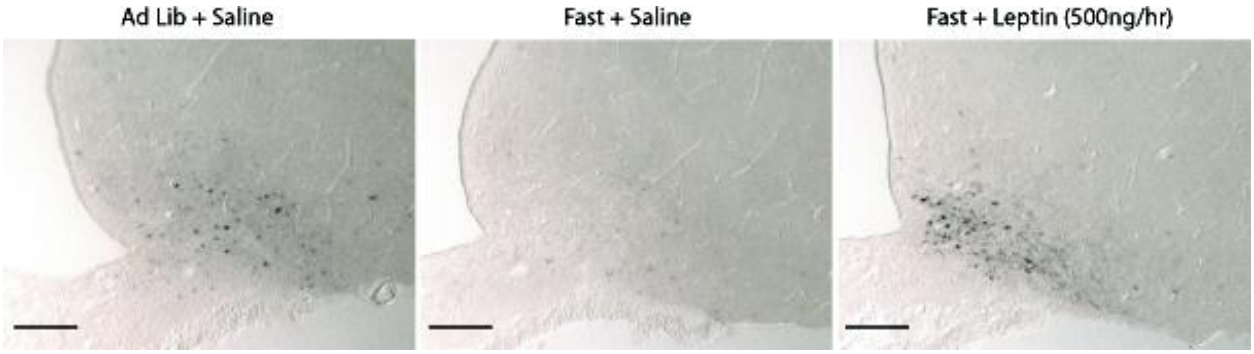


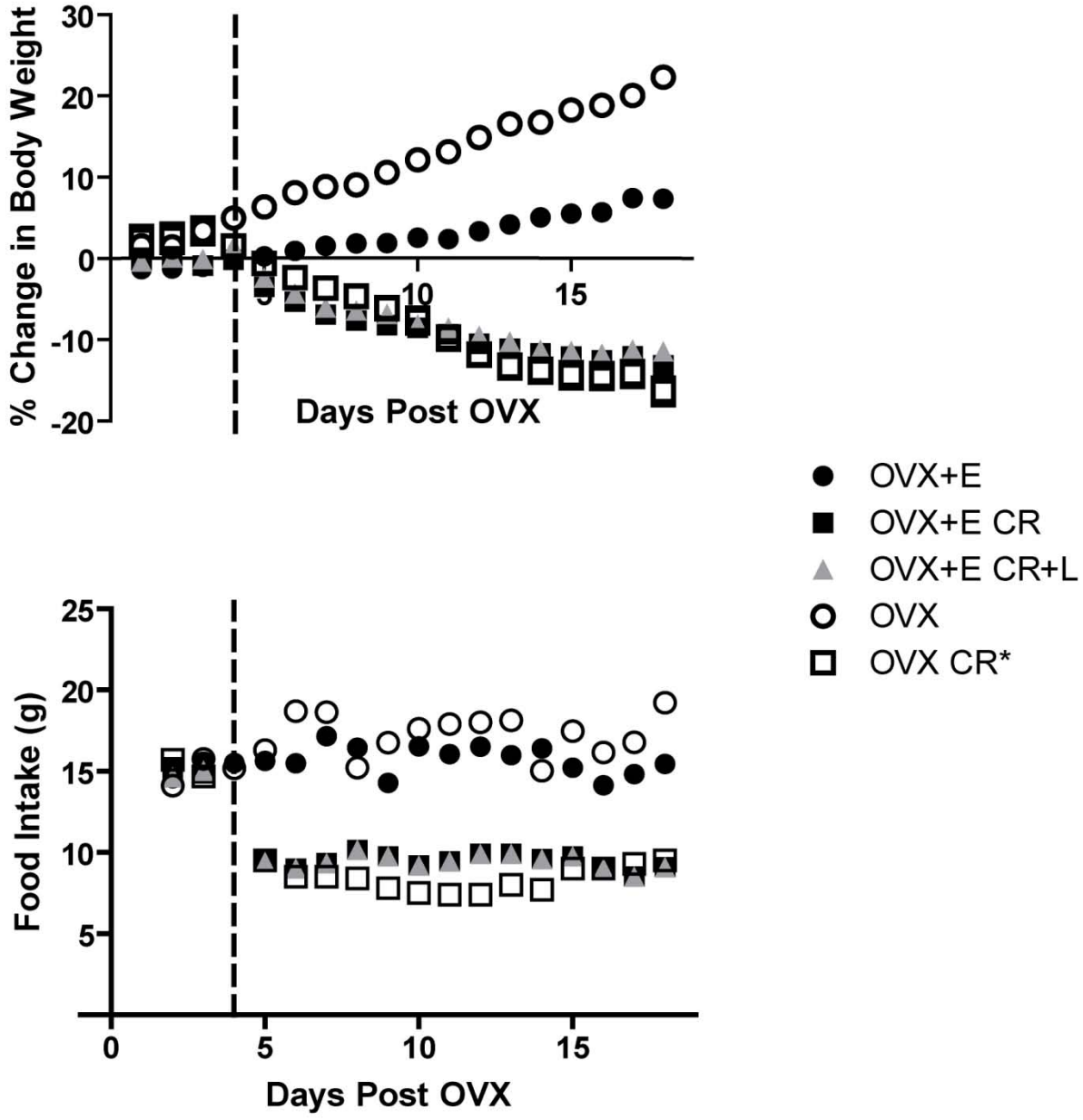
Figure 6. pSTAT3 and Kiss1 immunohistochemistry following acute pharmacological leptin administration.

A) pSTAT3-ir (NiDAB, dark brown nuclear staining) in the hypothalamus following saline (left) and leptin (right) treatment. B) ARH Kiss1-ir cells (DAB, brown staining, arrows) predominantly lacked pSTAT3-ir. The number colocalized Kiss1/pSTAT3-ir cells were not different between saline and leptin-treated groups (right). Scale bar represents 100 µm.

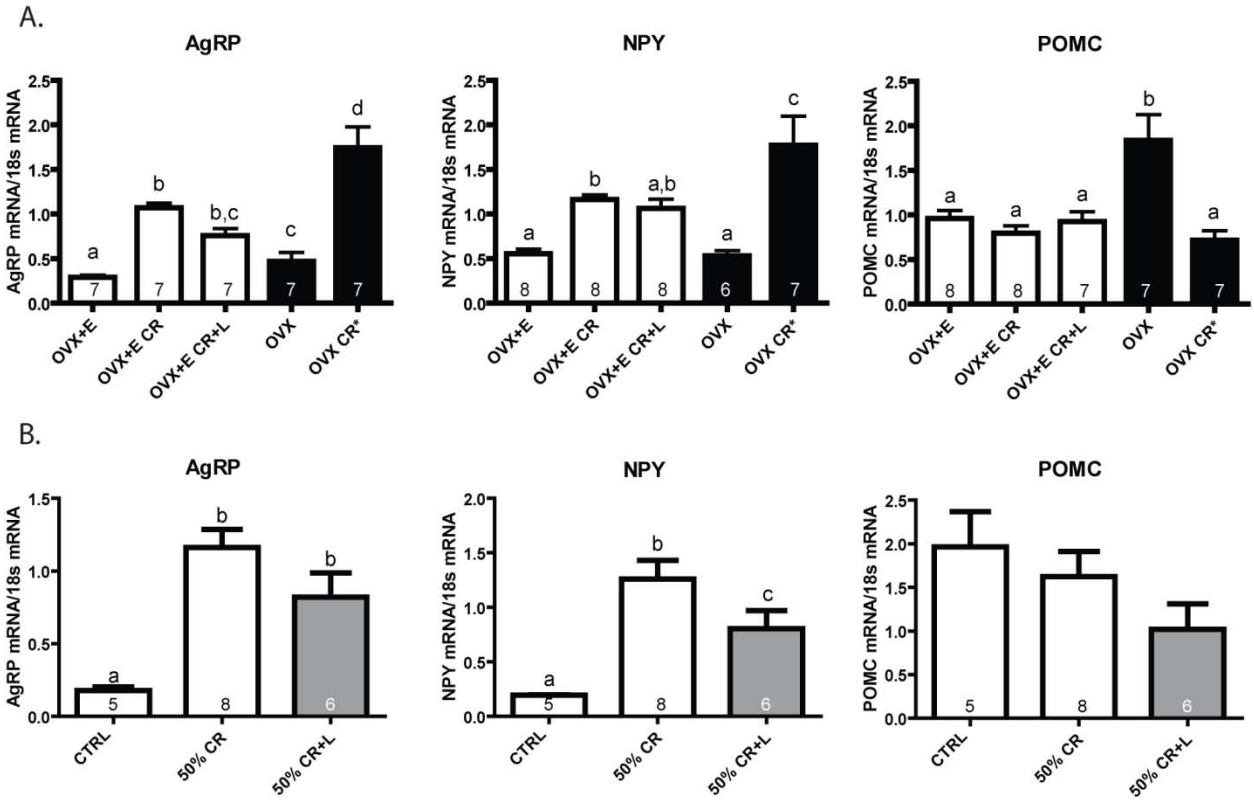


Supplemental Figure 1. ARH pSTAT3 immunohistochemistry in fasted animals following leptin minipump implantation.

pSTAT3-ir is shown for three groups: OVX+E females ad libitum fed receiving saline minipumps, OVX+E females fasted for 48 hours receiving saline minipumps, and OVX+E females fasted and receiving leptin (500ng/hr) minpumps. Minipumps were implanted 2 hours prior to the end of a 48 hour fast. Perfusion and immunohistochemistry (rabbit anti-pSTAT3, 1:250; Cell Signaling, 9145; NiDAB) were performed as described for Experiment 4, with the exception that borate-buffered 4% paraformaldehyde was used for fixation. pSTAT3-ir was increased with leptin treatment in fasted animals, compared to fasted animals receiving saline, suggesting leptin treatment was biologically active in the brain. Scale bars represent 100 µm.



Supplemental Figure 2. Daily changes in body weight and food intake during 40% CR.
Animals were OVX on Day 0 and 40% CR began on Day 4 (dotted line). Changes in body weight for OVX+E CR and OVX+E CR+L groups were significantly different from the control OVX+E group beginning on Day 6, as determined by a two-way ANOVA ($p<0.05$).



Supplemental Figure 3. ARH feeding neuropeptide mRNA levels in response to 40% and 50% CR.
A) ARH mRNA levels for NPY, AgRP and POMC following 40% CR. B) ARH mRNA levels for NPY, AgRP and POMC following 50% CR. Columns with different letters are significantly different, $p<0.05$.

Chapter 4- The role of cocaine- and amphetamine-regulated transcript in metabolically-driven GnRH inhibition

ABSTRACT

Cocaine- and amphetamine-regulated transcript (CART) is a hypothalamic neuropeptide implicated in both metabolic and reproductive function, suggesting CART may play a role in reproductive inhibition during negative metabolic conditions. Recent research demonstrated CART directly depolarizes GnRH neurons; therefore, we hypothesized CART may be inhibited with negative energy balance. The aim of the current study was to investigate CART protein and mRNA levels in two different models of negative energy balance: caloric restriction and lactation. CART protein and mRNA levels were suppressed in the arcuate nucleus (ARH) and CART mRNA was suppressed in the anteroventral periventricular region (AVPV) with caloric restriction. In the lactation model, where energy intake is insufficient for the excessive energy expended in milk production, negative energy balance did not suppress either ARH or AVPV CART mRNA expression levels. Surprisingly, lactation did results in a significant increase in AVPV CART cell number by immunohistochemistry. Given that AVPV CART mRNA levels were unaffected, this increase in cell number may represent inhibition of protein release. Immunohistochemistry also revealed that CART fibers were found making close appositions to GnRH neurons as well as ARH and AVPV Kiss1 neurons. The majority of CART fibers making close contact on Kiss1 cells appeared to coexpress the POMC cleavage product α-MSH, indicating that the ARH is likely the predominant source of these CART projections. In conclusion, CART contacts onto GnRH and Kiss1 cells indicate that CART might play both a direct and indirect role in GnRH regulation. While CART levels are largely unaffected with lactation, inhibition of stimulatory CART expression in the ARH and AVPV during caloric restriction might contribute to both Kiss1 and GnRH inhibition.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the final output signal of the hypothalamus governing regulation of the ovarian/steroid cycle. Negative metabolic states, either from undernutrition or overexertion, result in GnRH inhibition in all female mammals studied to date. This inhibition of GnRH release results in impaired reproductive function, but the pathway underlying this metabolically driven anovulatory state remains poorly understood. Particularly the metabolic signals and neural circuits contributing to GnRH inhibition remain elusive. One clear player in metabolically-driven GnRH inhibition, is the powerful GnRH secretagogue, Kisspeptin (Kiss1). Inhibition of hypothalamic Kiss1 mRNA levels has been observed in multiple models of negative energy balance and in numerous species (Backholer et al., 2010; Castellano et al., 2005; Castellano et al., 2006b; Forbes et al., 2009; Kalamatianos et al., 2008; True et al., 2011b; True et al., 2011c; Wahab et al., 2011). This data suggests inhibition of the stimulatory Kiss1 signal may be a conserved mechanism contributing to decreased GnRH release during negative metabolic states.

Both hormonal and neuronal metabolic signals have been proposed to contribute to GnRH inhibition during negative energy balance. While significant evidence exists for the permissive role of adipocyte hormone leptin in signaling sufficient energy stores for pubertal maturation, conflicting evidence exists on the role of this peripheral hormone in mediating negative energy balance-induced GnRH inhibition during adulthood (Ahima et al., 1996; Szymanski et al., 2007; True et al., 2011c; Welt et al., 2004). While physiological levels of leptin can restore LH levels in women, physiologically-relevant levels of leptin appear less effective at restoring LH release in rats, suggesting additional signals of negative energy balance are likely required for GnRH inhibition in this species (Schurgin et al., 2004; True et al., 2011c; Welt et al., 2004). In addition to leptin, there is a wealth of evidence that many of the hypothalamic feeding neuropeptides are metabolically-regulated and may also modulate GnRH release.

One such neuropeptide is cocaine- and amphetamine-regulated transcript (CART). The role of hypothalamic CART in regulating food intake is somewhat controversial. Third ventricle application of CART decreases food intake, but CART administration into specific hypothalamic feeding nuclei paradoxically increases food intake (Abbott et al., 2001; Kristensen et al., 1998). These opposing findings have not fully been reconciled, although there is some evidence to suggest that CART's anorexigenic effects may be secondary to a motor abnormality observed with large doses of CART (Abbott et al., 2001; Aja et al., 2001). However, ARH CART levels are inhibited with fasting, consistent with an anorexigenic role for this neuropeptide (Adam et al., 2002; Kristensen et al., 1998; Robson et al., 2002), although it is unclear whether this inhibition is found in other conditions of negative energy balance. In addition it is also unknown whether the other hypothalamic CART populations are sensitive to conditions of negative energy balance. In addition to CART's effects on regulating food intake, there is also data to suggest that CART may stimulate thermogenesis and thus increase energy expenditure (Elias et al., 1998; Kong et al., 2003). CART is also colocalized with the well-documented anorexigenic neuropeptide proopiomelanocortin (POMC) in the ARH (Elias et al., 1998). Based on these findings there is sufficient evidence to suggest CART may act in part as a satiety signal for metabolic homeostasis.

CART has also been implicated in the regulation of GnRH. CART fibers were noted making close appositions to GnRH cells in both hamsters and rats (Leslie et al., 2001; Rondini et al., 2004). Furthermore, these appositions were found on GnRH cells expressing c-Fos on the afternoon of proestrus, suggesting CART might be involved in regulation of the GnRH surge (Rondini et al., 2004). While little is known about how CART might regulate GnRH cells, one group has demonstrated that CART can increase GnRH pulse frequency in a hypothalamic explant preparation (Lebrethon et al., 2007; Lebrethon et al., 2000). Importantly, recent work from our laboratory found that CART appears to depolarize roughly 75% of GnRH neurons, and increase firing frequency in a subset of neurons, and this stimulatory effect persists in the presence of the voltage-gated sodium channel blocker tetrodotoxin

(manuscript in preparation). Thus it appears that CART might act post-synaptically at GnRH neurons to increase neuronal excitability.

While it appears clear that CART plays a role in both metabolic and reproductive regulation, it is unknown whether CART provides a link between these two neural circuits for metabolically-driven GnRH inhibition. The goal of the current study was to determine what CART populations might be involved in negative energy balance-driven GnRH inhibition by examining protein and mRNA levels across several different CART populations. In addition, we also investigated whether the ARH CART population might be the source for CART projections found near GnRH neurons. Finally we investigated whether CART might also indirectly regulate GnRH and reproductive function by providing input to Kiss1 populations.

MATERIALS AND METHODS

Animals

Adult female Wistar rats (Simonsen, Gilroy, CA), weighing between 200-220 grams, were used in all histological studies. Animals were singly housed and maintained on a 12-hour light (0600 hr) and dark (1800 hr) cycle throughout the experiment and allowed water ad libitum. All protocols were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Lactation Model

The lactation model has been described previously (Xu et al., 2009b). Briefly all animals were ovariectomized and had silastic implants containing 30 µg/mL estradiol (1 cm in length for every 100 grams of body weight; OVX+E) implanted subcutaneously on Day 2 post partum for lactating animals and on a random day of the cycle for virgin controls. All litters were also adjusted to 8 pups on Day 2. All animals received 5 mg/kg carprofen subcutaneous at 24 hrs and 48 hrs post surgery for analgesic pain relief. Animals were euthanized 8/9 days after ovariectomy, corresponding to Day 10/11 post partum in lactating animals, and tissue was collected as according to either the IHC or ISH protocol.

Caloric Restriction Model

The caloric restriction model has also been described in publication previously (True et al., 2011c). Briefly, animals were OVX+E and given analgesic treatment similar to above. Four days later animals were randomized into either the ad libitum fed control group or the 50% caloric restriction (CR) group. 50% CR was based on average food intake of ad libitum fed animals on the previous day. Food intake was measured in controls and food was given in the CR group between 0700-0800 every morning. Body weights were also measured at this time. Animals were euthanized 12 days after group assignment, with tissue collection and euthanization occurring between 0700-1000. It should be noted that 9 days after ovariectomy and estradiol implantation, animals were briefly anesthetized and silastic implants were moved to a new subcutaneous location. Implants were moved to prevent scar tissue encapsulation of the implants and impaired estradiol release which occurs with longer implantation durations.

Immunohistochemistry

For immunohistochemistry, animals were sedated with tribromoethanol and perfused transcardially with 0.9% saline followed by 4% paraformaldehye. Brains were then post-fixed in paraformaldehyde for 24 hours and cryoprotected in 25% sucrose potassium phosphate buffer for an additional 24 hours before being frozen on dry ice. Fixed tissue was cut by sliding microtome at 25 µm into a 1-in-6 series and kept in cryoprotectant at -20 ^{o}C.

Tissue was rinsed in potassium phosphate buffer and blocked in normal donkey serum prior to incubation in primary antibodies at room temperature overnight. Primary antibodies and concentrations were as follows: Rabbit anti-CART (Phoenix H-003-62, 1:500,000), rabbit anti-Kisspeptin (Millipore AB9754, 1:1000) and sheep anti-α-melanin stimulating hormone (Millipore AB5087, 1:5000). CART staining was performed first, and after overnight incubation in primary antibody at room temperature, biotinylated tyramide amplification was performed as described in Hoffman et al., (2008).

Tissue was rinsed, incubated at room temperature in donkey biotinylated anti-rabbit antibody (Jackson Immunoresearch, 711-065-152, 1:5000) for 60 minutes, A/B solution (Vector, PK-6200) for 30 minutes, biotinylated tyramide (Perkin Elmer, SAT7000) and hydrogen peroxide for twenty minutes and finally Alexa Fluor 488 Streptavidin (Invitrogen, 11223, 1:1000) for 2-3 hours at 37 ^{o}C. For additional staining, tissue was rinsed in buffer for 2 hours followed by incubation in anti-kisspeptin and anti-α-MSH antibodies overnight at room temperature, followed by rinse and one hour in Alexa Fluor 568 Anti-Rabbit (1:1000) and Donkey anti-sheep Cy5 (1:200) secondary antibodies. Tissue was mounted onto subbed slides and coverslipped using SlowFade. For triple-label CART/α-mSH/GnRH (GnRH antibody was raised in mouse, 1:5000, HU4H, a gift from Dr. Henryk Urbanski) staining in rostral AVPV sections, the same CART protocol was used except all primary antibodies were coincubated overnight at room temperature and following Streptavidin 488 incubation, tissue was washed and rinsed in a Alexa Fluor 568 Anti Mouse and Donkey anti-sheep Cy5 secondary antibodies.

The two primary antibodies raised in rabbit raised the possibility of cross-reactivity of secondary antibodies. This was avoided by titrating down the rabbit anti-CART antibody to very dilute concentrations for use with tyramide amplification. The dilution of 1:500,000 used for the CART antibody resulted in a complete absence of staining with direct secondary detection. Additionally when the entire double-label protocol was carried out with either the CART or kisspeptin antibody omitted, there was a complete lack of staining in the given fluorophore channel, suggesting that there was little cross-reactivity of these antibodies due to the sequential staining and very dilute concentration of the CART antibody used.

***In Situ* Hybridization**

For *in situ* hybridization animals were briefly sedated with isoflurane and then decapitated. Brains were rapidly removed and frozen on dry ice. Tissue was sectioned into a 1-in-3 series of 20 µM slices using a cryostat. Tissue was stored on slides at -80 ^{o}C. *In situ* hybridization was carried out as

described previously (True et al., 2011b; Xu et al., 2009b). One series of fresh frozen tissue per animal was briefly fixed in a phosphate-buffered paraformaldehyde solution and subsequently treated with 0.25% acetic anhydride in 0.1 M triethanolamine. Tissue was then taken through two washes in sodium saline citrate and dehydrated in a series of ethanol solutions, followed by delipidation in a choloroform wash. Tissue was then rehydrated through a reverse series of ethanol solutions and air-dried.

The CART probe (a gift from Drs Carol Elias and Joel Elmquist) has been described previously (Douglass et al., 1995; Elias et al., 2001). Briefly, the CART probe was transcribed using a T3 polymerase in the presence of ^{35}S. The radioactively-labeled probe was heat shocked and then diluted in hybridization buffer (50% formamide, 6.25% dextran sulphate, 0.7% Ficoll, and 0.7% polyvinylpyrolidone) and counted for final radioactive concentration. The CART-^{35}S probe was used at a concentration of 3.5 million counts per minute/ 100 µL. Slides were incubated in this diluted radioactive probe overnight in humidified chambers at 55 ^{o}C. After incubation, slides were washed in 4 X SSC, ribonuclease A at 37 ^{o}C and in 0.1 X SSC at 60 ^{o}C. Slides were then taken through a series of alcohols for dehydration. For quantification of mRNA levels, in situ hybridization slides were dipped in Kodak NTB emulsion (Eastman Kodak, Rochester, NY, USA) diluted in 600 mM ammonium acetate (Fisher Scientific Co., Pittsburgh, PA, USA) and placed in light-tight boxes containing desiccant at 4 ^{o}C. Slides were left to develop for 6 days. After development, slides were dehydrated in an alcohol series followed by washes in xylene and coverslipping with Permount.

Confocal Analysis

All immunofluorescence analysis was performed on images taken with a Leica SP5 confocal microscope with Acousto-Optical Beam Splitter (AOBS; Buffalo Grove, Illinois). For AVPV, DMH and ARH CART cell count analyses photomicrographs were taken with a 20X objective at 512 x 512 pixel resolution and at a speed of 400 Hz. For each animal CART cell counts were determined from 3 AVPV sections, 2 DMH sections, and 4 ARH sections per animal. For analysis of CART fiber close appositions to

AVPV Kiss1 and ARH Kiss1 cells photomicrographs were taken at a higher magnification 40X objection at 1024 x 1024 pixel resolution and at a speed of 700 Hz. Focal planes were 1 µM apart for this analysis and 2 AVPV, 3 ARH sections were analyzed per animal. Stacks were analyzed using ImageJ software and the Image5D plugin for easy visualization between numerous fluorophore channels in a given stack.

Silver Grain Analysis

Dark-field silver grain analysis was performed using Metamorph Imagining software. Pictures of the ARH and AVPV were taken at constant exposures, and a common threshold for silver grain detection was used for analysis at each nucleus. A separate bounding region was created for the AVPV, DMH and ARH to ensure integrated intensities were taken from the same area of tissue in all animals. Background measurements were also taken for each section, and the integrated intensity of the background was subtracted from the area of interest. The integrated intensity was averaged across 4 AVPV sections, 3 DMH sections and 9 ARH sections per animal.

Statistical Analysis

Comparisons of cell numbers and ISH integrated intensity were done by a Student's *t*-test. Group mean numbers are presented ± standard deviations.

RESULTS

Experiment 1. Differential regulation of CART populations during caloric restriction

CART protein and mRNA levels were examined by IHC and ISH, respectively, in the ARH, DMH and AVPV following a 12 day 50% CR. ARH CART cell numbers determined by IHC in 4 sections per animal were significantly lower in CR animals compared to ad libitum fed controls (cell count: ad libitum 218 ± 43, CR 139.5 ± 22, *t*-test p=0.004; Figure 1, A). ARH CART mRNA levels averaged across 9 sections per animal were significantly decreased in CR animals compared to ad libitum fed controls (integrated intensity: ad libitum 11932 ± 6623, CR 2527 ± 1286, *t*-test $p < 0.005$; Figure 1, B). In the 2 DMH sections analyzed per animal there were very few CART cells although fiber staining was abundant. There was no

significant difference in the number of DMH CART cells between ad libitum fed and CR animals (cell count: ad libitum 9 ± 4, CR 13 ± 4, *t*-test p=0.15; Figure 2, A). *In situ* hybridization also found no significant difference in DMH mRNA levels, averaged across 3 sections, although there was a trend toward decreased levels with CR (integrated intensity: Ad Libitum 3791 ± 2925, CR 1722 ± 873, *t*-test p=0.08; Figure 2, B). In the AVPV nucleus, CART fibers were abundant, but once again cells bodies were not frequently observed by immunohistochemistry (Figure 3, A). AVPV CART cell numbers counted in 2 sections per animal were not significantly different between ad libitum fed and CR animals (cell count: ad libitum 17.6 ± 4.5, CR 15.33 ±5, *t*-test p=0.45). *In situ* hybridization found that AVPV CART mRNA levels averaged across 4 sections per animal were significantly lower in CR animals compared to ad libitum fed controls (integrated intensity: ad libitum 1262±756, CR 281.3±300, *t*-test p=0.04; Figure 3, B).

Experiment 2. Differential regulation of CART populations during lactation

In the ARH nucleus there was a trend toward decreased CART cell numbers with lactation, but this was not significantly different from virgin controls (cell count: virgin 151.7 ± 25, lactation 130.3 ± 10, *t*-test p=0.08; Figure 4, A). Similarly, *in situ* hybridization found no significant difference in ARH CART mRNA levels during lactation (integrated intensity: virgin 6439 ± 4517, lactation 5044 ± 3325, *t*-test p=0.55; Figure 4, B). There was also no significant difference in the number of CART cells in the DMH between virgin and lactating animals (cell count: virgin 7 ± 8, lactation 5.7 ± 3, *t*-test p=0.72; Figure 5, A). Interestingly, there was a trend toward decreased CART mRNA levels in the DMH during lactation, but this did not reach statistical significance (integrated intensity: virgin 1243 ± 840, lactation 3210 ± 2325, *t*-test p=0.06; Figure 5, B). Immunohistochemistry for AVPV CART cell numbers revealed a dramatic greater-than three-fold increase in the number of CART-ir cells during lactation compared to virgin controls (cell count: virgin 43.5 ± 15, lactation 145.3 ± 46, *t*-test p<0.001; Figure 6, A). This increase in CART protein was accompanied by a decrease in AVPV CART mRNA levels during lactation, although this did not reach statistical significance (integrated intensity: virgin 672.6 ± 445, lactation 381.2 ± 195, *t*-test

p=0.17; Figure 6, B). The dramatic increase in AVPV CART IHC and decrease in mRNA during lactation is similar to previous results reported for AVPV Kiss1 during lactation (True et al., 2011b); therefore, to determine if the two neuropeptides were coexpressed in the same cells of the AVPV double-label immunohistochemistry was performed. Notably AVPV CART and Kiss1 showed no colocalization of cell bodies during lactation (Figure 7) or control conditions (data not shown), with the most abundant CART cells being located in the most rostral sections of the AVPV, while Kiss1 cells were concentrated in more caudal sections containing the periventricular region (Figure 7).

Experiment 3. Investigation of CART fibers near GnRH and Kiss1 populations

To determine whether ARH CART cells may be capable of directly regulating GnRH release, triple-label immunohistochemistry was used to determine if CART/αMSH-ir fibers are in close contact with GnRH cells. Indeed coexpressing CART/α-MSH fibers make close appositions onto 50% of GnRH cells found in sections just proceeding the beginning of the AVPV and at the level of the most rostral AVPV sections, pertaining to the regions of the medial septum and the nucleus of the diagonal band (Figure 8). While Given CART and POMC, the α-MSH precursor protein, are only colocalized in the ARH nucleus, this suggests that a portion of CART fibers contacting GnRH cells likely originate in this nucleus. A similar triple-label IHC protocol was used to determine whether CART fibers make any contact with Kiss1 populations. Single-labeled CART-ir fibers were observed to make close appositions to 35% of AVPV Kiss1 cells. In the ARH nucleus a similar analysis found that roughly 65% of Kiss1 cells were also found to have frequent close appositions from CART cells. Triple label IHC revealed that a large proportion of CART fibers contacting ARH and AVPV Kiss1 cells coexpress α-MSH, with 30% of AVPV Kiss1 cells having close appositions from CART/α-MSH fibers while 55% of ARH Kiss1 had similar close appositions (Figure 8). Single-labeled CART-ir fibers were also found to make close appositions to Kiss1-ir cells, although this was infrequent compared to double-labeled CART/α-MSH-ir contacts.

DISCUSSION

The current results indicate that CART may be an important regulator of GnRH neurons and potentially be involved in the metabolic regulation of the neuroendocrine hypothalamic-pituitary-gonadal axis. Consistent with this hypothesis, ARH and AVPV CART populations were both differentially regulated during CR. Particularly mRNA levels were significantly decreased in the ARH and AVPV CART populations, leading to the hypothesis that inhibition of this GnRH-stimulating signal during negative metabolic states might be a factor contributing to decreased GnRH release and subsequent acyclicity. In addition to direct effects on GnRH, we have also provided the first morphological evidence that CART might also regulate Kiss1 populations, and thus indirectly contribute to GnRH regulation as well.

Previous work has demonstrated that CART fibers make close appositions to GnRH neurons in the rat (Rondini et al., 2004) and hamster (Leslie et al., 2001). Tracing studies have further determined that these CART fibers may have several different origins, since retrograde labeling out of the POA labeled CART cells in the ventral premammillary nucleus, dorsomedial hypothalamus, the ARH and the AVPV (Rondini et al., 2004). Given the current evidence that both ARH and AVPV CART populations appear to be inhibited with CR, this might result in a decrease in stimulatory tone directly at GnRH neurons, and contribute to the known suppression of pulsatile GnRH release during negative energy balance. It appears that the role of CART in the DMH is limited during negative energy balance-induced GnRH inhibition since no significant changes in protein or mRNA was noted in either CR or lactation.

Despite significant suppression of both ARH and AVPV CART mRNA expression with CR, these populations were relatively unaffected in the lactation model of negative energy balance. This may indicate that CART inhibition is not common to all models of negative energy balance. Another possibility is that perhaps CART is only inhibited in conditions of undernutrition and not conditions of excessive energy expenditure, as experienced during lactation. Consistent with this hypothesis, previous results observed an inhibition of ARH CART mRNA with a short term fast, indicating that inhibition of this

population is common to at least two models of undernutrition (current study and Kristensen et al., 1998). While lactation offers the benefit of a naturally occurring, and thus physiologically relevant model of negative energy balance, there are a myriad of other physiological changes that occur during lactation not related to the negative metabolic condition. In addition to the negative energy balance, it is also thought that the suckling stimulus of lactation contributes to GnRH inhibition during lactation, given previous results that suckling pups in the absence of milk production maintain lowered LH levels (Brogan et al., 1999). An alternative explanation for unaltered CART levels during lactation then may be that inhibition of CART is not necessary during lactation given the multitude of other lactation-specific signals potentially contributing to GnRH inhibition.

The only affected change noted in the CART system during lactation was an increase in AVPV CART cell numbers during lactation. This protein accumulation in AVPV CART cells during lactation is strikingly similar to results observed in AVPV Kiss1 cells during lactation (True et al., 2011b). While AVPV CART mRNA was not significantly decreased during lactation, as has been reported for AVPV *Kiss1*, it is clear that increases in mRNA production do not account for increased AVPV CART cell numbers. We hypothesize that like AVPV Kiss1 cells, AVPV CART cells might have inhibited protein release during lactation, leading to accumulation of protein in cell bodies (True et al., 2011b). Importantly, this might indicate there are two different modes by which AVPV CART cells are inhibited: one in which mRNA is decreased but protein levels are unchanged and another where mRNA is unaffected but protein release is inhibited leading to a build-up in the soma. The physiological significance of two distinct modes of inhibition is unclear, but perhaps the accumulation of protein represents a more abrupt cessation of protein release followed by a delayed and more modest decrease in mRNA production during lactation. As mentioned above, the suckling stimulus of lactation has been shown to contribute to GnRH inhibition. Somatosensory signals directly activated by suckling are thought to result in activation of many brainstem neuronal populations that then project to many other areas of the brain including the

hypothalamus (Li et al., 1998; 1999a; c). It is tempting then to speculate that during lactation, inhibition of AVPV CART protein release occurs abruptly by mostly neural cues derived from the suckling stimulus, while inhibition during CR relies on longer term changes in hormones like leptin and transcriptional inhibition of CART mRNA production. Future work will be needed to address this hypothesis, but the current evidence highlights an importance of the AVPV nucleus in lactation, given two populations have now been shown to have increased protein accumulation with a lack of increased mRNA in this nucleus.

Inhibition of AVPV CART mRNA levels observed during CR indicates inhibition of these cells, although it is unclear what functional significance this may have when protein levels are unaltered. The disagreement between protein and mRNA in the current study might be due to differences in sensitivity of the detection methods. Silver grain analysis of integrated intensity is a more sensitive method of detection for mRNA than immunoreactive cell counts for IHC data. In addition, AVPV CART cell numbers were noticeably lower than would be expected from mRNA levels, indicating AVPV CART protein may be transported and released too quickly for sufficient accumulation in cell bodies for detection by IHC. Similar results are frequently observed with the ARH neuropeptide Y (NPY) and AVPV Kiss1 populations in the rat, in which cell bodies are rarely observed by IHC without colchicine treatment, despite high mRNA levels (Pelletier et al., 1984). Interestingly, both CART and Kiss1 cells are easily detected by IHC in the ARH nucleus, despite few immunoreactive cells observed in the AVPV, indicating perhaps a similar physiologic role of these neuropeptides across the hypothalamus. This discrepancy of CART IHC protein build up between the ARH and AVPV is also potentially reflective of a different mechanism or speed of protein release. This hypothesis is consistent with different effects of negative energy balance on the two populations observed in the current study.

Experiments investigating the upstream mechanism for CART inhibition during negative energy balance, specifically the metabolic hormones or neural cues causing this decrease in mRNA, will be an important next step in understanding metabolic regulation of reproduction. One metabolic cue known

to regulate the ARH CART population is the adipocyte hormone leptin. CART cells in the ARH, as well as the ventral premammillary nucleus and DMH, express the long form of the leptin receptor and are activated by leptin administration as demonstrated by c-Fos labeling (Elias et al., 2000; Elias et al., 2001). ARH CART levels are also low in leptin-deficient ob/ob mice and exogenous leptin can normalize CART levels (Kristensen et al., 1998). Serum leptin levels are decreased by negative energy balance, including the CR model (True et al., 2011c). Therefore, it is conceivable that hypoleptinemia during negative energy balance contributes to CART inhibition. Consistent with this hypothesis, low leptin is also thought to contribute to Kiss1 and GnRH inhibition during negative energy balance. However, recent evidence in the CR model found that restoring leptin to normal physiological levels at the end of CR could not restore Kiss1 mRNA or serum LH levels, suggesting hypoleptinemia is not the only critical signal for inhibition of reproduction (True et al., 2011c). Future work is needed to determine what if any effect restoring leptin to physiological levels has to restore ARH and AVPV CART levels during CR. Interestingly, CART cells in the AVPV do not appear to express leptin receptors, so inhibition of these cells is likely transmitted indirectly or by an alternative signal.

The current study provides evidence that the ARH CART population coexpressing POMC appears to send direct projections to GnRH cells as well as both hypothalamic Kiss1 populations. The former finding is consistent with tracing studies in which ARH CART cells were found to make projections to the area of GnRH neurons (Rondini et al., 2004); however, this is the first study to demonstrate that these ARH CART fibers make close appositions to GnRH neurons in this area. In addition to CART's ability to directly regulate GnRH release, it was also investigated whether CART might regulate the powerful upstream GnRH-secretagogue Kiss1. The current study finds evidence that CART cells may in fact regulate both Kiss1 populations, based on the observation of CART fibers in close apposition to Kiss1 cells. Electron microscopy will be needed to verify whether CART fibers indeed make synaptic contact onto Kiss1 cells. In addition, electrophysiological recordings from newly created Kiss1-Cre mice may be

used to determine if CART has any direct post-synaptic effects on Kiss1 neuronal excitability. The current results demonstrate that colocalized CART/α-MSH fibers account for the majority of CART contacts on to Kiss1 cells in the AVPV and ARH nucleus. This result was somewhat surprising, particularly for the AVPV Kiss1 population given its close proximity to AVPV CART cells, which do not express POMC. However, anatomical organization demonstrated that the densest and most easily observed Kiss1 cells, and those used in contact analysis, are located more dorsal and caudal to the concentration of AVPV CART cells.

An interesting finding was a large quantity of single-labeled α-MSH in both the ARH and AVPV. One potential explanation is that these fibers originate from the nucleus of the solitary tract (NTS) POMC population, which to date has not been shown to coexpress CART (reviewed in Cone, 2005). However, two previous studies looking at knife-cut deafferentation as well as excitotoxic lesion of ARH POMC cells indicated that projections from NTS POMC cells to the hypothalamus are minimal (Joseph and Michael, 1988; Pilcher and Joseph, 1986). Another possible source of single-labeled α-MSH cells is the roughly 10% of ARH POMC cells which do not coexpress CART (Elias et al., 1998). Technically it must also be considered that although CART and POMC are coexpressed in the soma, this may not always be reflected in fiber projections from these cells. This could possibly be due to differential transport of the neuropeptides down the axon or perhaps differences in antibody sensitivities, potentially leading to false negatives in which ARH CART/α-MSH fibers appear single-labeled and thus were not counted in analysis. Therefore, while it is clear that double-label CART/α-MSH fibers do frequently make close appositions to ARH and AVPV Kiss1 cells, as well as to GnRH neurons, the percentages of contacts presented here may be an underestimate. The high number of cells that were observed to have CART/α-MSH-ir close appositions in the current study indicates the important regulatory role for the ARH CART population in reproductive regulation.

It is possible that CART indirectly regulates GnRH neurons by modulating Kiss1 release, but there may also be interactions of Kiss1 and CART postsynaptically at GnRH cells (Figure 10). Future work in which CART and Kiss1 are applied together could begin to determine if there are any synergistic depolarizing effects at GnRH cells. Importantly, the inhibition of both CART and Kiss1 levels during CR could result in a dramatic decrease in stimulatory tone to GnRH cells and be a large component of the known inhibition of pulsatile GnRH release during negative energy balance (Figure 10). Overall the current study provides compelling evidence that CART may be an important component of reproductive neuroendocrine circuits. In particular, given CART's known role in metabolic regulation and the observed inhibition of specific CART populations with negative energy balance, it appears that CART may be an important signal for relaying information regarding negative metabolic states to both Kiss1 and GnRH neural populations.

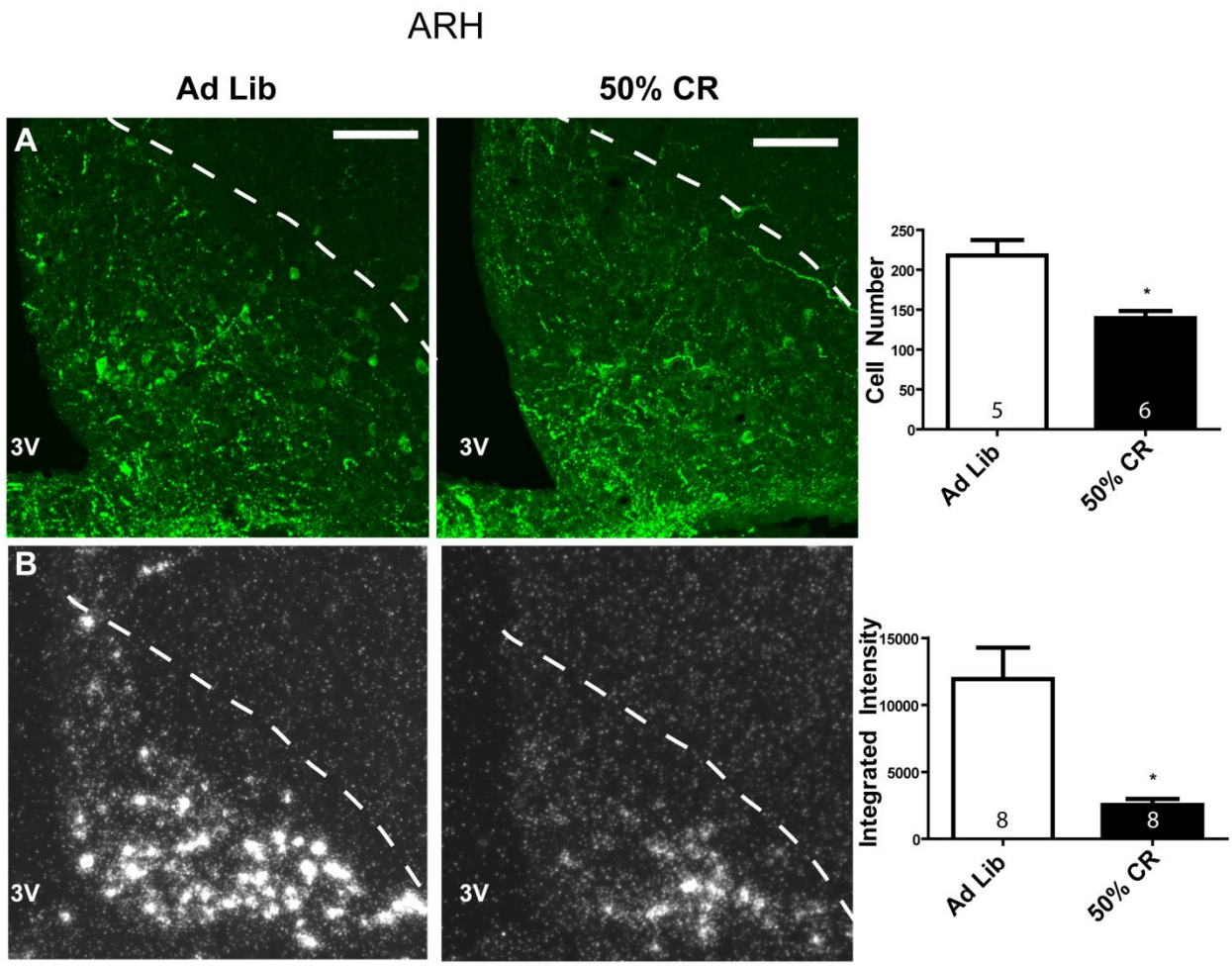


Figure 1. Arcuate nucleus CART protein and mRNA levels during caloric restriction (CR).
A) Confocal photomicrograph of immunohistochemistry for CART protein (Phoenix antibody, H-003-62) in the arcuate nucleus (ARH, location denoted by dotted line) in ab libitum fed and 50% CR animals (100 µM scale bar provided). Quantification of ARH CART cell numbers is provided in the bar graph. B) Dark field silver grain photomicrograph of *in situ* hybridization for ^{35}S-CART mRNA probe. Quantification of ARH CART mRNA integrated intensities provided in the histogram. Sample group size is given within eachnhistogram. 3V abbreviation for third ventricle.

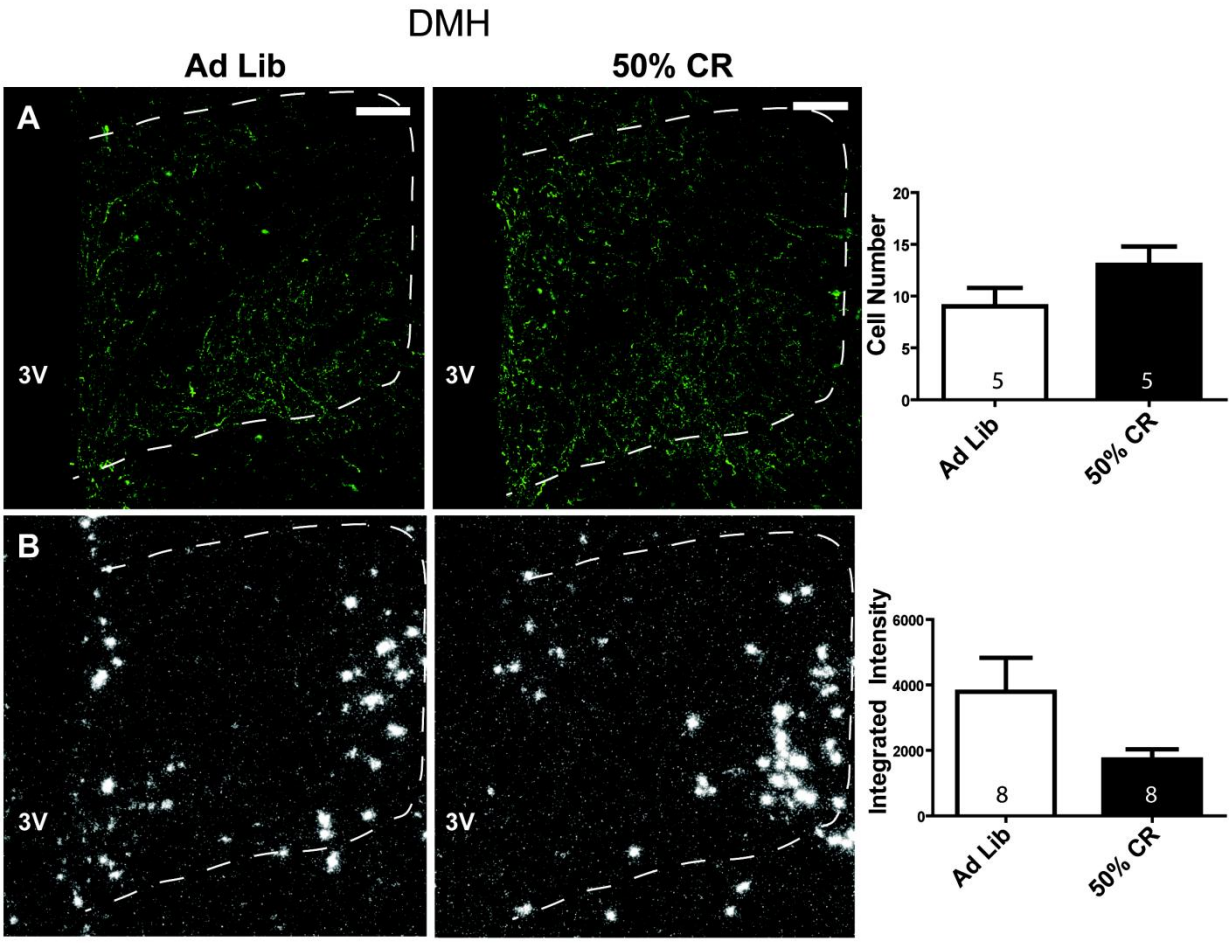


Figure 2. Dorsomedial hypothalamus CART protein and mRNA levels during caloric restriction (CR). A) Confocal photomicrograph of immunohistochemistry for CART protein in the dorsomedial hypothalamus (DMH, location denoted by dotted line) in ab libitum fed and 50% CR animals (100 µM scale bar provided). Quantification of DMH CART cell numbers is provided in the bar graph. B) Dark field silver grain photomicrograph of *in situ* hybridization for ^{35}S-CART mRNA probe. Quantification of DMH CART mRNA integrated intensities provided in the histogram. Sample group size is given within each histogram. 3V abbreviation for third ventricle.

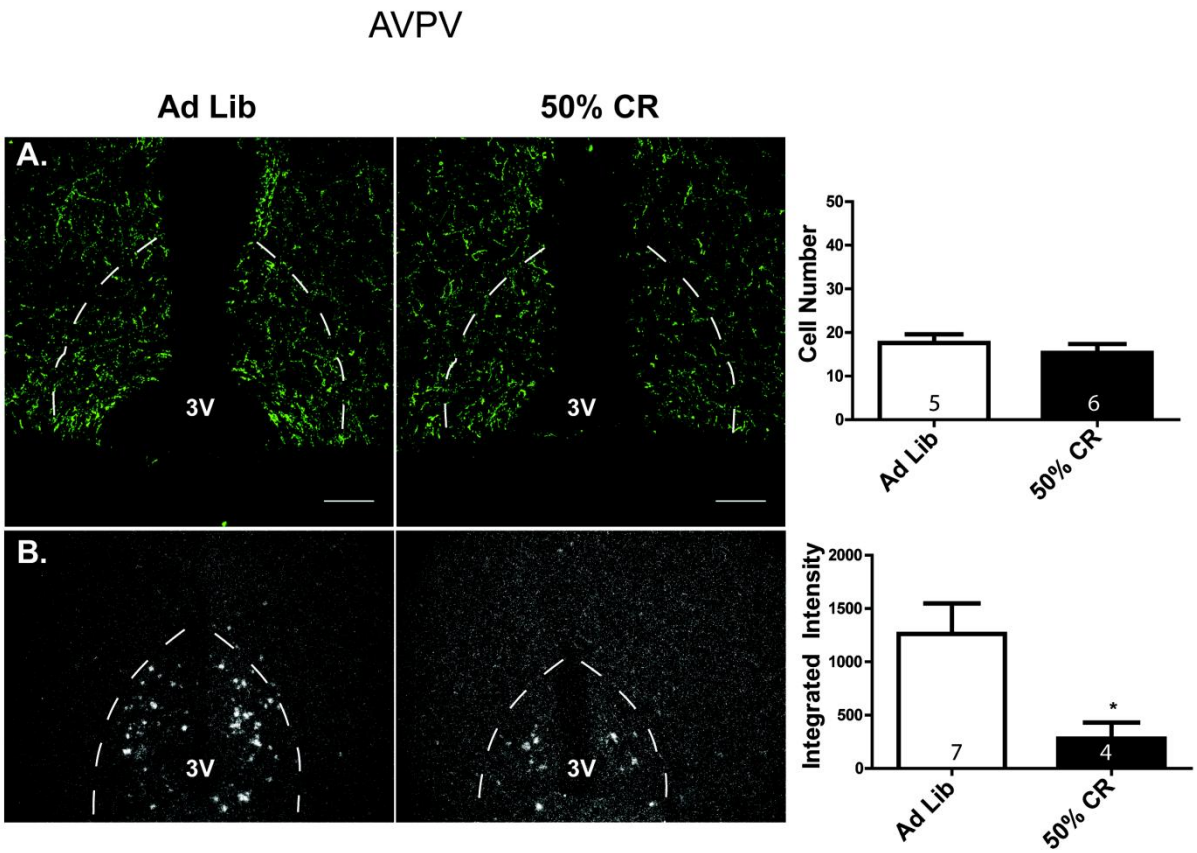


Figure 3. Anteroventral periventricular nucleus CART protein and mRNA levels during caloric restriction (CR).

A) Confocal photomicrograph of immunohistochemistry for CART protein in the AVPV (location denoted by dotted line) in ab libitum fed and 50% CR animals (100 µM scale bar provided). Quantification of AVPV CART cell numbers is provided in the bar graph. B) Dark field silver grain photomicrograph of *in situ* hybridization for ^{35}S-CART mRNA probe. Quantification of AVPV CART mRNA integrated intensities provided in the histogram. Sample group size is given within each histogram. Abbreviations 3V, third ventricle and OC, optic chiasm.

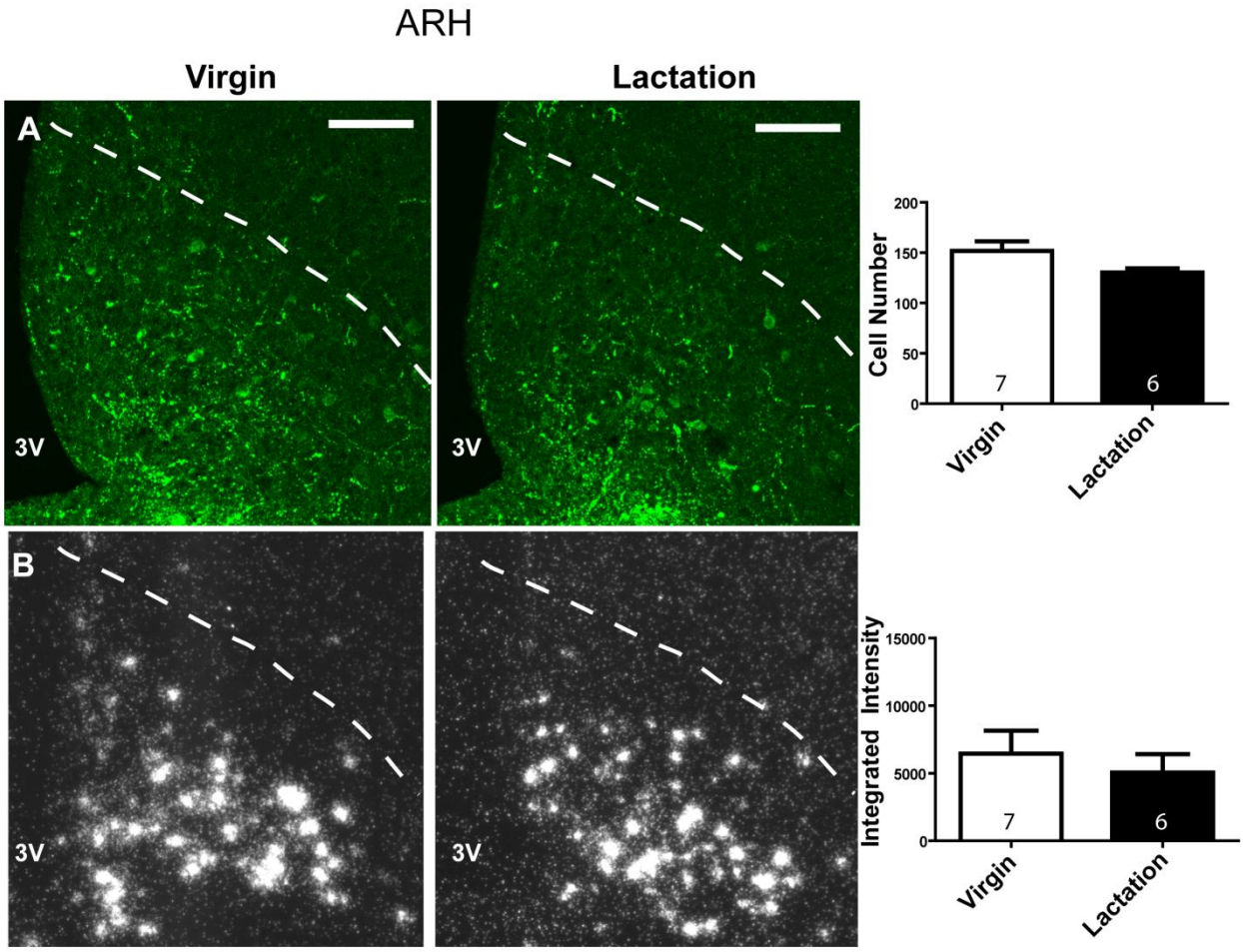


Figure 4. Arcuate nucleus CART protein and mRNA levels during lactation.
A) Confocal photomicrograph of immunohistochemistry for CART protein in the ARH (location denoted by dotted line) during virgin control and lactation conditions (100 µM scale bar provided). Quantification of ARH CART cell numbers is provided in the bar graph. B) Dark field silver grain photomicrograph of in situ hybridization for 35S-CART mRNA probe. Quantification of ARH CART mRNA integrated intensities provided in the histogram. Sample group size is given within each histogram. 3V abbreviation for third ventricle.

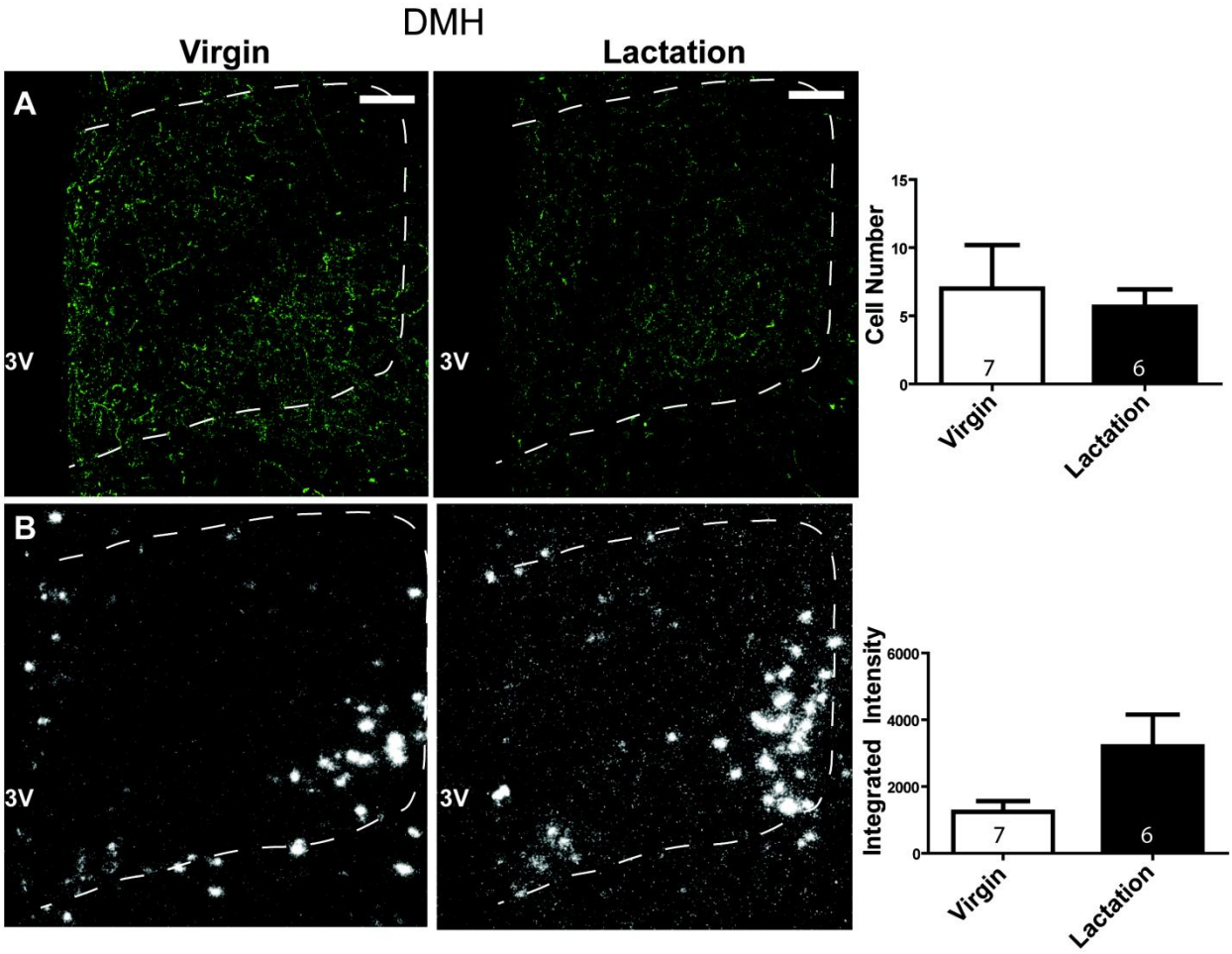


Figure 5. Dorsomedial hypothalamus nucleus CART protein and mRNA levels during lactation.

A) Confocal photomicrograph of immunohistochemistry for CART protein in the DMH (location denoted by dotted line) during virgin control and lactation conditions (100 µM scale bar provided). Quantification of DMH CART cell numbers is provided in the bar graph. B) Dark field silver grain photomicrograph of in situ hybridization for 35S-CART mRNA probe. Quantification of DMH CART mRNA integrated intensities provided in the histogram. Sample group size is given within each histogram. 3V abbreviation for third ventricle.

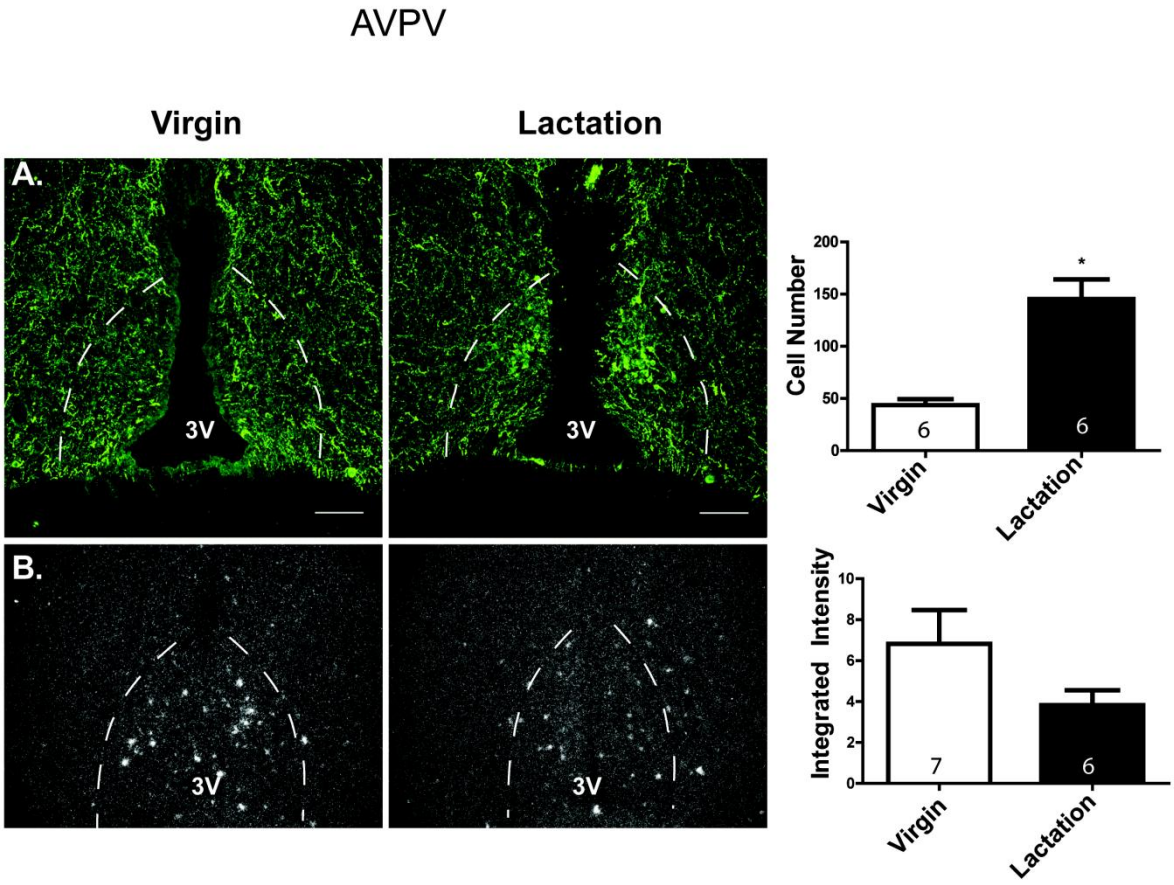


Figure 6. Anteroventral periventricular nucleus CART protein and mRNA levels during lactation.
A) Confocal photomicrograph of immunohistochemistry for CART protein in the AVPV (location denoted by dotted line) during virgin control and lactation conditions (100 µM scale bar provided). Quantification of AVPV CART cell numbers is provided in the bar graph. B) Dark field silver grain photomicrograph of *in situ* hybridization for ^{35}S-CART mRNA probe. Quantification of AVPV CART mRNA integrated intensities provided in the histogram. Sample group size is given within each histogram. 3V abbreviation for third ventricle.

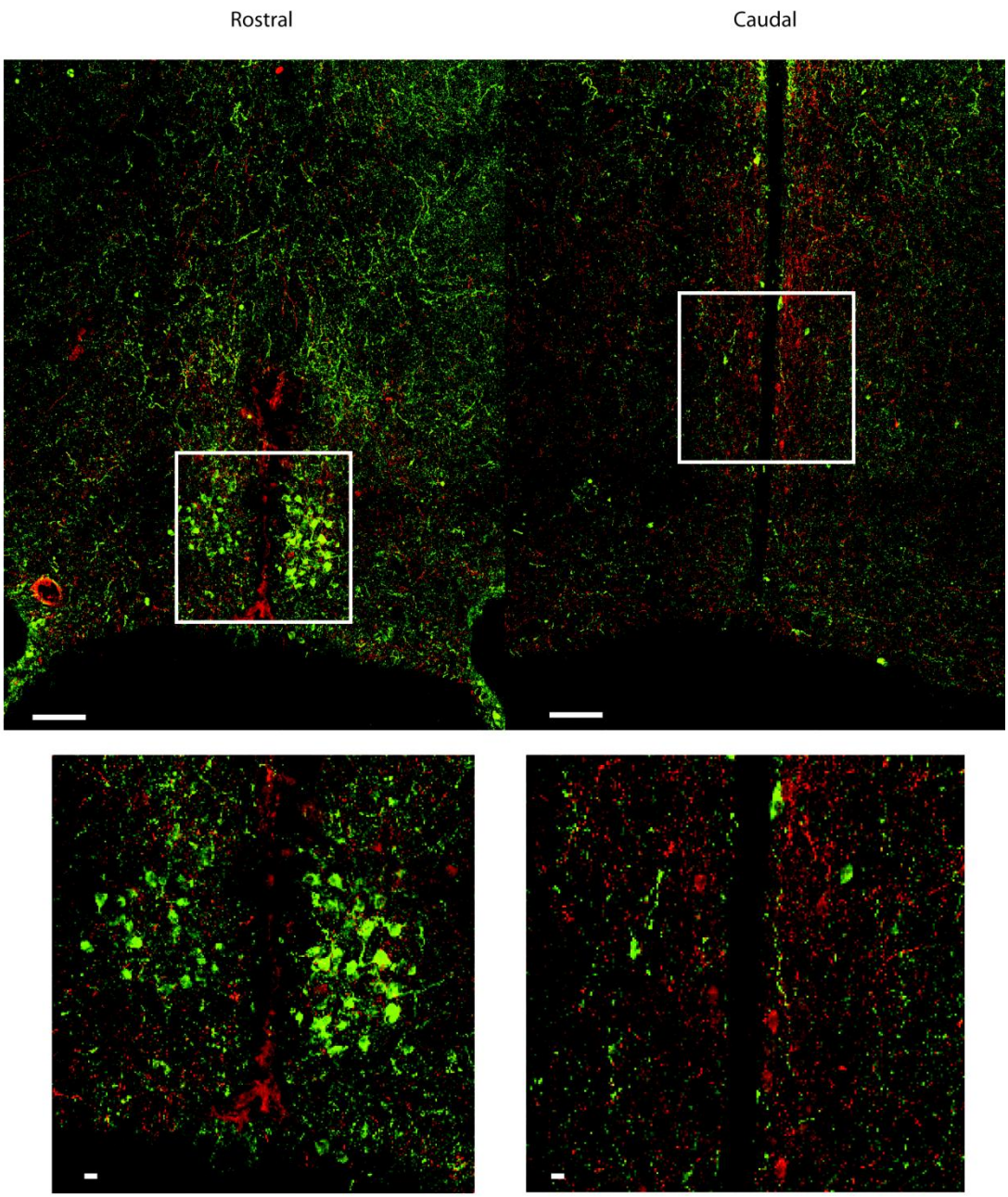


Figure 7. Distribution of CART and Kiss1 cells in the periventricular region and AVPV during lactation. Photomicrographs of immunohistochemistry for CART-ir (green) and Kiss1-ir (red) in the rostral AVPV (left column) and slightly caudal periventricular region (right column) at low magnification (top panel). White boxes represent approximate location of high magnification insets (bottom panel). 100 µM scale bars are given for low magnification images, 10 µM scale bars are given for high magnification images.

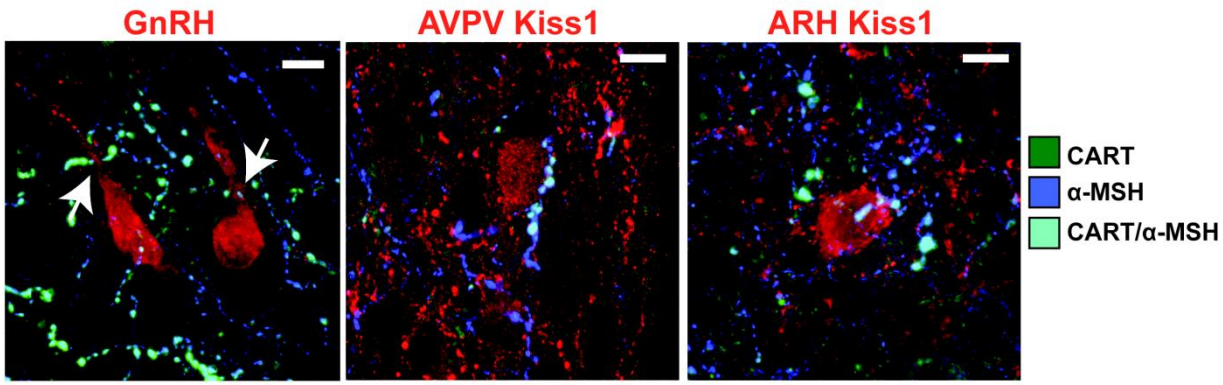


Figure 8. CART close appositions onto GnRH cells near the AVPV and ARH and AVPV Kiss1 cells. Photomicrograph of triple-label immunohistochemistry for CART (green), α-MSH (blue) and either GnRH (red, left panel) or Kiss1 (middle and right panel, red) immunoreactivity. Coexpressing CART/α-MSH (cyan) fibers were found in close apposition to GnRH-ir cells near the AVPV (left panel, close appositions marked with white arrows) as well as AVPV Kiss1-ir cells (middle) and ARH Kiss1-ir cells (right panel, red). Confocal single optical slice analysis at 1 µM focal planes was used to determine if CART/α-MSH fibers made close appositions Kiss1 cells in the same focal plane. 10 µM scale bars are given.

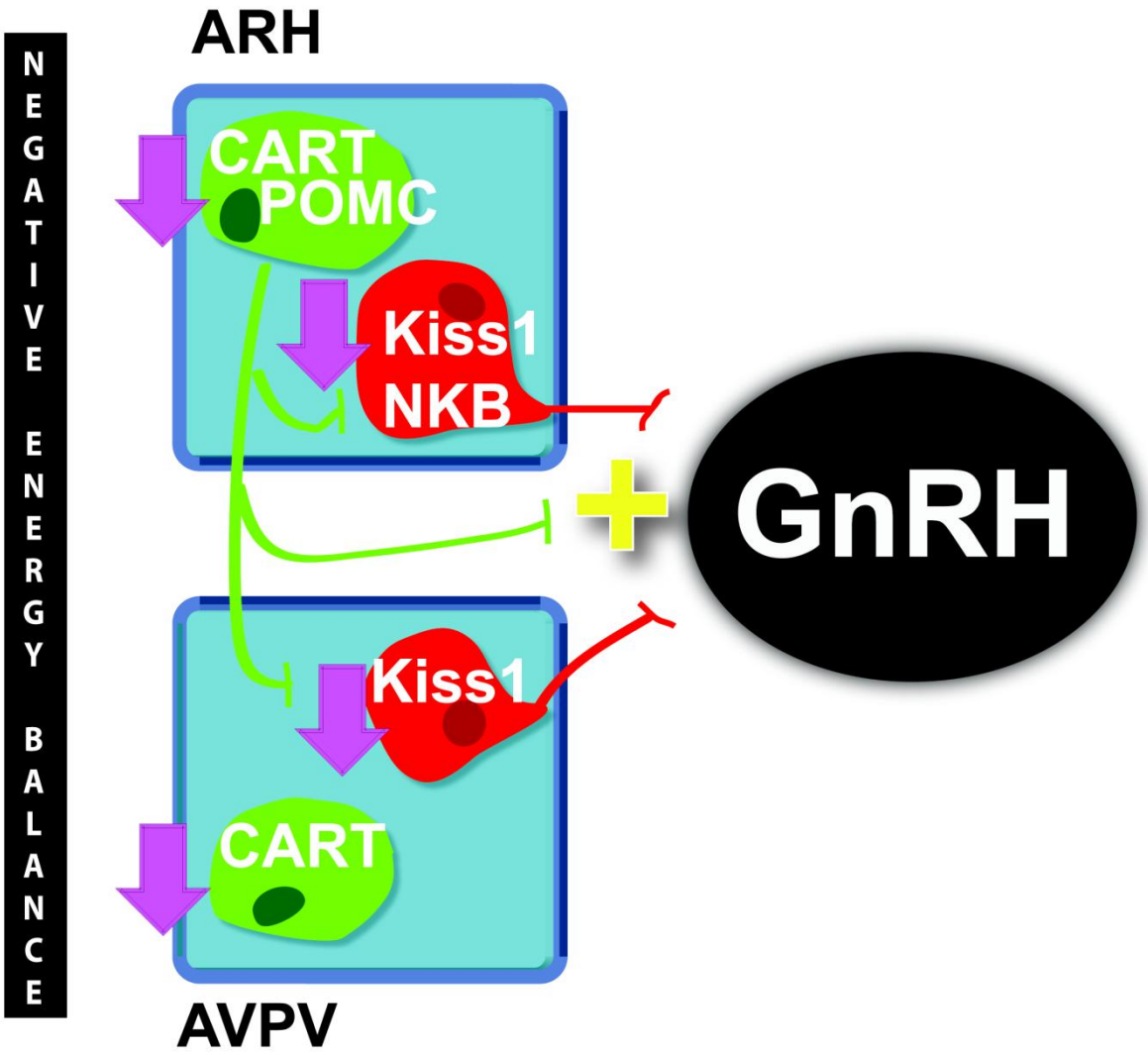


Figure 9. Working model of the role of CART and Kiss1 for negative energy balance-induced GnRH inhibition.
CART populations (green) in the ARH and AVPV nucleus are inhibited (red downward arrow) with CR similar to previous results for ARH and AVPV Kiss1 mRNA levels. ARH CART fibers appear to send direct projections to ARH and AVPV Kiss1 wells as well as GnRH neurons directly. Given the stimulatory role of CART and Kiss1 for GnRH excitability, inhibition of these populations during CR might be a contributing factor in inhibited pulstaile GnRH release that is the hallmark of negative energy balance.

Chapter 5- DISCUSSION

Pages 130-144 of this discussion are published as:

True C, Grove K, Smith MS. 2011. Beyond leptin: emerging candidates for the integration of metabolic and reproductive function during negative energy balance. Frontiers in Endocrinology 2.

The current study examined the role of two hormonal signals and two neuronal populations in mediating metabolic regulation of GnRH release from the hypothalamus. We provide evidence that circulating levels of estradiol appear required for negative energy balance-induced GnRH inhibition, perhaps by dampening certain GnRH stimulatory signals. These conclusions are based on findings that in OVX animals without circulating estradiol, similar weight loss to OVX+E animals does not produce the same degree of inhibition of LH levels. In addition, hypoleptinemia is not the only critical signal for GnRH inhibition during negative energy balance, since restoration of leptin to values within the physiological range could not restore LH levels. Leptin could also not restore Kiss1 levels, a neuropeptide that we observe to be consistently inhibited during negative metabolic conditions, particularly the ARH Kiss1 population. Finally, the current work indicates that CART likely plays an important role in reproductive regulation, and particularly inhibition of both CART and Kiss1 during negative energy balance might result in decreased stimulatory tone at GnRH cells, contributing to the observed anovulatory state.

1. Beyond leptin: emerging candidates for the integration of metabolic and reproductive function

Metabolic status is a known regulator of reproductive function, with both over- and under-nutrition resulting in reproductive dysfunction. In female mammals, this is frequently observed as a disruption of reproductive cycling leading to anovulation. Despite years of intense study in this field, many key questions remain unanswered in our understanding of how changes in metabolic status result in disruption of the hypothalamic-pituitary-gonadal axis. One well-studied candidate for the integration of metabolic and reproductive function is the adipocyte hormone leptin (for reviews see Bluher and Mantzoros, 2007; Clarke and Henry, 1999; Cunningham et al., 1999; Hill et al., 2008; Tena-Sempere, 2007). Circulating leptin levels directly correlate to adipose stores and are sensitive to changes in

metabolic status, making it an ideal candidate to signal changes in energy balance to central and peripheral systems (Blache et al., 2000; Maffei et al., 1995). Within the hypothalamic-pituitary-gonadal axis, leptin acts to stimulate GnRH release through an intermediate cell population, rather than through direct actions on GnRH neurons (Quennell et al., 2009; Watanobe, 2002; Yu et al., 1997). The candidates for this intermediate cell population include arcuate nucleus kisspeptin cells (discussed below) and also glutamate cells in the ventral premammillary nucleus. The latter population has only been described recently for its role in leptin's reproductive regulation, based on the high number of cells expressing leptin receptors in this area, and direct projections from these cells to GnRH neurons (Leshan et al., 2009; Louis et al., 2011; Patterson et al., 2011). In addition to the morphological evidence, *in vivo* studies found that lesions to the ventral premammilary nucleus prevented leptin-induced LH stimulation (Donato et al., 2011; Donato et al., 2009). Given these results, glumate cells in the ventral premammillary nucleus are clearly exciting new candidates in understanding leptin's role in reproduction.

A large portion of research has focused on the regulatory influence of leptin for the initiation of puberty. There is evidence of a developmental increase in leptin levels between postnatal day 20 and 40 in the rat, the latter date corresponding to vaginal opening and followed soon after by the first estrus cycle (Gruaz et al., 1998). Similar results have been found in humans, suggesting increases in leptin signaling are necessary to stimulate normal pubertal development (Mantzoros et al., 1997). There is an abundance of data to support this hypothesis, most notably the evidence that leptin deficient ob/ob mice do not undergo puberty and are infertile, a phenotype rescued with exogenous leptin treatment (Barash et al., 1996; Chehab et al., 1996; Ingalls et al., 1950; Swerdloff et al., 1976). Zucker Fatty Rats lacking a functional leptin receptor also have delayed pubertal development and reduced LH levels (Phillips et al., 1996; Saiduddin et al., 1973; Todd et al., 2003; Zucker and Zucker, 1961). These transgenic studies recapitulate findings in humans in which genetic mutations in the leptin signaling

system have been reported to result in both dramatically delayed and absent pubertal development (Clement et al., 1998; Strobel et al., 1998). In addition, overexpression of leptin, or exogenous leptin treatment of wild-type mice results in early onset of puberty (Ahima et al., 1997; Chehab et al., 1997). Given this evidence it is clear that leptin plays a critical role in signaling sufficient metabolic energy stores required for the initiation of GnRH release and puberty in rodents and humans. Notably, puberty in the non-human primate is not proceeded by a rise in circulating leptin, suggesting other signals are responsible for the initiation of puberty in this species (Plant and Durrant, 1997).

1.2 Negative energy balance-induced acyclicity and hypoleptinemia

To understand the role of leptin for the integration of energy balance and reproductive function, investigators have relied in part on animal models of negative energy balance, where energy output exceeds energy input. Negative energy balance-induced reproductive acyclicity is a highly conserved phenomenon, present in all female mammals investigated to date. It is well understood that the halting of ovarian cycling in this case likely occurs through inhibition of GnRH release from the hypothalamus, since exogenous GnRH rescues cyclic reproductive function (Aloi et al., 1997; Bergendahl et al., 1991; Bronson, 1986; Cameron and Nosbisch, 1991; Kile et al., 1991). Given the proposed stimulatory role of leptin in GnRH release, the prevailing hypothesis in the field was that reproductive dysfunction during negative energy balance occurs due to hypoleptinemia and thus a decrease in stimulatory drive for GnRH release. This hypothesis is supported by multiple studies demonstrating that exogenous leptin treatment during fasting models of negative energy balance stimulates GnRH release as measured by circulating LH levels (Ahima et al., 1996; Nagatani et al., 1998; Nagatani et al., 2000). However, these studies used pharmacological doses of leptin that resulted in levels around 50-fold higher than normal circulating levels (Ahima et al., 1996). Even with pharmacological doses of leptin replacement, Ahima et al. (1996) found only partial restoration of LH levels, demonstrating that a continued inhibitory source

for GnRH release was still present. This latter finding indicates that while hypoleptinemia may play a role in suppression of GnRH during negative energy balance in rodents, other players are also involved.

Recent research suggests that metabolic signaling beyond leptin is also critical for the reversal of GnRH inhibition upon exit from negative energy balance back to a normal metabolic state. Previous research from our lab examined the effects of restoring leptin to physiological levels during lactation, a naturally occurring condition of negative energy balance in which the energy requirement for milk production exceeds energy intake. Restoring leptin during mid-lactation had no effect to restore LH levels (Xu et al., 2009b). However, it could be argued that lactation is a complicated physiological model, with other known sources of GnRH inhibition (Brogan et al., 1999); therefore, it is possible that leptin's effects were masked by continued inhibitory inputs specific to lactation (Li et al., 1999c). To test this hypothesis a long-term caloric restriction (CR) model was developed to mimic the duration and intensity of lactation-induced negative energy balance, but once again restoration of leptin to physiological levels did not normalize mean LH levels (True et al., 2011d). This data, presented in Chapter 3, was in direct contrast to previous results; however, these earlier studies used a short-term fasting model of negative energy balance and also employed much higher pharmacological levels of leptin replacement (Ahima et al., 1996; Nagatani et al., 1998). To determine whether these discrepancies were due to the different models of negative energy balance or to the dose of leptin, leptin was replaced in a 48-hr fasting model to both physiological and pharmacological levels. Despite partial normalization of LH levels with the pharmacological dose of leptin, consistent with previous results (Ahima et al., 1996; Nagatani et al., 1998), restoration of physiological levels of leptin had no effect on fasting-suppressed LH levels (True et al., 2011d). Additional research arguing against a critical role for leptin in the restoration of LH upon exit from negative energy balance comes from the lean ewe model. Szymanski et al. (2007) demonstrated that when lean acyclic ewes are refed, LH levels rise quickly, and importantly this increase occurs prior to any increase in circulating leptin levels. Together,

these studies confirm that while hypoleptinemia may play a permissive role in negative energy balance-induced reproductive dysfunction it does not appear to be a critical trigger for GnRH inhibition (Smith et al., 2010b). It should be noted that long-term treatment with high physiological levels of leptin is more effective at restoring LH levels in humans, pointing to important species differences in this field (Welt et al., 2004). In the rodent many other candidates involved in the integration of metabolic and reproductive function exist, and there are many reviews on the role of appetitive hormones and hypothalamic peptides in this process (Fernandez-Fernandez et al., 2006; Garcia et al., 2007; Hill et al., 2008; Kalra and Kalra, 1996; Schioth and Watanobe, 2002; Smith and Grove, 2002; Smith et al., 2010b; Tena-Sempere, 2008). More recent candidates have emerged in the metabolic and reproductive fields that may play a role in negative energy balance-induced GnRH inhibition.

1.3 Inhibition of kisspeptin as a central mechanism of GnRH inhibition

It would be remiss to discuss regulation of GnRH release without mention of the neuropeptide Kiss1. Work presented here indicates that Kiss1 is regulated by metabolic conditions suggesting it may also be important for negative energy balance-induced GnRH suppression. As presented in Chapters 2 and 3, both lactating and CR models result in suppression of Kiss1 levels in both the ARH and AVPV (True et al., 2011d; Xu et al., 2009b; Yamada et al., 2007). While studies reporting the effect of fasting on Kiss1 levels have been inconsistent, there are reports of inhibition in both nuclei in this model as well (Backholer et al., 2010; Castellano et al., 2005; Forbes et al., 2009; Luque et al., 2007; True et al., 2011c). These results suggest that inhibition of GnRH release during negative energy balance occurs upstream at Kiss1 populations, which in turn results in decreased stimulatory drive for GnRH (Castellano et al., 2010; Castellano et al., 2009; Hill et al., 2008; Roa et al., 2008a).

Surprisingly, very little is known about afferent signals that regulate AVPV and ARH Kiss1 cells. Numerous studies provide evidence for a stimulatory role of leptin for Kiss1 expression (Backholer et al., 2010; Castellano et al., 2010; Castellano et al., 2006b; Luque et al., 2007). Leptin receptor expression is

colocalized with arcuate nucleus Kiss1 cells in the mouse and sheep as shown by double-label *in situ* hybridization found (Backholer et al., 2010; Smith et al., 2006a), and leptin treatment results in rapid depolarizations of ARH Kiss1 cells in guinea pigs, suggesting a direct regulatory relationship (Qiu et al., 2011). However, studies using the leptin receptor-green fluorescent protein (GFP) transgenic mice show virtually no colocalization of GFP with Kiss1-immunoreactivity (Louis et al., 2011). Further work in mice and work presented in Chapter 3 in rats has demonstrated a lack of pSTAT3, a signal transducer and activator of transcription stimulated by leptin receptor signaling, in Kiss1 cells after treatment with high levels of leptin (Louis et al., 2011; Quennell et al., 2011; True et al., 2011d); however, it remains possible that leptin signaling in Kiss1 cells is through cascades not involved in gene transcription and pSTAT3 activation, as indicated by rapid electrophysiological responses (Qiu et al., 2011). Importantly, restoring leptin to physiological levels does not restore ARH Kiss1 levels in either lactation or CR, demonstrating that hypoleptinemia is not a required signal for ARH Kiss1 inhibition during negative energy balance (True et al., 2011d; Xu et al., 2009b). Given the important role of Kiss1 in GnRH regulation, and its implicated involvement in negative energy balance, understanding the regulatory afferent inputs for Kiss1 cells will be critical to our understanding of the integration of energy balance and reproduction.

1.4 Regulation of neurokinin B and dynorphin as a central mechanism of GnRH inhibition

ARH Kiss1 cells also express the tachykinin neuropeptide, neurokinin B (NKB) (Goodman et al., 2007; True et al., 2011b). Similar to Kiss1, NKB is also critical for development of reproductive function since mutations in the genes encoding NKB and the NKB receptor NK3, also result in hypogonadotropic hypogonadism in humans (Topaloglu et al., 2009). NKB levels are inhibited by estradiol in several species and there is strong evidence that NKB plays a role in negative steroid feedback (Danzer et al., 1999; Goubillon et al., 2000; Navarro et al., 2011a; Pillon et al., 2003; Rance, 2009; Rance and Young, 1991). For many years results were inconsistent as to whether NKB was stimulatory or inhibitory for GnRH release (Corander et al., 2010; Sahu and Kalra, 1992; Sandoval-Guzman and Rance, 2004), but

more recent evidence has supported a stimulatory role, consistent with reproductive dysfunction in humans with mutations in the NKB system (Billings et al., 2010; Navarro et al., 2011a; Ramaswamy et al., 2010; Wakabayashi et al., 2010). There is evidence in the rat that NKB may directly stimulate GnRH release through fiber contacts in the external zone of the median eminence (Krajewski et al., 2005). In addition to direct regulation of GnRH cells, there is also evidence that NKB may indirectly regulate GnRH release through stimulating-autoregulatory actions on ARH Kiss1/NKB cells (Navarro et al., 2011a; Navarro et al., 2009; Wakabayashi et al., 2010).

Our laboratory has investigated whether NKB may play a role in negative-energy balance-induced inhibition of GnRH release. Real-time PCR analysis presented in Chapter 3 demonstrated that ARH NKB mRNA expression is decreased during CR, consistent with previous findings of ARH NKB inhibition during lactation (True et al., 2011d; Xu et al., 2009b). This contrasts to a lack of inhibition during more moderate 40% CR and a 48-hr fast (True et al., 2011d). Based on these findings, we hypothesize that ARH NKB inhibition is only involved in more severe conditions of negative energy balance to shut off cyclic reproductive function. Similar to Kiss1, little is known about the upstream regulatory input for ARH NKB expression, although work from our group observed no evidence that leptin regulates NKB expression during negative energy balance (True et al., 2011d; Xu et al., 2009b). It is of interest to note that ARH Kiss1/NKB cells also express dynorphin, yet another neuropeptide involved in reproductive regulation (Burke et al., 2006; Goodman et al., 2007). Similar to NKB, dynorphin receptors are also found on arcuate nucleus Kiss1/NKB/dynorphin (KNDy) cells suggesting a potential autoregulatory action (Navarro et al., 2009). However, dynorphin is thought to be inhibitory for GnRH release and unlike NKB, dynorphin is not differentially regulated during negative energy balance (Kinoshita et al., 1982; Leadem and Kalra, 1985; Schulz et al., 1981; True et al., 2011d; Xu et al., 2009b). A similar example of juxtaposed coexpressing neuropeptides exists in the hypothalamic feeding cells containing α-melanocyte-stimulating hormone and β-endorphin, which are stimulatory and

inhibitory for food intake, respectively (Imura et al., 1985; Kim et al., 2000; Tsujii and Bray, 1989). It remains unclear how coexpression of these two counteracting neuropeptides may be coordinated to control food intake in the hypothalamus (Hughes et al., 1988). In the case of KNDy neurons it is tempting to speculate that differential expression of NKB and dynorphin may tightly regulate the release of Kiss1 into the median eminence. Furthermore, precisely timed Kiss1 release may be physiologically significant for the regulation of basal pulsatile GnRH release.

1.5 Gonadotropin-inhibitory hormone

While Kiss1 and NKB are important stimulatory signals for GnRH and gonadotropin release, research over the past decade has uncovered a similarly important inhibitory signal aptly named gonadotropin-inhibitory hormone (GnIH). GnIH was first isolated from avian brains and characterized by its ability to inhibit gonadotropin release from pituitary explants (Tsutsui et al., 2000). Soon after the mammalian homologue termed RF-amide related protein 3 (RFRP3) was characterized (Hinuma et al., 2000; Johnson et al., 2007; Kriegsfeld et al., 2006; Ubuka et al., 2009; Yano et al., 2003). Like its avian counterpart, RFRP3 has also been localized to the median eminence and shown to inhibit gonadotropin release from the pituitary (Clarke et al., 2008). In addition to pituitary actions, there is also evidence that GnIH/RFRP3 can inhibit GnRH cell firing (Ducret et al., 2009; Wu et al., 2009b), demonstrating that there may also be hypothalamic actions of GnIH/RFRP3. This finding is supported by immunohistochemical data of GnIH/RFRP3-ir fibers in close contact with GnRH cell bodies in birds, rats, sheep and non-human primates (Johnson et al., 2007; Smith et al., 2008a; Smith et al., 2010a; Ubuka et al., 2008). In fact, results demonstrating a lack of hypophysiotropic effects of RFRP3 in the rat have lead to the hypothesis that RFRP3 action may be solely hypothalamic in this species (Anderson et al., 2009; Rizwan et al., 2009). More research is needed to understand the site of action of RFRP3 in the rat given contradictory evidence regarding 1) the presence of RFRP3 fibers in the external zone of the median eminence (Bentley et al., 2010; Johnson et al., 2007; Rizwan et al., 2009) and 2) *in vivo* actions of

intracerebral ventricular RFRP3 administration on LH release (Anderson et al., 2009; Johnson et al., 2007; Murakami et al., 2008; Rizwan et al., 2009).

Given the inhibitory action of GnIH/RFRP3 on gonadotropin release, many studies have investigated the role of this peptide during conditions of GnRH suppression (Clarke, 2011; Smith and Clarke, 2010). In both birds and sheep GnIH/RFRP3 is higher in the non-breeding season suggesting a potential role in seasonal regulation of reproductive function (Bentley et al., 2003; Clarke and Smith, 2010; Smith et al., 2008a). GnIH/RFRP3 also inhibits sexual behavior in birds and rats (Bentley et al., 2006; Johnson et al., 2007). Although GnIH/RFRP3 is implicated in the inhibition of reproductive function, the role of this peptide in governing cyclic fluctuations of gonadotropins during ovarian cycling in mammals is still not well understood. Exogenous RFRP3 reduces c-Fos activity in GnRH and AVPV cells at the time of an induced-LH surge in the rat, but RFRP3 has no affect on basal pulsatile GnRH release (Anderson et al., 2009). In the hamster c-Fos activation is decreased in RFRP3 cells at the time of the LH surge (Gibson et al., 2008). Differential regulation of RFRP3 during the ovarian cycle occurs in hamsters and monkeys, but in opposite directions. While RFRP3 is high during diestrus in the hamster (Gibson et al., 2008), non-human primates have elevated levels immediately prior to the GnRH/LH surge (Smith et al., 2010a). The latter finding is surprising since high levels of RFRP3 are expected to suppress GnRH/LH levels; therefore, it is possible RFRP3's role in ovarian cyclicity is not always strongly inhibitory.

Studies focusing on the reproductive aspects of the GnIH/RFRP3 system have also noted a potential role for GnIH/RFRP3 in appetite regulation. Exogenous administration of GnIH in chicks modestly stimulates food intake, potentially through an opioid receptor system (Tachibana et al., 2008; Tachibana et al., 2005). RFRP3 was also found to stimulate food intake in rats, resulting in a doubling of food consumption during the light-phase of the day (Johnson et al., 2007; Murakami et al., 2008), consistent with the well-established role of RF-amide related proteins and appetite regulation (Dockray, 2004). The evidence for a regulatory role of GnIH/RFRP3 in both reproduction and appetite highlights

the potential importance of this neuropeptide for the integration of energy balance and reproductive function (Clarke, 2011; Smith and Clarke, 2010). The characteristics of inhibitory effects on reproductive function and stimulatory effects on food intake makes it tempting to hypothesize that RFRP3 will be upregulated during negative energy balance. If RFRP3 is elevated during negative energy balance it could work to both conserve energy output through inhibition of reproductive cycling and increase energy input through food intake, and future research will undoubtedly be aimed at answering this question. Additional future directions for GnIH research may be electrophysiological and anatomical studies to determine if RFRP3 has any direct influence on Kiss1 release, since fiber distribution analysis suggests RFRP3 terminals are in the region of both the AVPV and ARH Kiss1 populations (Rizwan et al., 2009). Additionally, investigations using GnIH antagonists could determine whether blocking a potential negative energy balance-induced rise in GnIH/RFRP3 prevents inhibition of GnRH release.

1.6 Alarin

Galanin-like peptide (GALP) has been previously linked to the integration of energy balance and reproduction (Kageyama et al., 2005; Krasnow et al., 2003; Lawrence and Fraley, 2010). Recent evidence suggests a splice variant of the GALP gene, termed alarin (Santic et al., 2006), may have a similar regulatory function. Alarin appears orexigenic in rodents since intracerebral ventricular injections of alarin result in a five-fold acute increase in food intake and increased body weight gain after chronic administration (Boughton et al., 2010; Van Der Kolk et al., 2010). Alarin has also been implicated in reproductive regulation due to its expression in sexually important nuclei such as the preoptic area and other hypothalamic regions including the ARH (Eberhard et al., 2011). Furthermore, work in male rodents indicates that alarin may stimulate LH release through a GnRH-dependent mechanism (Boughton et al., 2010; Van Der Kolk et al., 2010). Further research is needed to understand the role of alarin in regulation of reproductive function in females, since past studies have focused only on males. This need for studies in females will also be important in determining whether steroid

hormone environment affects the direction of alarin's regulatory action on LH release as it does for many other orexigenic neuropeptides (Crowley and Kalra, 1987; Pu et al., 1998).

The apparent stimulatory action of alarin on LH release observed in males is in contrast to the inhibitory reproductive effects found with most other orexigenic neuropeptides, such as NPY, MCH and orexin (Chiocchio et al., 2001; Kalra and Kalra, 1996; Kohsaka et al., 2001; Murray et al., 2000; Pu et al., 1998; Small et al., 2003; Tamura et al., 1999; Wu et al., 2009a). There is significant evidence that these well-studied orexigenic neuropeptides regulate GnRH release through direct projections to GnRH cell bodies (Campbell et al., 2003; Iqbal et al., 2001; Li et al., 1999b; Small et al., 2003; Williamson-Hughes et al., 2005). Therefore, direct inhibitory effects on GnRH cells would be greatly enhanced during states of negative energy balance when their activities are upregulated (Rondini et al., 2010; Smith et al., 2010b; Xu et al., 2009a). However, it is unclear how alarin may influence GnRH regulation. One study found alarin-like immunoreactivity in the preoptic area (Eberhard et al., 2011), but it has not been examined whether this immunoreactivity is in close proximity to GnRH cell bodies. It is also unclear how alarin might be differentially regulated during negative energy balance, since orexigenic drive should be high while LH stimulation would be expected to be low. Future research may elucidate how alarin contributes to the integration of energy balance and reproductive function, and what if any role this neuropeptide has in negative energy balance-induced acyclicity.

1.7 Brainstem glucose-sensing populations

Certain neuronal populations are capable of detecting changes in glucose levels. This characteristic offers an attractive and relatively simple mechanism through which metabolic status may be acutely sensed in the brain and potentially relayed to reproductive circuits. There are two types of glucose-sensitive cells: cell that increase firing in response to rising glucose levels, termed glucose-responsive (GR) cells, and cells that decrease firing in response to rising glucose, termed glucose-sensing (GS) cells respectively. One such glucose-sensing population is hypothesized to exist in the ventrolateral

medulla of the brainstem (for review see Ritter et al., 2006). Glucose-deprivation achieved with administration of 2-deoxy-D-glucose, a glucose molecule unable to undergo glycolysis, results in c-Fos activation in the catecholaminergic A1/C1 subregion of the ventrolateral medulla (Ritter et al., 1998). To determine if the A1/C1 catecholamine neurons contribute to the reproductive inhibition associated with glucoprivation this population was selectively ablated by injecting a conjugated dopamine-beta hydroxylase-saporin toxin complex into the A1/C1 region (I'Anson et al., 2003). Interestingly, when A1/C1 catecholamine neurons were destroyed this prevented 2-deoxy-D-glucose-induced reproductive acyclicity, strongly implicating a role for this population in the integration of energy balance and reproductive function. Microdialysis during 2-deoxy-D-glucose administration reveal increases in noradrenaline release in the hypothalamus, and intervention to block this noradrenaline rise prevents inhibition of LH (Nagatani et al., 1996). In addition, it appears that noradrenaline's role in LH suppression is not specific to the 2-deoxy-D-glucose model of negative energy balance given similar results were observed in fasting animals (Cagampang et al., 1992; Maeda et al., 1994). These studies suggest that increases in noradrenaline, potentially from the A1 subregion, during negative energy balance may be critical for inhibition of LH release. This inhibitory role for catecholamines, and noradrenaline specifically, on LH release is consistent with previous literature (Maeda et al., 1994; Nagatani et al., 1996; Tsukamura et al., 1994), and electrophysiological recordings as well as neuroanatomical results indicate this inhibitory action may be exerted directly upon GnRH cells (Campbell and Herbison, 2007; Han and Herbison, 2008; Todman et al., 2005). However, noradrenaline is also stimulatory for LH release, and these contradictory regulatory influences are thought to be dependent on steroid hormone levels, with noradrenaline stimulatory for LH in the presence of high levels of estradiol and inhibitory in the presence of low levels of estradiol (Cagampang et al., 1992; Gallo and Drouva, 1979; Havern et al., 1991; Herbison, 1997; Leung et al., 1982; Meyer and Goodman, 1985; Robinson and Kendrick, 1992), as would be associated with negative energy balance.

In the lactation model of negative energy balance the A1 noradrenergic region is activated, as indicated by c-Fos activation, in response to pup suckling (Li et al., 1999c). Pup suckling is strongly implicated in LH inhibition during negative energy balance (Brogan et al., 1999; Smith and Grove, 2002; Smith et al., 2010b), once again supporting an inhibitory role for noradrenaline during negative energy balance. Noradrenergic neurons of A1 activated by the suckling stimulus also project to the ARH (Li et al., 1999a). However, contradictory evidence finding a lack of A1 noradrenaline projections to GnRH cells also exists, suggesting it is possible that an intermediary population may be involved in mediating noradrenaline's effects on GnRH (Campbell and Herbison, 2007; Simonian et al., 1999; Wright and Jennes, 1993). Given the projection to the ARH, we sought to determine whether noradrenaline might regulate the upstream ARH Kiss1/NKB population. Immunohistochemistry presented here demonstrates that ARH Kiss1/NKB and AVPV Kiss1 neurons appear to have close appositions from DBH/NPY positive fibers (Figure 1). This finding indicates Kiss1 populations may receive regulatory inputs from brainstem catecholamine neurons coexpressing NPY (Bai et al., 1985; Everitt et al., 1984). It remains to be confirmed whether brainstem catecholamine fibers contacting Kiss1 cells are from the ventrolateral medulla A1 region and future tract-tracing studies will be aimed at defining the source of this catecholaminergic input.

It is clear that although leptin is undoubtedly involved in the regulation of reproductive and metabolic status, leptin's role in negative energy balance is not causative for the severe inhibition of GnRH release. The finding that restoration of leptin to normal physiological levels does not restore GnRH release in multiple models of negative energy balance supports this conclusion. Given this evidence, the search continues for signals that may be critical for integrating reproductive and metabolic function. One obvious candidate not previously discussed here is the orexigenic gut hormone ghrelin, which has already been extensively reviewed for its potential role in the integration of energy balance and reproduction (Fernandez-Fernandez et al., 2006; Garcia et al., 2007; Tena-Sempere, 2008). Similar

to leptin, ghrelin is a predominantly peripheral-derived hormone capable of sensing changes in metabolic state, but unlike leptin ghrelin is orexigenic and elevated during negative energy balance and it has also been found to be inhibitory for GnRH release in several species (Ariyasu et al., 2001; Fernandez-Fernandez et al., 2005a; Fernandez-Fernandez et al., 2004; Fernandez-Fernandez et al., 2005b; Furuta et al., 2001; Iqbal et al., 2006; Tschop et al., 2000; Vulliemoz et al., 2004; Wren et al., 2000). In looking beyond leptin, future research investigating the circuits and modes through which ghrelin regulates GnRH release may prove critical for our understanding of negative energy balance-induced reproductive dysfunction. One potential intermediary for ghrelin's actions on GnRH release may be Kiss1 (Forbes et al., 2009). Kiss1 has been strongly implicated in the integration of energy balance and reproduction given arcuate nucleus Kiss1/NKB and AVPV Kiss1 expression levels are low in various states of negative energy balance. Significantly, arcuate nucleus Kiss1/NKB and AVPV Kiss1 levels were also not restored with physiological leptin treatment during negative energy balance in data presented in Chapter 3, and methods aimed at preventing negative energy balance-induced hyperghrelinemia may elucidate whether this rise in ghrelin is critical for Kiss1 inhibition. Given the importance of the Kiss1 system for negative energy balance acycility, the search for other metabolic signals influencing this neuronal population is a leading research question in this field.

GnIH/RFRP3, alarin and glucose-sensing ventrolateral medulla neurons are just a few of the emerging candidates that have been linked to reproductive and metabolic regulation and as such may play a role in the integration of these systems. These three candidates are diverse in both their mechanism of metabolic sensing and their site of action in reproductive neuroendocrine circuits (Figure 2). GnIH/RFRP3 and noradrenergic ventrolateral medulla cells are both inhibitory signals for GnRH release that are increased during negative energy balance. While noradrenergic A1 cells are likely activated by low glucose levels, it is unclear how GnIH/RFRP3 might be upregulated during negative energy balance. However, once activated GnIH/RFRP3 cells likely contribute to increased orexigenic

drive during negative energy balance, and it will be of interest to understand how GnIH may integrate with other well known metabolic/food intake systems of the hypothalamus, such as NPY, MCH and orexin, to contribute to this orexigenic drive. Although GnIH/RFRP3 and the A1 populations may both be inhibitory for GnRH release, there is evidence that they may differ in their site of action within the hypothalamic-pituitary-gonadal axis. There is strong evidence that GnIH/RFRP3 acts directly at GnRH cells. Data presented here demonstrates brainstem noradrenergic input to ARH Kiss1/NKB and AVPV Kiss1 cells, and future research will be aimed at determining whether the source of these inputs is in fact the noradrenergic A1 population. GnIH/RFRP3 fibers are also observed in the ARH indicating that this population may regulate Kiss1/NKB as well, although more detailed histological research will be needed to test this hypothesis. Unlike GnIH and the A1 noradrenergic population, there is little research on the role of alarin for reproductive regulation during negative energy balance given how recently this neuropeptide was discovered. However, the limited amount of research available on alarin suggests it plays a role in both food intake and reproductive regulation, much like its sister-gene product, GALP. Research examining the effects of alarin on reproductive parameters in females will be a crucial next step in determining what role this neuropeptide might play in the larger neuroendocrine pathway, including the regulatory direction of alarin's effect on LH release and the potential site of action.

1.8. Adiponectin

Just as there are both increases in orexigenic signals and decreases in anorexigenic signals regulating food intake during negative energy balance, there may be dual changes required for restoration of GnRH upon exit from negative energy balance. It is possible that there is a threshold level of leptin required for the restoration of GnRH but that this signal by itself is not sufficient to increase GnRH release. In addition to increased leptin levels, which are thought to provide indirect stimulation to GnRH cells, there may be an added requirement for decreased inhibitory signals for full restoration of reproductive function upon exit from negative metabolic conditions. In the adipocyte there is another

hormone released termed adiponectin, which is thought to function in the opposite manner of leptin. Unlike leptin levels, adiponectin levels are inversely correlated with body mass index (Matsubara et al., 2002; Yang et al., 2001). Although adiponectin levels did not change in humans fasted 48 hours (Gavrila et al., 2003), adiponectin levels are elevated after weight loss and fat loss in obese humans (Yang et al., 2001). Adiponectin levels are also high in patients with anorexia nervosa, although correlations between BMI and adiponectin do not exist in this extreme negative metabolic condition (Bosy-Westphal et al., 2005; Iwahashi et al., 2003). To our knowledge no studies have looked at changes in adiponectin levels during negative energy balance in rodents; however, given the substantial weight loss and fat loss in the CR model described in Chapter 3, it is conceivable that adiponectin levels may be increased with long-term CR.

Recent work provides evidence that adiponectin may act at the pituitary to decrease LH release (Lu et al., 2008; Rodriguez-Pacheco et al., 2007); however, the effect of adiponectin on hypothalamic GnRH release *in vivo* has not been studied to date. Given that adiponectin may be elevated during negative energy balance and contribute to decreased sensitivity to GnRH release at the pituitary, it is possible that this inhibitory signal masked the effects of leptin restoration during CR. More work is needed to understand the role of adiponectin in metabolically-driven GnRH inhibition to determine if it may in fact be an important inhibitory signal for GnRH release during CR.

1.9 The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is generally described as the stress pathway, but many aspects of this circuit also appear to affect food intake as well as reproduction. In the HPA axis corticotropin-releasing hormone (CRH) is secreted from neurons in the paraventricular nucleus (PVN) of the hypothalamus into the hypophyseal portal blood, leading to pituitary stimulation of adrenocortiotropic hormone (ACTH) release, and downstream glucocorticoid (GC) release from the adrenal gland. However, CRH is also thought to contribute to metabolic regulation through actions in

the CNS. Exogenous CRH, and related stress-peptide urocortin-1, administration result in decreased food intake, consistent with stress-induced decreases in food intake observed in rodents (Arase et al., 1988; Gosnell et al., 1983; Levine et al., 1983; Spina et al., 1996). In addition, it appears that CRH levels in the PVN increase with leptin administration and CRH antagonists can partially block leptin's anorexigenic effects (Gardner et al., 1998; Schwartz et al., 1996; Uehara et al., 1998). CRH also increases activity and metabolic rate, and this appears to be largely due to direct action of CRH in the brain and independent of peripheral increases in ACTH and GC release (Arase et al., 1988; Brown et al., 1982; Eaves et al., 1985). Paradoxically, leptin-deficient ob/ob mice have elevated glucocorticoid levels and hyperphagia, and adrenalectomy reverses the obese phenotype and restores melanocortin levels in these mice (Makimura et al., 2000). Clearly CRH likely plays a complicated role in the regulation of food intake and energy homeostasis.

Unlike most anorexigenic signals, CRH and GC's are thought to be inhibitory for GnRH release. There is evidence in both primates and rodents that CRH inhibits gonadotropin release, and there is evidence at least in the rodent that this may be through regulation of hypothalamic GnRH release (Gambacciani et al., 1986; Olster and Ferin, 1987; Rivier and Vale, 1984). In addition to CRH effects on GnRH cells, there is additional evidence that downstream glucocorticoids decrease pituitary responsiveness to GnRH release (Breen and Karsch, 2004). HPA activation may then result in inhibition of the HPG axis both at the level of the hypothalamus and the pituitary. This dual role of CRH to inhibit food intake but also inhibit GnRH release makes its role in negative energy balance unclear, since anorexigenic drive should be inhibited during negative energy balance while GnRH-inhibitory signals would be expected to be increased.

Consistent with the inhibitory role of CRH and GCs for reproductive function, there is significant evidence that HPA activation and perceived stress is an important component of acyclicity in patients with hypothalamic amenorrhea (Berga et al., 1997; Biller et al., 1990; Giles and Berga, 1993; Marcus et

al., 2001). Indeed many clinical patients with hypothalamic amenorrhea are "weight stable" with BMI's that fall in the lower range of the "normal weight" classification, indicating metabolic factors are not likely to fully account for the observed infertility (Laughlin et al., 1998; Meczekalski et al., 2000). These findings have been interpreted as evidence that mild stress and metabolic signals likely act in concert to cause GnRH inhibition. This observation in humans may also be true in other species, since work in the non-human primate has also found that a combination of mild stress, modest diet and a regular exercise regime can result in acyclity in a subpopulation of "stress-sensitive" monkeys (Williams et al., 2007).

There is also significant evidence in rodent models of HPA activation in conditions of metabolic stress. Particularly it appears that increased levels of CRH and GCs may play a direct role in GnRH inhibition during fasting (reviewed in Maeda et al., 1994; Maeda and Tsukamura, 1996; Tsukamura et al., 1994), although it remains unclear how critical this HPA activation is for subsequent GnRH inhibition (Nagatani et al., 2001). Interestingly, others have found evidence of decreased CRH levels during starvation, consistent with CRH's anorexigenic role in food intake (Brady et al., 1990; Suemaru et al., 1986). Relevant to the current study, one study found that a 2 week caloric restriction in female rats resulted in a decrease in CRH, indicating that activation of these neurons might not play a significant role in GnRH inhibition (Brady et al., 1990). It is also possible, however, that low levels of CRH during starvation reflect negative feedback from elevated GC levels (Chowers et al., 1969; Suemaru et al., 1986). These conflicting reports make it difficult to speculate at this time on the level of HPA activation that may have occurred during CR reported in Chapters 3 and 4.

If CR does result in HPA activation it is possible that this may 1) contribute to Kiss1, CART and GnRH inhibition, 2) mask effects of leptin restoration on GnRH release and 3) account for discrepancies in the changes in Kiss1 and CART observed between lactation and CR models of negative energy balance. While it is conceivable that CR results in HPA axis activation based on previous results in fasting, current evidence indicates that during lactation there is a characteristic hyporesponsiveness of the HPA axis to

external stressors. Several studies have noted that during lactation the stress response in rodents, both behaviorally and measured at many different neuroanatomical levels, is markedly decreased compared to non-lactating animals (da Costa et al., 1996; Lightman and Young, 1989; Stern et al., 1973; Thoman et al., 1970). Importantly, one study found that exercise-induced GC increases were attenuated in lactating women, compared to non-lactating controls, indicating a specific hyporesponsiveness of the HPA axis to metabolic stressors during lactation (Altemus et al., 1995). Based on these observations it is possible that HPA response to metabolic stress is similarly blunted in lactating rats, and thus HPA-activated signals may play a role in GnRH inhibition during CR but not lactation. This difference could potentially account for the differences in AVPV Kiss1 and CART regulation between lactation and CR models, as well as potentially play a role in both ARH CART inhibition only found during CR and not lactation.

As more and more candidates involved in both reproductive and metabolic regulatory systems emerge, it is becoming clear that there are likely multiple signals and mechanisms working in concert to tightly couple the regulation of these two critical physiological processes. More specifically, negative energy balance results in wide spread changes in the hypothalamus, including increases in numerous orexigenic neuropeptides known to regulate GnRH release, increases in signals inhibitory for reproduction, and decreases in anorexigenic neuropeptides and other signals excitatory for reproduction (Figure 2). With such a myriad of changes in these two regulatory systems, it is unlikely any one factor is solely responsible for the subsequent inhibition of GnRH release. Similarly, there may be many metabolic signals acting at different time frames, for example acute decreases in glucose levels, intermediary increases in the gut orexigenic hormone ghrelin and long-term decreases in circulating leptin might all be required for full suppression of reproductive function during negative metabolic states. The most beneficial work of the future will likely be those studies that attempt to understand how multiple signals work in concert to control GnRH release during negative energy balance.

2. Discrepancies in studies investigating Kiss1 projections

While there is large agreement that AVPV Kiss1 cells send direct projections to GnRH neurons, the site of ARH Kiss1 regulation of GnRH neurons remains controversial. While our studies presented in Chapter 2 have indicated that ARH Kiss1 cells send only a minor projection to the POA this has recently been disputed in mouse studies performed by Herbison and colleagues (Yeo and Herbison, 2011). In this work it was demonstrated that both anterograde tracing out of the ARH and retrograde tracing from the POA provide evidence for ARH Kiss1 projections into the POA where GnRH cell bodies are found (Yeo and Herbison, 2011). However, since the time of our publication another study investigating Kiss1/NKB-ir projections in the rat recapitulated the current results of infrequent Kiss1/NKB fibers being found in close apposition to GnRH neurons (Kallo et al., 2012). This discrepancy may be due to species differences or differences in sensitivity of the techniques employed. Another study has recently indicated that anteograde tracing out of the ARH nucleus in rats has also found evidence of ARH KNDy projections to the POA where GnRH neurons are found; however, these results were not shown or quantified making it difficult to determine if that study observed more significant projections than that described in Chapter 2 (Krajewski et al., 2010). Quantification was provided in this study in which lesions of ARH nucleus were found to result in a roughly 50% decrease in NKB labeled fibers in regions containing GnRH cell bodies, indicating that there may in fact be significant ARH NKB projections to this region.

2.1 Dual-label immunohistochemistry for projection mapping

It is possible that differences between studies investigating ARH KNDy projections are due to differences in techniques employed to determine projections out of these cells. For our studies, presented in both Chapter 2 and Chapter 4, we have used double-label IHC of two coexpressed neuropeptides as a method to map projections from the nucleus of interest. This simplistic approach is only possible for cell populations coexpressing two neuropeptides at a high percentage, but it offers

considerable ease compared to the traditional tract-tracing approach of injecting labels into discrete brain regions. Particularly tract-tracing studies can be confounded when the injection site is not strictly limited to the area of interest. This is particularly important when this technique is employed to look at abundantly expressed neuropeptides, such as CART and NKB, since leakage of the tracer into adjacent nuclei could label cells presumably outside the nucleus being investigated. To limit diffusion of the injected tracer into adjacent nuclei, small quantities of the tracer are usually injected leading to only few cells taking up the dye which prevents quantification of projections, adding another limitation to this technique. Despite these potential difficulties and drawbacks to the tracing approach, it offers a uniquely definitive method to determine neuronal projections.

Dual-label IHC offers an easier methodological approach as discussed above, but it remains to be experimentally confirmed. Yeo and Herbison argued that although our method using double-label Kiss1/NKB immunohistochemistry undoubtedly positively identified projections from the ARH, that perhaps our method resulted in a significant number of false negatives. That is to say that fibers originating from the ARH could have possibly appeared single-labeled for either Kiss1 or NKB by IHC and thus were not counted in our analysis. This could potentially be due to differences in sensitivity of the antibodies used or more likely differential transport of neuropeptides in neuronal processes. Antecdotally, we observed beaded fibers with alternating beads of Kiss1- and NKB-ir and occasional double Kiss1/NKB-ir beads as well. This suggests that perhaps Kiss1 and NKB transport down axons is not entirely homogenous, and thus immunohistochemistry alone may be less sensitive than tract-tracing in positively identifying projections.

This potential differential transport may also be supported by findings in Chapter 4, in which we noted a significant number of single-labled α-MSH fibers. The primary and most-studied POMC population is found in the ARH, where 90% of POMC cells coexpress CART. However, there is also an infrequently studied population of POMC cells in the NTS, and no evidence has been found that these

cells coexpress CART. It is unclear at this time whether the NTS sends major projections to hypothalamic nuclei and can account for the abundance of single-labled α-MSH observed (discussed in Chapter 4, Disucssion). It is also conceivable that these single-labeled α-MSH fibers originate in the ARH but did not have significant CART-ir for detection by IHC. This observation has no significant effect for interpretation of the tracing studies in Chapter 4, since the majority of CART contacts did coexpress α-MSH and were thus undoubtedly from the ARH nucleus. However, this finding does point to a further need to validate the sensitivity of the dual-label immunohistochemistry method as a means of projection mapping.

To address this concern, future work in the lab is investigating the presence of double-label Kiss1/NKB fibers as well as single-labeled Kiss1 fibers near GnRH neurons in the male rat. Previous work in the mouse has observed a virtual absence of Kiss1 mRNA levels in the AVPV of males. However, similar to the female the ARH Kiss1 population is believed to coexpress NKB in the male. Therefore, based on our past findings it would be hypothesized that if ARH Kiss1/NKB cells do not send projections rostrally to the level of GnRH neurons then there would likely be very few Kiss1 fibers in this area in the male rodent. Although this work has not been quantified, the preliminary results indicate that both infrequent double-label Kiss1/NKB fibers as well as single-labeled Kiss1 fibers are observed in the POA near GnRH neurons in male rats, similar to the females. In addition, preliminary results in our lab have also found electrophysiological evidence that Kiss1 tonically stimulates GnRH neurons in male rats, again similar to the female, indicating that GnRH cells do likely receive input from Kiss1 projections at the soma. The Kiss1-ir fibers near GnRH neurons likely originate either in the ARH nucleus (and do not coexpress NKB due to the insensitivity of the technique), or the newly observed amygdala Kiss1 population, which has higher expression in male rats than diestrus females and has not been found to coexpress NKB (Kim et al., 2011). If the former, this suggests that IHC may produce false negatives, in which ARH KNDy fibers appear to only express Kiss1 or NKB in far reaching projections to GnRH neurons.

As a final form of validation, tracing studies from the ARH nucleus of female rats are currently underway to address this question. Specifically, brains in which the anteograde tracer biotinylated dextran (BDA) was injected into ARH are currently being labeled with NKB to determine if ARH KNDy neurons do in fact send significant projections to GnRH neurons, or whether discrepancies between our work and Yeo and Herbison is due to species differences. However, it should be noted that while the study by Yeo and Herbison found evidence for rostral ARH Kiss1 projections to the POA, based on the technique they were unable to confirm whether these projections make close contact to GnRH cells and thus the physiologically significance of this finding has yet to be validated. An interesting observation from the double-label IHC method was evidence of rostral KNDy projections that appeared to dissipate prior to the level of GnRH cell bodies as discussed in Chapter 2. If tracing studies confirm that these projections do in fact diminish prior to rostral location of GnRH cell bodies it will be of future research interest to determine the targets and physiological role of these rostral Kiss1 projections.

2.2 Kiss1 regulation of GnRH at the median eminence

If our previous results are confirmed that ARH Kiss1/NKB cells do not project towards GnRH cell bodies, this raises the question of how ARH Kiss1/NKB directly regulates GnRH release. While Kiss1/NKB-ir fibers are observed in the ME this is found almost exclusively in the internal zone, and there is an absence of Kiss1/NKB-ir staining in the external zone where GnRH fibers terminate in both rats and monkeys (Ramaswamy et al., 2008; True et al., 2011b). Since our original results were published work has been conducted to determine if there are any synaptic connections between Kiss1 and GnRH fibers in the ME. Although Kiss1-ir fibers are located near GnRH fibers in the ME, there was no evidence of functional synapses as determined by electron microscopy (Uenoyama et al., 2012). However, the same study found that Kiss1 could stimulate GnRH release from a ME explant that lacked GnRH cell bodies, similar to previous reports carried out in rodents and sheep (d'Anglemont de Tassigny et al., 2008; Smith et al., 2011). This evidence strongly points to a regulatory role of Kiss1 in the ME, but

future research will be needed to determine if this is through an indirect cell population or is directly mediated by volume transmission instead of the traditional synaptic connection.

3. Interpreting incongruity between protein and mRNA levels

Consistently throughout the work presented here we have noted discrepancies between protein and mRNA levels in different treatment groups. In fact a change in protein and mRNA levels in the same direction appears to the exception rather than the rule when it comes to investigating Kiss1 and CART cell populations. These findings highlight the importance of quantifying both protein and mRNA levels before conclusions are drawn about the direction of differential regulation. As an example, studies investigating protein levels alone would conclude that AVPV Kiss1 cells are activated during lactation; however, based on the decrease in mRNA we have hypothesized the opposite to occur and that in fact protein release may be inhibited. As another example, if only CART mRNA levels had been investigated by ISH during lactation, it would have been concluded that this population is not affected by lactation. As demonstrated in Chapter 4 there is a dramatic increase in CART-ir cell number with lactation, suggesting some change in CART regulation is indeed occurring during lactation and this could contribute to downstream GnRH inhibition.

It should be noted that even in the absence of changes in protein and mRNA levels it is possible that neuropeptide release may be affected, and this could be missed with traditional IHC and ISH methods. This appears to be the case with GnRH cells, since mRNA and protein levels are largely unaffected during the surge when there is a large increase in GnRH release (Finn et al., 1998b) as well as during negative energy balance when there is a strong suppression of pulsatile GnRH release (Bergendahl et al., 1992; Gruenewald and Matsumoto, 1993; Marks et al., 1993). In the future, it will be helpful to employ functional hypothalamic explants studies to determine how changes in metabolic status might affect Kiss1 and CART release from the hypothalamus. In addition, electrophysiological recordings provide a method by which electrical excitability and firing rates may be measured in

neurons. This approach offer a more direct method of measurement of changes in protein release than examination of protein or mRNA levels at a single time point as is the case during IHC and ISH. This change in electrical excitability appears to be true for GnRH neurons since increased inhibitory presynaptic inputs during lactation result in hyperpolarization and contribute to decreased GnRH release in the absence of changes in mRNA levels (Marks et al., 1993; Xu et al., 2009a).

While we have interpreted discrepancies between protein and mRNA to reflect changes in the rates of protein release, it is possible that other factors account for these differences. For instance, it is possible the peptidase activity is decreased in the AVPV CART and Kiss1 cells during lactation, leading to decreased degradation rates and protein buildup. The physiological significance of such changes in protein clearance rates is unclear, but IHC and ISH experiments could be used to determine if in fact peptidase levels are altered in AVPV cells during lactation Identification of other conditions and cell populations demonstrating this increase in protein and decrease in mRNA may help unravel the potential physiological significance of such changes.

4. Similarities between Kiss1 and CART populations for GnRH regulation

While it has long been established that Kiss1 is a potent and important regulator of GnRH release, the work presented here in Chapter 4 provides evidence that CART may be another important regulator of the GnRH network. Specifically, there appear to be significant similarities in the Kiss1 and CART system, which may reflect an interrelated role for these two neuropeptides in regulating GnRH release. Given evidence that CART fibers make contact with Kiss1 neurons, it is also possible that changes in CART drive downstream changes in Kiss1 and that this is the reason for similar changes in the two neuropeptides during negative energy balance. Given the importance of the Kiss1 system in GnRH release, the overlap between CART and Kiss1 systems implies CART is a new critical player in hypothalamic regulation of reproduction.

Consistent with previous results we have demonstrated that large CART populations are located in both the ARH and AVPV nucleus, similar to the distribution of Kiss1 cells (Douglass et al., 1995; Koylu et al., 1997). While previous work has demonstrated a lack of colocalized CART and Kiss1 immunoreactivity in the ARH, as demonstrated by studies investigating ARH POMC and Kiss1 interactions in the sheep and mouse (Backholer et al., 2010; Fu and van den Pol, 2010), we now have evidence that AVPV CART and Kiss1 cells are also separate populations, indicating that while these neuropeptides may act in concert to regulate GnRH release, they are not coreleased from the same cells. We also have provided evidence that similar to AVPV Kiss1 cells in the rat, AVPV CART cells are infrequently observed by IHC in control conditions. It is important to note that this lack of cell body staining did not account for a lack of colocalized Kiss1/CART-ir cells in the AVPV since colocalized Kiss1/CART-ir fibers were also not observed. The lack of Kiss1 and CART cell body staining is somewhat surprising given the abundant number of cells detected by in situ hybridization for both neuropeptides in the AVPV. We hypothesize that there may be a common mechanism that prevents both CART and Kiss1 protein build-up in the AVPV, but not in ARH populations where cell bodies are easily observable for both neuropeptides. Perhaps this common low level of somatic protein underlies a common physiological role for Kiss1 and CART, which requires rapid release of protein from the AVPV and thus an absence of protein build-up. Given the known role of the AVPV in regulating positive feedback, it may be that this rapid release of Kiss1 and CART contributes to the GnRH surge, although this would theoretically only require rapid release during proestrus, when the surge occurs, and not in other stages of the estrus cycle.

Previous work in our lab demonstrated that not only can CART depolarize roughly 75% of GnRH neurons, but it should be noted that maximum depolarization achieved by CART is only roughly 6 mV less than the maximum depolarization achieved by Kiss1 under similar TTX conditions (manuscript in preparation). Given CART's apparent stimulatory role in driving GnRH depolarization, it is not surprising

that CART levels are decreased with the negative energy balance model of CR. Interestingly, CART mRNA levels are decreased in the ARH and AVPV similar to Kiss1 inhibition in these nuclei during CR as well. Inhibition of ARH and AVPV stimulatory tone in the form of decreased Kiss1 and CART might represent a significant decrease in tonic stimulatory input to GnRH and contribute to decreased pulsatile frequency observed in many models of negative energy balance.

Another striking similarity between CART and Kiss1 is the increase in CART and Kiss1 cell numbers in the AVPV during lactation. This increase in cell number as detected by IHC is dramatic and in both cases represents an over three-fold increase in the number of detectable immunoreactive cells. Interestingly, in both cases this increase in protein is not accompanied by an increase in corresponding mRNA, and in fact mRNA levels are decreased during lactation although this decrease did not reach significance for CART mRNA. We have interpreted these findings as an inhibition of protein release leading to increased protein accumulation in cell bodies that cannot be accounted for by increased mRNA production. This also appears to be a phenomenon specific to lactation since changes in AVPV CART and Kiss1 immunoreactive cell numbers are not observed during CR. The specificity to lactation may indicate that the suckling stimulus plays a role in stimulating this change in AVPV CART and Kiss1 protein levels. Another potential explanation is that factors other than changes in protein release rates account for differences in protein and mRNA, as discussed previously.

We have also provided morphological evidence that CART cells may regulate Kiss1 populations in the AVPV and ARH nucleus. Importantly, close appositions observed in 1 µM confocal slices do not prove a functional synapse exists. To better determine if CART fibers in fact synapse on Kiss1 cells, electron miscroscopy needs to be performed to look for synaptic morphology in CART fibers near Kiss1 cells. While it appears that the majority of these fibers coexpress CART and the POMC cleavage product α-MSH, single-labeled CART fibers were infrequently observed near Kiss1 cells; therefore, it is possible that other CART populations play a role in Kiss1 regulation. Another important CART population is

located in the ventral premammillary nucleus (PMv). CART cells in the PMv have been previously shown to make direct projections to the area where GnRH neurons are found (Rondini et al., 2004). The PMv has also been implicated strongly in mediating both leptin's stimulatory effect for reproduction during puberty as well as negative energy balance-induced GnRH inhibition (Donato et al., 2011; Donato et al., 2009). While it is known that PMv CART cells are leptin responsive (Elias et al., 2000), it is still unknown whether CART PMv cells are those involved in leptin-mediated GnRH regulation. Future studies investigating the PMv CART population's role in stimulating GnRH and Kiss1 cells will likely be of great interest to further our understanding of the role of hypothalamic CART in reproductive regulation.

To begin to understand what if any role CART plays in Kiss1 regulation, it will be necessary to first determine what if any effect CART has on Kiss1 neuronal excitability. Recently generated Kiss1-Cre-GFP mice will be used to make electrophysiological recordings from Kiss1 cells in the presence of CART. However, for fully meaningful analysis, the morphological evidence presented in Chapter 4 would need to be re-evaluated in the mouse to ensure that in fact CART fibers make close appositions to Kiss1 cells in this species as well. Even with these factors taken into consideration, interpretation of experiments of CART's effects on Kiss1 membrane potentials will be limited by our lack of knowledge of CART's post-synaptic effects. Without identification of the CART receptor it remains difficult to categorize CART antagonists, and thus determine what happens if CART levels are artificially blocked during normal metabolic conditions and if this affects Kiss1 and GnRH release. However, it would be of interest to compare CART's effect on Kiss1 cells to those on GnRH cells to determine if in fact there is a common receptor and post-synaptic response to CART in two different cell populations. In addition, electrophysiological studies can begin to identify the type of GPCR CART activates as well as potential signaling pathways involved to begin to increase our understanding of the CART post-synaptic receptor.

4.1. Kiss1 and CART involvement in estradiol-mediated GnRH inhibition during CR

Consistent with previous results, the studies reported in Chapter 3 provide evidence for a requirement for estradiol in undernutrition-induced GnRH inhibition. During CR, OVX animals had normal LH levels despite equivalent weight loss to OVX+E treated animals. This suggests that a primary mechanism of estradiol is likely required for GnRH inhibition and this is not due to secondary effects of estradiol to enhance negative energy balance through increased energy expenditure. An obvious hypothesis is that without estradiol present, certain GnRH stimulators are left unchecked and thus full inhibition during negative energy balance cannot be achieved. An obvious candidate that is stimulatory for GnRH release and inhibited by estradiol is the ARH Kiss1 population; however, work presented in Chapter 3 demonstrates in OVX CR animals ARH Kiss1 levels were significantly inhibited while LH levels were normal; therefore, while estradiol is required for GnRH inhibition it does not appear to be required for ARH Kiss1 inhibition. This suggests that estradiol adds to GnRH inhibition through an ARH Kiss1-independent mechanism.

Very little is known about the role CART populations might play in regulating steroid feedback. It has previously been established that ARH POMC levels appear regulated by circulating estradiol levels and estradiol can also directly regulate membrane potentials in ARH POMC cells, 90% of which coexpress CART (Bohler et al., 1991; Cheung and Hammer, 1995; Gao et al., 2007; Qiu et al., 2003). However, to our knowledge studies looking at estradiol effects on ARH CART expression have not yet been performed. Similarly, although it is known that ERα is abundantly expressed in the AVPV (Wintermantel et al., 2006), and particularly on Kiss1 neurons in this nucleus (Smith et al., 2005), it is unknown at this time whether AVPV CART cells can be directly regulated by estradiol. Given the important role of ARH and AVPV Kiss1 cells in negative and positive steroid feedback respectively, it will be of particular interest to determine if CART populations in these areas are also implicated in this aspect of GnRH regulation.

The potential site for estradiol action for GnRH inhibition is likely numerous. In addition, while previous work has found no evidence for the classic ER's on GnRH neurons, it remains possible that estradiol's inhibitory effects during negative energy balance may occur directly at GnRH cells. Previous work has indicated the likely presence of an unidentified estradiol membrane receptor that appears to directly modulate GnRH cell excitability (reviewed in Kelly and Levin, 2001; Kelly et al., 2002; Lagrange et al., 1995; Zhang et al., 2010). This would suggest that an intermediate cell population may not be required to relay changes in circulating estradiol to functional changes in GnRH neuronal firing and release. Finally, estradiol likely regulates many of the hypothalamic feeding neuropeptides as well as peripheral metabolic sites, such as adipose tissue and the liver, indicating that there are likely many potential sites for estradiol to contribute to metabolically-driven GnRH inhibition.

5. Conclusions

Overall the current dissertation has advanced our understanding of the neuronal populations mitigating metabolically-driven GnRH inhibition as well as providing further evidence on the role of two hormones in regulating these neurocircuits. We have carefully examined regulation of both ARH and AVPV Kiss1 populations and provide evidence that ARH Kiss1 inhibition appears to be common to most models of negative energy balance while AVPV Kiss1 inhibition is likely only achieved in more severe conditions. Additionally, we have demonstrated that coexpressed NKB in ARH KNDy neurons also appears differentially regulated in negative metabolic conditions while DYN levels were consistently unaffected by metabolic disturbances. These findings indicate a potentially important role for differential changes in these three coexpressed neuropeptides for potential autoregulatory functions at KNDy cells.

We also provide further evidence and clarify for the first time that estradiol is likely required for LH inhibition during negative energy balance independent of the it's metabolic effects. By carefully weight-matching OVX animals to OVX+E animals during CR we were able to determine that a 14-day CR

paradigm could inhibit LH levels only in OVX+E animals. Interestingly, estradiol was not required for ARH Kiss1 inhibition during CR, indicating estradiol is required at other parts of the circuitry to contribute to GnRH inhibition. The physiological significance of this finding is not entirely clear, since OVX-like conditions of absent circulating estradiol only naturally occur during menopause when ovarian cycling is already compromised. However, this finding does suggest that estradiol is required to keep GnRH release dampened and without this negative feedback brake it is difficult for full GnRH inhibition to be achieved. This would then indicate that there are likely important cell populations negatively regulated by estradiol that are important stimulators of GnRH release.

In addition to the hormonal effects of estradiol, it was also investigated whether low levels of the adipocyte hormone leptin were a critical signal for Kiss1 and GnRH inhibition during negative energy balance. Surprisingly the work presented here indicates that restoration of leptin to physiological levels had little effect to restore reproductive function. This finding contributes to our overarching hypothesis that many hormonal and neuronal changes are likely required to act in concert to contribute to negative energy balance-induced reproductive inhibition. This includes the inhibition of reproductive-promoting /anorexigenic signals as well as the activation of reproductive-inhibiting/orexigenic signals during negative energy balance. Therefore, estradiol and low leptin are likely just a few of the signals that interact to drive energy conservation in the form of gonadal suppression. The neuronal circuitry likely involves many of the hypothalamic feeding neuropeptides, which importantly includes CART. Work presented in Chapter 4 provides the firsts evidence that ARH CART cells may regulate both GnRH and Kiss1 populations, indicating a potential direct and indirect role for CART in the regulation of GnRH release. We also provide evidence that both CART and Kiss1 levels are inhibited in the hypothalamus during CR, supporting the hypothesis that decreases in these reproductively stimulating signals contribute to metabolically-driven GnRH inhibition.

The complexity of the interaction of metabolic and reproductive hypothalamic circuits exists at multiple levels. First is an observation that many of the hypothalamic feeding and reproductive cell populations coexpress numerous signals involved for regulating each system. For feeding, orexigenic NPY cells in the ARH coexpress the endogenous melanocortin receptor antagonist AgRP and anorexigenic ARH CART cells coexpress POMC. POMC produces the two cleavage products α-MSH and β-END, which are thought to have stimulatory and inhibitory effects on GnRH release, respectively. Similarly, ARH KNDy cells express GnRH stimulating, Kiss1 and NKB, and GnRH inhibiting, DYN, factors. These examples of coexpression of neuropeptides with opposing roles may be required for fine-tuned regulation of target populations and these findings highlight the need for more physiological experiments investigating the functional significance of coexpressed neuropeptides. For instance little is known about how corelease of α-MSH and β-END might effect GnRH release, does one signal overpower the other? In addition, relatively little is known about how differences in firing frequencies might change the release from one neuropeptide to another. Are coexpressed neuropeptides packaged into separate secretory vesicles? And if so are they transported to distinct synapses from one another? We have only begun to scratch the surface on understanding how coexpression might physiologically affect downstream target cells.

Just in the past two years our knowledge of Kiss1 populations has expanded significantly and it now appears that a subpopulation of both ARH KNDy and AVPV Kiss1 cells coexpress galanin, another feeding neuropeptide indicating the ability of Kiss1 cells to potentially play a direct role in metabolic regulation (Porteous et al., 2011). Additional studies have demonstrated that AVPV Kiss1 cells are coexpressed in both dopamine neurons and cells expressing met-enkphalin, which is implicated in GnRH inhibition (Clarkson and Herbison, 2011; Porteous et al., 2011). Therefore, AVPV Kiss1 cells may play a role in both GnRH stimulation and inhibition. This hypothesis is consistent with recent evidence of both symmetrical and asymmetrical Kiss1 synapses on GnRH neurons as well as evidence that Kiss1 is

coexpressed with both glutamate and GABA in both ARH and AVPV nuclei (Cravo et al., 2011; Kallo et al., 2012). This provides further evidence for a likely important and integrative role for these neurons in brain function. These cells likely have multiple roles in both metabolism and reproduction and it may be oversimplified to consider them as only "stimulatory" or "inhibitory" for GnRH release.

In addition to numerous types of neuropeptides expressed in many of these cells, the system is further integrated in view of reports that there is likely cross-talk between many of the key players. ARH Kiss1 cells appear to receive direct inputs from NPY and POMC cells and a subset of Kiss1 cells in both nuclei appear to express the POMC melanocortin receptor 4 (Cravo et al., 2011; Fu and van den Pol, 2010). Further research has provided evidence that Kiss1 may in turn act to stimulate POMC cells and indirectly inhibit NPY cells (Fu and van den Pol, 2010). While these types of electrophysiological studies in which Kiss1 is applied to and saturates an entire brain slice are not representative of physiological conditions, and thus may highlight connections that are of minimal relevance *in vivo,* it still provides evidence of the direction of regulation increased Kiss1 release may have on hypothalamic feeding. GnRH neurons also have projections that make close contact with Kiss1 cells in the non-human primate (Ramaswamy et al., 2008), indicating that this "output" signal may also feedback and regulate the circuit internally. In addition, most of the neuronal populations are thought to be regulated directly or indirectly by the same metabolic hormones such as estradiol, insulin and leptin. It may be of little use to even ponder the first order responders and second order effectors of such cues given that these signals likely affect the circuits in multiple ways and create a change in global output versus discrete changes in one particular cell type.

In conclusion it appears that few neuropeptides or hormones can be categorized as purely reproductive or purely metabolic. Instead, the systems appear intertwined to such a degree that these previous characterizations are uninformative. Future studies will be needed to examine what happens on a global level to numerous hypothalamic populations to begin to understand how these signals might

be integrated to ultimately control energy homeostasis and GnRH release. In addition these efforts may one day lead to the development of multi-level treatment paradigms for conditions of hypothalamic amenorrhea, and potentially other forms of infertility, given the important role these signals likely play in endogenous GnRH regulation as well as metabolically-perturbed conditions.

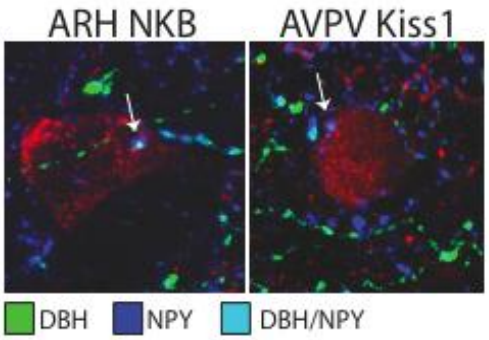


Figure 1. Close appositions of brainstem catecholamine fibers on arcuate nucleus (ARH) NKB and AVPV Kiss1cells.
Immunohistochemistry of colocalized NPY (AB1583, Millipore; 1:5000; blue) and dopamine beta hydroxylase (MAB308, Millipore; 1:5000; green) fibers represents projections from brainstem catecholamine populations that coexpress NPY (for detailed methods see True et al., 2011). Colocalized NPY/DBH fibers (light blue) were found in close contact with ARH NKB cells (3/61, a gift from Dr. Phillipe Ciofi; 1:4000; red, left photomicrograph) and AVPV Kiss1 cells (AB9745, Millipore; 1:1000; red, right photomicrograph).

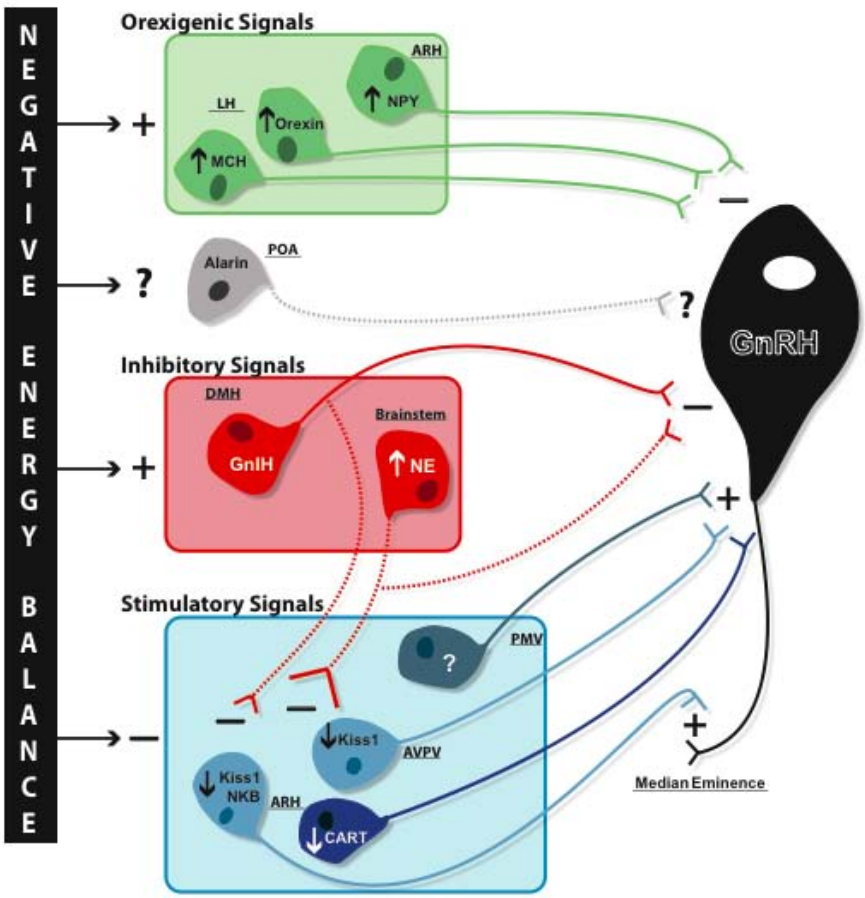


Figure 2. Proposed schematic of negative energy balance-induced changes in reproductive and metabolic circuits contributing to GnRH inhibition.

Negative energy balance results in differential regulation of systems both stimulatory and inhibitory for GnRH release. Orexigenic neuropeptides (green) melanin-concentrating hormone (MCH) and orexin in the lateral hypothalamus (LH) and neuropeptide Y (NPY) in the arcuate nucleus (ARH) are all stimulated (plus sign) with negative energy balance and inhibit (minus sign) GnRH (black) release through direct regulation at cell bodies. It is unknown whether alarin is differentially regulated during negative energy balance, but it is proposed these neurons may also project to GnRH cell bodies (hypothesized regulatory influence and projections represented with dashed lines). Negative energy balance stimulates additional cell populations that are inhibitory for GnRH release (red), namely the dorsomedial hypothalamus (DMH) GnIH and brainstem noradrenaline (NE) populations. While GnIH, and potentially brainstem NE, cells project to GnRH cell bodies, it is hypothesized these cells may also inhibit upstream stimulatory Kiss1 populations. ARH kisspeptin/neurokinin B (Kiss1/NKB) and anteroventral periventricular nucleus (AVPV) Kiss1 populations (blue), which stimulate GnRH terminals and cell bodies, respectively, are inhibited during negative energy balance. ARH CART is also stimulatory for GnRH release and inhibited in conditions of negative energy balance. In addition, negative energy balance is also proposed to inhibit an unknown stimulatory cell population in the ventral premammillary nucleus (PMV) which has direct projections to GnRH cell bodies.

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