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RFamide Peptides and Ovulation: Circadian Control and Environmental Disruptors

By

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Wilbur Putney Williams, III

Abstract

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Successful reproduction depends upon a highly orchestrated cascade of events during optimal environmental conditions in order to appropriately time ovulation. In spontaneously ovulating rodents, neurons in the hypothalamus secreting gonadotropin-releasing hormone (GnRH) are triggered by a timed, stimulatory signal originating from the circadian clock in the suprachiasmatic nucleus (SCN), initiating a cascade of events that ultimately leads to the release of a mature egg from it's follicle. Reproductive viability is maintained in part by the temporal precision of GnRH signaling, and ageing-related declines in ovulatory function are a direct result of the dysregulation of circadian control of GnRH. The transition to reproductive senescence is characterized by irregular estrous cycles prior to any decline in ovarian reserves, suggesting the central mechanisms controlling GnRH are initially responsible for determining reproductive quiescence.

In addition to the circadian control of the HPG axis, most mammals are markedly affected by the availability of energy with regard to reproductive function. In most natural habitats, food availability and energetic status fluctuate markedly, requiring individuals within a species to prioritize survival via foraging and ingestive behaviors over behaviors that perpetuate the individual during periods of low available energy. Consequently, the mechanisms monitoring available energy also regulate the reproductive axis, primarily through inhibiting or permitting the release of GnRH under specific energetic conditions.

The specific mechanisms connecting the circadian clock and energy balance to the reproductive axis remain to be fully characterized. Two related RFamide (Arg-PHe-NH_2), kisspeptin and RFamide-related peptide-3 (RFRP-3) stimulate and inhibit GnRH, respectively, and may bridge the gap between the circadian system, energy balance and reproduction. All studies utilized female Syrian hamsters, which exhibit precise 4-day estrous cycles and are considered to be ideal model systems for understanding reproductive cyclicity. In the first series of experiments, the role of kisspeptin in the circadian control of ovulation was examined. Kisspeptin neurons exhibit a circadian pattern of activation and are regulated by the SCN via vasopressin (AVP) efferents. AVP administration upregulates kisspeptin indiscriminately, but the GnRH response is restricted to the afternoon. Furthermore, GnRH neurons exhibited a timed sensitivity to

kisspeptin administration, suggesting these neurons act as a gating site for upstream signals in order to properly time ovulation.

The circadian control of RFamide peptides may be critical to maintain ovulatory function in aging animals. The second set of experiments investigated whether or not the transition to reproductive acyclicity is characterized by disruptions in the timing of RFamide activation. Middle-aged, female Syrian hamsters exhibiting irregular estrous cycles displayed alterations in the timing of RFRP-3 activation, compared to young and middle-aged, regularly cycling hamsters. Furthermore, middle-aged hamsters exhibit abnormally high levels of kisspeptin immunoreactivity around the time of the LH surge, which may be indicative of improper kisspeptin release during the periovulatory period.

The final set of experiments aimed to determine whether or not the neural circuitry detecting energy status communicates to RFRP-3, and if metabolic challenge, such as food deprivation, alters the activational state of this inhibitory peptide. RFRP-3 activation increases following 48 hours of food deprivation, and may downregulate the HPG axis during periods of negative energy balance. Furthermore, immense innervation of RFRP-3 by the orexigenic peptide, NPY, provides a mechanism of control between these two systems. Finally, mild food restriction over 4-12 days increases RFRP-3 activation, as well as motivation to engage in food hoarding over sexual behaviors. Interestingly, the food restriction paradigm does not alter food intake or estrous cyclicity, suggesting a novel role of RFRP-3 in motivated behaviors.

To My Family

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Chapter 1

Introduction

In order to survive and successfully reproduce, animals in their natural environments must satisfy a variety of competing behavioral and physiological demands on a daily basis. Because selection pressures vary over the course of the day, and all requirements cannot be filled concomitantly, the most successful individuals of a species are those whose behavioral and physiological functions are organized temporally. As a result, organisms have evolved in ways that restrict behavioral activities such as foraging, defending territory, seeking and competing for mates, and sleeping, each to its own specific temporal niche. Not surprisingly, the internal central and peripheral physiological processes subserving these behaviors exhibit the same systematic orchestration. Furthermore, salient environmental pressures, including the availability of food, markedly impact the reproductive axis, such that energetically costly processes, including gestation and lactation, only occur during periods that optimally ensure the survival of offspring. The harmony maintained among organismal events and the environment is coordinated by a clock in the brain, located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, and various fuel detection systems that convey energetic status to the reproductive axis. In mammals, disruptions in circadian function as well as the availability of oxidizable fuels markedly impact reproduction.

Despite the importance of these processes in maintaining reproductive success, the specific mechanisms underlying the circadian control and energetic impact of the reproductive axis remain to be fully characterized. This series of studies explores the contribution of two novel RFamide peptides, kisspeptin and RFRP-3, in the circadian control of ovulation as well as the impact of internal and external factors, including aging and metabolic challenge, on these peptides. Because precise temporal coordination of the endocrine system and the energetic costs of pregnancy and lactation are particularly crucial for mediating reproduction in females, this dissertation focuses on the role of circadian timing and energetic impact in this sex, using Syrian hamsters (*Mesocricetus Auratus*) as a model system to explore the regulation of the ovulatory cycle. Uncovering the relative contribution of RFamide peptides in the circadian control of ovulation, as well as the impact of external and internal disruptors on these peptides, may provide insight into a suite of reproductive maladies that occur in humans. Given that ovulatory pathologies, decreased libido and infertility are associated with circadian disruptions, including chronic jet lag and shift work, eating disorders such as anorexia nervosa, improper dieting or excessive exercise, and age-related disorders such as premature ovarian failure, understanding the mechanisms contributing to RFamide control of ovulation may provide potential targets for ameliorating an array of reproductive health issues.

Initiation of Ovulation

Neurons in the mediobasal hypothalamus that secrete gonadotropin-releasing hormone (GnRH) represent the final common pathway for the central control of reproduction in mammals. When properly stimulated, these neurons release GnRH into the median eminence and act on GnRH receptors in the anterior pituitary to release the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The gonadotropins act on the ovaries to stimulate sex steroid production and gametogenesis, respectively. GnRH neurons integrate various signals from disparate brain regions, incorporating myriad signals from the environment that collectively control the release of GnRH, and therefore, the onset of ovulation. Disruptions in the secretory pattern of GnRH neurons, or upstream signals that stimulate these cells, leads to ovulatory deficits and suboptimal fertility. Characterizing the timing mechanism responsible for triggering GnRH release, as well as the ubiquitous and predictable disruptions that lead to submaximal GnRH secretion, including aging and low energy balance, will lend insight into the neuronal cascade of events that regulate GnRH and fertility under normal conditions.

The release of GnRH into the median eminence is regulated over the course of most of the ovulatory cycle by the negative feedback effects of estrogen from maturing ovarian follicles. In contrast, when levels of estradiol are highest, positive feedback effects of estradiol induce a surge in GnRH release that ultimately leads to ovulation. A surge of progesterone enhances the effects of estrogen and leads to the induction of behavioral estrus, during which female rodents are highly motivated to engage in sexual behavior that maximizes reproductive success. Despite the relative conservation of the effects of estradiol amongst spontaneously ovulating species, the mechanisms underlying feedback mechanisms of estradiol remain incompletely characterized, though evidence points to the importance of ERα receptors in mediating estradiol negative and positive feedback in regions upstream of the GnRH system, especially in the AVPV (Herbison and Theodosis, 1992b; Dorling et al., 2003; Wintermantel et al., 2006). Estrogen may exert some effects at the level of the pituitary gland as well, as sensitivity to GnRH within the adenohypophysis is markedly increased on the day of proestrous, suggesting a priming effect of this sex steroid, contributing to the surge in gonadotropins (Cooper et al., 1974). Despite the established role of estrogen feedback for the GnRH surge, it is unclear whether a single system switches from negative to positive feedback, whether two independent systems (one positive and one negative) differentially dominate throughout the cycle, or a combination of the two mechanisms mediate the switch from negative to positive feedback, to allow for the LH surge.

The relative necessity of estrogen in permitting GnRH onset differs among species. In spontaneous ovulating rodents, rats and mice require high levels of estrogen in order to elicit a proestrous-level GnRH surge, and ovariectomized rats or mice fail to produce an LH surge without exogenous treatment of estradiol (Legan and Karsch, 1975; Christian et al., 2005). The necessity of estrogen in Syrian hamsters, on the other hand, differs depending on the specific photoperiod. In 'long day' photoperiods consisting of at least 14 hours of light per 24 hours, estrogen is necessary to induce the LH surge. In

intact models, Syrian hamsters only exhibit an LH surge on proestrous and remain in a state of persistent anestrous following ovariectomy (Norman et al., 1973; Seegal and Goldman, 1975). However, female Syrian hamsters held in 10 hours of light exhibit timed, daily LH surges following ovariectomy, suggesting liberation from this sex steroid under certain photoperiodic conditions (Seegal and Goldman, 1975; Bittman and Zucker, 1977). It remains unclear how photoperiod dictates the relative level of control that estrogen exerts on the GnRH system, but suggest extra-steroidal mechanisms of ovulatory control within this species. Despite this difference among short- and long-day Syrian hamsters, the temporal precision of the LH surge in this species provides an ideal model system to investigate the neural mechanisms underlying the GnRH surge.

Circadian Control of Ovulatory Function

Ovulation in many species exhibits a precise temporal component that occurs in the late afternoon near the transition to darkness, a time preceding the period of sexual receptivity in nocturnal rodents (Nequin et al., 1975), thus increasing the likelihood of successful pregnancy (Sarkar et al., 1976). The precision of the estrous cycle was initially postulated to be governed by a central, 24-hour clock by Everett and Sawyer, who described the 'neurogenic activation of the hypophysis' as a necessary component of ovulation. They discovered brief injections (30-45 minutes) of sodium pentobarbital were capable of delaying ovulation in female rats by exactly 24 hours. Administration outside of a critical time window, relative to the light-dark cycle, failed to prevent ovulation and indicated that this phenomenon was governed by a central, circadian clock (Everett and Sawyer, 1950). Work over subsequent decades determined that the critical signal triggering ovulation involved an endogenous time-keeping mechanism, rather than a response to external time cues. Using Syrian hamsters (*Mesocricetus auratus*) as a model system, the nature of this central regulation of gonadotropin release was thoroughly characterized as an endogenous, multioscillator system that can be entrained by the external environment, but is capable of regulating estrous cycles through an internal time-keeping mechanism. The temporal precision of estrous cyclicity is abundantly clear in female Syrian hamsters held in photostimulatory light schedules (16:8), where estrus onset occurs every 96 hours (Alleva et al., 1971). However, the free-running period of LH onset in female hamsters held in constant conditions provided the first definitive evidence of an endogenous timekeeper regulating mammalian estrous cycles. Under these conditions, a process regulated by an endogenous clock should "free run," revealing the endogenous nature of the estrous cycle ‘clock’. In contrast, if the external environment participates in the LH surge, then this rhythmicity should be abolished in constant conditions. The estrous cycle of female hamsters held in constant conditions (constant light or 'LL'), as measured by behavioral heat onset, exhibited free-running rhythms longer than 96 hrs, reflecting the internal rhythm of the circadian clock in the absence of entraining photic information (Alleva et al., 1971). This reveals the estrous cycle period is governed by a central clock with an endogenous rhythm that does not necessitate an externally-mediated time cue. Furthermore, the same clock regulating estrous onset was postulated to regulate other processes that exhibit a free-running rhythm in the absence of external cues, such as locomotor activity (Aschoff, 1960; Pittendrigh, 1960; Decoursey, 1964).

The development of the radioimmunoassay by Rosalin Yalow and Solomon Berson in the 1950s allowed for the measurement of rhythmic hormonal release. The free running periodicity of estrous cycles, measured by heat onset (i.e. susceptibility to lordosis, gonadotropin surge and vaginal cytology), occurs in increments that are exactly 4 times the free running period of locomotor onset, and can be predicted by daily shifts in locomotor behavior under constant conditions (Fitzgerald and Zucker, 1976). The coupling of estrous onset and activity onset in both LD and LL conditions suggested that either the LH surge and locomotor activity onset are controlled by a single, endogenous oscillator, or a coupled, multioscillator system that regulates the rhythms of each process independently (Fitzgerald and Zucker, 1976). Subjecting female hamsters to various LD schedules effectively uncouples locomotor and estrous onset phase relationships, such that the time lag between estrous and locomotor onset shifts among various lighting schedules, suggested multiple oscillators, or differential output mechanisms, may regulate each process independently (Carmichael et al., 1981). The nature of the circadian control of behavioral and hormonal rhythms was further characterized by exploiting "splitting" phenomena in hamsters held in prolonged, constant LL. Under these conditions, hamsters may exhibit periods of activity that "split" into two bouts of locomotor activity occurring 12 hours apart- suggesting the dissociation of at least two independent oscillators. Female, ovariectomized (OVX) hamsters treated with estrogen exhibited two LH surges in a 24-hour period, each phase-locked to an individual activity bout (Swann and Turek, 1985). It is now clear that estradiol is permissive for the circadian clock to trigger the GnRH/LH surge at a precise time point, a phenomenon observed in hamsters (Norman et al., 1973), rats (Legan and Karsch, 1975), and later, mice (Christian et al., 2005).

The central mechanism underlying circadian control of estrous cycles resides in the master circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. Ablation of the SCN abolishes circadian rhythms of myriad hormonal and behavioral processes (Moore and Eichler, 1972; Stephan and Zucker, 1972), including those responsible for the gonadal response in rodents (Stetson and Watson-Whitmyre, 1976; Brown-Grant and Raisman, 1977; Stetson and Watson-Whitmyre, 1977; Gray et al., 1978; Mosko and Moore, 1979b, a; Kawakami et al., 1980; Turek, 1988). Furthermore, knife cuts isolating the SCN from efferent targets disrupt ovulation, suggesting the SCN requires intact neuronal signaling in order to regulate reproductive processes (Nunez and Stephan, 1977; Gray et al., 1978; Mosko and Moore, 1979a; Wiegand and Terasawa, 1982). More recently, genetic techniques that alter the period of SCN clock genes via the *tau* mutation predictably shorten estrous periodicity (Lucas et al., 1999) and genetically disrupting the molecular components responsible for clock function at the cellular level (Miller et al., 2004) severely disrupts the LH surge, suggesting an intrinsic connection between the genomic machinery of the clock and reproductive competence. In addition to the effects on HPG axis activity following genetic or surgical disruptions of the SCN, several studies of unaltered functionality connect the central clock to reproduction. Tracing work indicates the SCN communicates rhythmic information to brain areas including hypothalamic cell phenotypes driving reproductive function, through extensive direct and indirect neural projections (DeVries et al., 1985; Watts and Swanson, 1987; Van der Beek et al., 1997). In 'split' hamsters held in constant conditions, each antiphase

locomotor bout and LH surge is accompanied by a "split" SCN- whereby unilateral activation, measured by the neuronal activation marker, FOS, within each hemisphere is associated with the timing of each bout of activity and estrous onset (de la Iglesia et al., 2000).

While it is apparent that the same circadian pacemaker regulates locomotor and estrous onset, the communication modalities mediating each process likely differs. Transplants of fetal SCN tissue into bilaterally SCN-lesioned hamsters restores locomotor but not estrous rhythmicity, suggesting an intact neural connection is required for endocrine but not behavioral rhythmicity (Silver et al., 1990; Meyer-Bernstein et al., 1999). Furthermore, the antiphase FOS activation of the SCN in split, female hamsters is concurrent with ipsilateral activation of the GnRH system, suggesting a functional, neural connection underlies the circadian control of reproduction (de la Iglesia et al., 2003; Gibson et al., 2008). Tracing studies corroborate the neural evidence for this activational response, as projections from the SCN to hypothalamic cell phenotypes driving reproductive function are predominately ipsilateral (DeVries et al., 1985; Watts and Swanson, 1987; Van der Beek et al., 1997). Despite the extensive evidence for the circadian control of reproduction in several spontaneous ovulators, the mechanisms underlying neuronal SCN control of ovulation remains enigmatic. Several SCN-derived peptides and intermediate neural systems have been strongly implicated in mediating the GnRH surge.

Direct and Indirect Circadian Control of Ovulation

A. VIP

Neurons synthesizing vasoactive intestinal polypeptide (VIP) are located in the retinorecipient, ventrolateral SCN (Ibata et al., 1989; Tanaka et al., 1993), and represent potential output signals conveying information about the external environment to SCN target systems. A monosynaptic pathway of SCN-derived VIPergic neurons projects directly to GnRH neurons (Van der Beek et al., 1997; Horvath et al., 1998) that express the VIP receptor VIP_2 (Smith et al., 2000). Several lines of evidence indicate an important role for this pathway in the timing of ovulation. GnRH neurons receiving VIPergic input preferentially express the neural activation marker, fos, during the afternoon of the LH surge on proestrous (van der Beek et al., 1994). In vivo antisense antagonism of VIP in the SCN abolishes GnRH/fos activation in OVX+E2 primed female rats, suggesting the necessity of VIP output in triggering the afternoon GnRH surge (Harney et al., 1996; Gerhold et al., 2005). Furthermore, blocking the VIP_2 receptor attenuates GnRH neuronal cell firing during the afternoon surge in female, estrogen treated mice (Christian and Moenter, 2008). The expression of VIP afferents on GnRH neurons is sexually dimorphic, with females rats exhibiting higher VIPergic innervation (Horvath et al., 1998) suggestive of a specific role for VIP estrous regulation. From a developmental standpoint, the number of VIP-GnRH contacts increases between prepubertal and adult female rats, and VIP innervated GnRH neurons exhibit lower activation levels in middle-aged female rats, suggesting this SCN peptide may be

partially responsible for the initiation and decline of reproductive competence in female rodents (Krajnak et al., 2001); Together, these lines of evidence suggest a positive role for VIP in the control of the GnRH surge.

Though it is likely essential, the direct VIPergic pathway from the SCN to GnRH is not sufficient for the generation of the LH surge in female rodents. Specifically, the positive feedback effects of estradiol necessary for surge generation likely target intermediate nuclei that express abundant ER (Pfaff and Keiner, 1973; Simerly et al., 1996; Shughrue et al., 1997). Furthermore, the pharmacological effects of VIP on the LH surge are equivocal, as some studies have implicated VIP as inhibitory (Alexander et al., 1985; Akema et al., 1988; Weick and Stobie, 1992) while other have indicated an excitatory role of VIP (Palm et al., 1999; Christian and Moenter, 2008). These conflicting pharmacological results have prevented definitive clarification of the specific role VIP plays in circadian surge generation.

B. AVP

A second pathway involves a multisynaptic, vasopressinergic (AVP) projection from the dorsomedial SCN to estrogen-responsive cells in the anteroventral periventricular nucleus (AVPV) (Hoorneman and Buijs, 1982; DeVries et al., 1985; de la Iglesia et al., 1995; Watson et al., 1995; Leak and Moore, 2001), an area that also expresses the vasopressin receptor, $V1_a$ (Ostrowski et al., 1994; Funabashi et al., 2000b). The convergence of circadian output signals and estrogen feedback within the AVPV has generated much interest in this nucleus as the integration site of these dual signals in the regulation of the GnRH surge. Vasopressin secretion in the SCN is directly controlled by circadian clock genes (Grace et al., 1999; Munoz et al., 2002) and is released in a circadian manner (Shinohara et al., 1994), with a peak just before the onset of the LH surge (Schwartz et al., 1983; Kalsbeek et al., 1995), providing a likely mechanism of SCN-derived control of the HPG axis. In POA and SCN cocultures, AVP but not VIP release synchronizes GnRH release in a circadian manner (Funabashi et al., 2000a), suggesting vasopressin may act as a circadian stimulator of GnRH resulting in the afternoon surge. Furthermore, AVP injections directed at the medial preoptic area (mPOA; the location of hypophysiotropic GnRH cells in rats) produces surge-like LH levels in SCN lesioned, ovariectomized, estradiol-treated rats (Palm et al., 1999). By contrast, central AVP receptor antagonists attenuate the LH surge in proestrous rats (Funabashi et al., 1999). Finally, the inability of *clock* mutant mice to generate an LH surge is associated with diminished *AVP* mRNA expression in the SCN, a phenotype that can be restored via central injections of AVP, linking this peptide to the circadian control of ovulation (Miller et al., 2004). Interestingly, in SCN-intact, OVX+E2 treated female rats, central injections of AVP are only capable of inducing a surge-like pattern of GnRH secretion during a narrow time window in the afternoon (Palm et al., 2001), suggesting the downstream targets of the SCN are differentially sensitive to this peptide- providing evidence of a multilevel gating mechanism restricting the GnRH surge to the afternoon in rodents.

C. The AVPV

While the existence of a monosynaptic connection between SCN-derived AVP and GnRH has not been reported, the regulation of GnRH secretion by AVP likely involves an indirect mechanism via the sexually dimorphic AVPV nucleus of the hypothalamus (Gu and Simerly, 1997; Herbison, 2008). mRNA for the vasopressin receptor, V1a, is exceedingly sparse in GnRH neurons (Kalamatianos et al., 2004), whereas neurons within the AVPV express high levels of V1a mRNA colocalized with estrogen receptors (Funabashi et al., 1999; Kalamatianos et al., 2004), providing a potential integration mechanism for circadian signals and positive feedback actions of estrogen within this nucleus. In agreement with this contention, anti-estrogens targeting the AVPV suppress the LH surge in ovariectomized, estradiol-treated rats (Petersen and Barraclough, 1989) and vasopressinergic efferents from the SCN directly target ER expressing cells within the AVPV (Watson et al., 1995).

The AVPV has been strongly implicated in the timing and stimulation of the LH surge, with monosynaptic projections from this region targeting GnRH cells, FOS expression in this nucleus coinciding with the LH surge, and lesions of the AVPV eliminating estrous cyclicity in both intact and OVX+E2 treated rats (Wiegand et al., 1980; Wiegand and Terasawa, 1982; Ronnekleiv and Kelly, 1988; Gu and Simerly, 1997; Le et al., 1999). Together with the extensive projections from the circadian clock (de la Iglesia et al., 1995) and abundance of neurons expressing estrogen receptors (Herbison and Theodosis, 1992a, b; Shughrue et al., 1997) that receive direct input from the SCN (Watson et al., 1995), the AVPV is a likely candidate region to transmit integrated circadian and steroidogenic signals to GnRH neurons and trigger the surge. The specific neuronal phenotype mediating this integration remains to be established, with a multitude of neuropeptides and neurotransmitters expressed within this relatively small nucleus, many of which express ER, including glutamate, GABA, galanin, dynorphin, enkephalin, substance P, neurotensin, and the RFamide peptide, kisspeptin (reviewed in (Herbison, 2008).

D. RFamide Peptides

Despite the established role of the circadian clock and estrogen in the regulation of GnRH secretion and, ultimately, ovulation, the specific targets and of these requisite signals remain unclear. The dearth of evidence for estrogenic actions on GnRH neurons and the established connection between the SCN and upstream nuclei, such as the AVPV, in regulating ovulation, suggests the integration of these signals occur via extra-GnRH mechanisms. The present studies focus on two related candidate peptides, RFRP-3 and kisspeptin, as crucial mediators of GnRH release. The contribution of RFamide peptides in ovulatory function and their regulation by the circadian clock, as well as the role of internal (aging) and external (negative energy balance) disruptors in contributing to suboptimal ovulatory function through these peptides was specifically assessed.

The RFamide family of peptides is characterized by an Arg-Phe-NH_2 motif at the C-terminus and consists of multiple members capable of acting as neurotransmitters, neuromodulators and hormonal signals in a host of invertebrate and vertebrate species. The first identified RFamide peptide was discovered in 1977 as a cardioexcitatory

neuropeptide in the molusk, *Macrocallista nimbosa* (Price and Greenberg, 1977). Subsequent work has identified myriad functions of related RFamide peptides across taxa, from feeding and growth hormone release in amphibians to analgesia via serotonergic and opioid regulation in mammals (Reviewed in (Tsutsui et al., 2010).

While the isolation of an RFamide peptide in vertebrates first occurred in 1983 (Dockray et al., 1983), it wasn't until 2000, when Tsutsui and colleagues isolated and characterized the dodecapeptide SIKPSAYLPLRFamide (with an identical C-terminus as the initially characterized peptide) from Japanese quail brain, that RFamides were discovered to play a role in reproductive processes. Tsutsui et al. determined its location to be in the hypothalamic-hypophyseal portal system, and using cultured quail anterior pituitaries, discovered a dose-dependent inhibition of gonadotropin release following administration of this novel peptide. Because of its direct inhibition on gonadotropin release, they named the novel peptide gonadotropin-inhibitory hormone, or GnIH (Tsutsui et al., 2000).

Subsequent studies have uncovered the mammalian ortholog of GnIH, RFRP-3, in cow, rat, hamster, mouse, sheep, monkey and human (reviewed in (Kriegsfeld et al., 2010; Tsutsui et al., 2010). In rats, hamsters and mice, RFRP-3 neuronal cell bodies are tightly localized to the dorsomedial hypothalamus (DMH) and project directly to the POA, forming close appositions to GnRH cells (Kriegsfeld et al., 2006). Central administration of RFRP-3 rapidly suppresses LH release and inhibits sexual behavior in males and females of multiple species (Kriegsfeld et al., 2006; Johnson et al., 2007; Anderson et al., 2009), though the specific mechanism of action requires further investigation. Application of RFRP to GnRH cells in brains slices from male and female mice decreased neural activity in a subset of cells (Ducret et al., 2009), supporting a suppressive role for this peptide via direct actions on GnRH. Furthermore, electrophysiological recordings suggest a direct postsynaptic inhibition of GnRH cell firing may occur via RFRP-3-mediated hyperpolarization of potassium (K+) channels in vGluT2-GnRH neurons (Wu et al., 2009). In addition to the direct action on GnRH, it is unclear whether RFRP acts at the level of the pituitary in mammals. Suggestive evidence for this possibility comes from studies showing that the RFRP receptor (GPR147) is localized to rat and Syrian hamster pituitaries (Hinuma et al., 2000; Gibson et al., 2008), and RFRP-ir fibers have been reported to extend into the external layer of the median eminence in hamsters (Gibson et al., 2008). Additionally, recent studies of rats suggest that RFRP inhibits GnRH-elicited release of LH at the level of the pituitary (Murakami et al., 2008). In other studies using a different antiserum to RFRP, RFRP-ir was not observed in the median eminence of rat brain, and intraperitoneal injections of the retrograde tracer Fluorogold only labeled a small number of RFRP cell bodies (Rizwan et al., 2009), suggesting that most of these cells do not reach the hypophyseal portal system. However, intravenously administered RFRP rapidly (within 3 minutes) inhibited GnRH-induced release of LH (Rizwan et al., 2009) as also occurs in birds (Osugi et al., 2004). These data indicate the potential for RFRP to act on the release of pituitary gonadotropin. Whether or not endogenous RFRP acts on the pituitary in addition to GnRH cells in mammalian species requires further investigation to clarify whether discrepant findings represent interspecific differences or result from technical variation across studies. The

availability of the RFRP-3 receptor antagonist, RF9, will provide further evidence into the specific signaling pathways mediating the effects of this peptide on the HPG axis.

RFRP-3 neurons may integrate steroidal and circadian feedback in order to properly regulate the GnRH surge. At least 40% of RFRP neurons express ERα in the DMH of hamsters (Kriegsfeld et al., 2006) and RFRP-3 activation decreases on proestrous, compared to other days of the estrous cycle in Syrian hamsters (Gibson et al., 2008), suggesting an effect of steroidal feedback on the activation of RFRP-3. Furthermore, estradiol downregulates *RFRP-3* mRNA in female mice, though the low expression of ERα in this species suggests possible indirect effects of estrogen on RFRP neurons (Molnar et al., 2011). Additionally, the SCN projects monosynaptically to RFRP-3 neurons in the DMH of Syrian hamsters, and split females exhibit ipsilateral inhibition of RFRP-3 concomitant with SCN and GnRH fos activation, indicative of a neural regulation by the circadian clock (Gibson et al., 2008). Therefore, in addition to triggering stimulatory signals that lead to the activation of the GnRH surge, the SCN may concurrently attenuate inhibitory signals, such as RFRP-3, in order to maximally elicit a GnRH response.

The discovery of kisspeptin, a related RFamide peptide with opposing actions on the HPG axis, has markedly impacted the field of reproductive biology since its initial isolation in human placenta and discovery as the endogenous ligand for the orphan G-protein coupled receptor, GPR-54 (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Although most research around kisspeptin focuses on its stimulatory effects on GnRH and role in puberty onset, its initial function was characterized as a tumor metastasis suppressor sequence, thus given the name metastin (Lee et al., 1996). The Hershey, Pennsylvania group credited for this discovery shortened the name of the gene encoding the metastin peptide to *KiSS1*, reflecting its suppressor sequence 'SS,' while 'Ki' was added as a ‘nod’ to the area's more famous discovery, the Hershey's Kiss (Lee et al., 1996). Typically, metastin is still used in the field of cancer biology, while reproductive biologists refer to the RFamide as kisspeptin. *Kiss1*-GPR-54 signaling was discovered to play a critical role in reproductive physiology in 2003, when two groups reported idiopathic hypogonadotropic hypogonadism in humans with a mutation of the GPR-54 receptor, while an identical phenotype was observed in mice with a targeted deletion in this receptor (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003). Since this initial discovery, kisspeptin has emerged as a critical regulator of reproduction in males and females across taxa (Reviewed in (Oakley et al., 2009)).

The gene *Kiss1* encodes a family of neuropeptides, kisspeptins, that play a significant role in positively regulating the reproductive axis. The initial product is a 145 amino acid propeptide, kisspeptin-145, which is cleaved into the active 54 amino acid peptide, kisspeptin-54. Three shorter products, kisspeptin 10, 13 and 14 are the result of further cleavage from the 54 amino acid sequence; all are biologically active, bind with similar affinity to the Kiss1 receptor, and share an RFamide sequence on their C termini (Kotani et al., 2001; Muir et al., 2001). In rodents, *Kiss1* mRNA expressing cells are localized to the AVPV and ARC nuclei, both of which highly express the estrogen receptor ERα (Gottsch et al., 2004; Smith et al., 2005b; Clarkson and Herbison, 2006;

Revel et al., 2006; Greives et al., 2007). The effects of estrogen on kisspeptin activity, however, varies by nucleus, with ovariectomy decreasing *Kiss1* mRNA in the AVPV and increasing *Kiss1* in the ARC, while estrogen treatment elicits the opposite effect in each nucleus (Smith et al., 2005a; Smith et al., 2005b). Therefore, the effects of estradiol on these nuclei are thought to mediate steroid positive and negative feedback, respectively. Exogenous kisspeptin administration potently induces LH release as well as upregulates FOS expression in GnRH neurons (Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Navarro et al., 2005a; Navarro et al., 2005b). In addition to direct actions of kisspeptin on GnRH cells (Irwig et al., 2004; Han et al., 2005; Kinoshita et al., 2005; Clarkson and Herbison, 2006; Smith et al., 2008), kisspeptin may stimulate the release of GnRH via direct actions on GnRH terminals in mice (d'Anglemont de Tassigny et al., 2008). The potent actions of kisspeptin on GnRH and the direct feedback effects of estrogen on these neurons make kisspeptin a likely candidate system in the induction of the GnRH surge. *Kiss1*/Fos expression is coordinated with LH surge in naturally cycling rats and in ovariectomized, estradiol-treated animals (Smith et al., 2006b; Adachi et al., 2007) and follows a circadian pattern of expression coincident with the LH surge in mice held in constant darkness (Robertson et al., 2009). These results implicate Kiss1 neurons in the AVPV as targets of estrogen positive feedback and potentially targets of the circadian clock, although the specific role of kisspeptin in the circadian control of the GnRH surge has not been explored.

Aging Disrupts the Circadian Control of Ovulation

Reproductive viability in female vertebrates requires precise, temporally controlled secretion of GnRH. With age, the precision of GnRH secretion decreases, leading to irregular cyclicity and eventually, reproductive senescence. The ubiquitous nature of this decline makes the transition to acyclicity an ideal model system for uncovering the dynamics of GnRH regulation, as well as discovering the specific mechanisms by which the SCN regulates ovulation under normal circumstances. Reproductive senescence in females is defined as the inability to produce viable oocytes. Historically, it was generally accepted that the transition to reproductive senescence resulted from a depletion of ovarian follicles and that changes in the CNS components of the reproductive axis resulted from reductions in ovarian estrogen (Mandl and Shelton, 1959; Nelson et al., 1987; Richardson et al., 1987). Whereas this mechanism drives the transition to menopause in primates, results from numerous studies suggest that aging causes changes in the brain that precede deficits at the level of the ovary in rodents (van der Schoot, 1976; Cooper et al., 1980; Sopelak and Butcher, 1982; Wise, 1982a; Felicio et al., 1983; Lloyd et al., 1994; Lu et al., 1994; Nelson et al., 1995). Thus, it is likely that the transition to reproductive senescence results from dysregulation of the reproductive axis at the levels of both the brain and the ovaries, at least in rodents (Rubin, 2000). Importantly, because ovarian follicles are the primary source of estrogen, many non-reproductive functions are also compromised following the transition to reproductive senescence, including: the regulation both of cardiovascular function and bone metabolism as well as protection from neurodegenerative diseases such as Alzheimer's

and Parkinson's disease (Downs and Wise, 2009). By unraveling the contributions of the brain in the transition to reproductive senescence, insight into the general process of the aging of the brain can be gained, as reproductive aging occurs in advance of many other pathological changes that tend to confound gerontological studies (Wise, 1999).

A. Peripheral Control of Reproductive Aging

As suggested above, reduction in the number of ovarian follicles is associated with reproductive aging in rats (Mandl and Shelton, 1959; LaPolt et al., 1998), mice (Nelson et al., 1987) and humans (Hansen et al., 2008), and is considered to be the hallmark of reproductive senescence. These peripheral changes in ovarian function can have a marked impact on the HPG axis. For example, when follicular reserves are experimentally reduced in young rats, reproductive deficiencies that are normally seen in aged animals emerge, including deficits in secretion of gonadotropin and abnormal estrous cycles (Meredith and Butcher, 1985). Conversely, slowing the loss of ovarian follicles through chronic treatment with progesterone (LaPolt et al., 1998) or through dietary restriction (Nelson et al., 1985) can delay age-related anomalies in estrous cycles. Moreover, experimental reduction in follicular reserves reduces the magnitude of the preovulatory LH surge, ovulation rate and estrous cyclicity in both young and middle-aged rats, further suggesting that with advancing age ovarian decline impacts hypothalamo-pituitary function (Anzalone et al., 1998). Importantly, animals with experimental reduction in follicular pools have unaltered estradiol profiles throughout their cycles, suggesting that the reduction in follicular pool-size affects the central neuroendocrine response independent of changes in gonadal steroids.

The molecular machinery that drives circadian rhythms in cells of the SCN also operates relatively ubiquitously throughout the brain and periphery (Hardin, 1994; Tosini and Menaker, 1996; Sun et al., 1997; Balsalobre et al., 1998; Zylka et al., 1998; Yamazaki et al., 2000). The intracellular clockwork consists of interacting transcriptional and translational feedback loops that operate with a period of about 24 hours (for review see (Okamura, 2007). Briefly, the transcription of three *Per* genes (*1-3)* and two *Cry* genes (*1 and 2)* is driven by the dimerization of the bHLH/PAS containing CLOCK and BMAL1 transcription factors (Gekakis et al., 1998; Takahata et al., 1998; Shearman et al., 2000). To complete the cycle, PER and CRY proteins translocate into the nucleus as multimeric complexes to discontinue CLOCK::BMAL-mediated transcription (Shearman et al., 2000). A number of cytoplasmic enzymes phosphorylate the PER and CRY proteins and play an important role in maintaining the precise timing of feedback loops (Lowrey et al., 2000). The SCN synchronizes independent oscillators in peripheral tissues, with loss of synchrony among independent cellular oscillators in the absence of SCN input (Welsh et al., 2004). Thus, the SCN acts as a "master clock" that synchronizes molecular rhythms in the periphery that, in turn, regulate the timing of a vast array of physiological and behavioral rhythms (Guo, 2006;Pando, 2002).

The ovaries have been shown to express daily rhythms in the clock genes *Per1* and *Per2* (Fahrenkrug et al., 2006; Karman and Tischkau, 2006). The integrity of these local clocks may be important for normal ovarian function; genes contributing to the

initiation of ovarian steroid production are rhythmic in the human ovary (Bao et al., 2003; Foster et al., 2005). Ovarian clock genes may also have a functional role in the synthesis of progesterone. In domestic chickens, the largest and most mature preovulatory follicle expresses diurnal changes in *Per2* and *Per3*, whereas smaller follicles do not exhibit changes in clock-gene expression (Nakao et al., 2007). These changes are concomitant with increases in StAR, a regulatory protein that increases synthesis of progesterone (Nakao et al., 2007). Importantly, StAR genes contain E-Box enhancers (Christenson and Strauss, 2001), DNA elements that are direct targets of the molecular clock. Although correlational, these studies suggest a potential role for local timing mechanisms in ovulatory control. Although previous work has confirmed the existence of rhythmically expressed clock genes in the ovarian cells of rats, the role of murine ovarian circadian clock genes is largely speculative (Fahrenkrug et al., 2006). Whether or not alterations in ovarian clock genes contribute to the maintenance of ovarian function and/or reproductive decline with age remains an open question.

B. Central Control of Reproductive Aging

Whereas alterations at the level of the ovary play an integral role in reproductive decline associated with advancing age, a number of lines of evidence suggest that central mechanisms also contribute to these changes. The timing of neural signals that control the release of gonadotropin exhibit marked changes in middle-aged women and non-human animals, and these modifications may accelerate the loss of follicles with advancing age (Nass et al., 1984; Wise, 1993; Hall et al., 2000; Gore et al., 2004). Transplanting the ovaries of old rats into young, ovariectomized rats restores follicular development and ovulation, indicating that aged ovaries maintain ovulatory function in some cases (Krohn, 1955; Peng and Huang, 1972). Likewise in young rats, electrolytic lesions of the medial preoptic area (POA), where GnRH neurons reside, result in irregular estrous cycling akin to that seen in older rats (Clemens and Bennett, 1977). Conversely, stimulation of the medial POA in old, acyclic rats results in an enhanced LH response (Wuttke and Meites, 1973). Together, these studies suggest that the brain contributes to aging of the reproductive axis and represents an important consideration for further investigation.

One caveat to consider when generalizing from rodent models to humans is that changes at the level of the brain may not be equivalent. In postmenopausal women, for example, gonadotropin levels are high from loss of negative feedback, whereas aged acyclic rats have normal levels of LH (Lu et al., 1994; Wise et al., 1999). Furthermore, follicular loss beginning in middle-aged women eventually leads to the complete depletion of ovarian follicles. In aged, acyclic rats, however, remaining follicles have been reported (Wise, 1999). These findings suggest that the contribution of hypothalamic decline versus the exhaustion of ovarian follicles to reproductive senescence likely differs between rodents and humans. Indeed, estrogen and progesterone administration in postmenopausal women can still generate or inhibit LH and FSH surges, depending on the timing and level of gonadal steroid treatment, suggesting the maintenance of positive and negative feedback in postmenopausal women (Gill et al., 2002; Ottowitz et al., 2008).

Despite these differences, rodent models can provide important insight into human menopause. In both rats and humans, for example, a rise in concentration of FSH remains a hallmark of impending reproductive decline (DePaolo, 1987; Klein et al., 1996), though a rise in human FSH has been primarily linked to a decrease in inhibin B, the main ovarian-derived negative feedback peptide in humans, through depletion in follicle numbers (Burger, 1993; Burger et al., 2000). The pattern of LH secretion also changes in both perimenopausal humans and in rats transitioning into acyclicity. The duration of LH pulses increases and the frequency decreases in both premenopausal women and middle-aged, regularly cycling rats (Scarbrough and Wise, 1990; Matt et al., 1998). The ability of estradiol to induce LH surges becomes attenuated in perimenopausal women (van Look et al., 1977) and middle-aged rats (Wise, 1984). The studies by van Look and colleagues and Matt and colleagues, however, take place in women that are well in to the transition to menopause and therefore have a marked decrease in follicle number, which alters levels of ovarian hormones such as inhibin B and may result in changes in cyclicity that precede any alterations in the brain's responsiveness to positive feedback. Therefore, while changes in the brain's ability to control the timing of the LH surge and respond to the negative and positive feedback of circulating estradiol contribute to the desynchrony of gonadotropin secretion in rats transitioning into senescence, the impact of changes in positive and negative feedback on the transition to menopause in humans remains controversial (Hall, 2007). This underscores the idea that the origin of the dysregulation of the brain's control of the HPG axis differs between rats and humans. In rats, changes in the hypothalamus contribute to reproductive senescence, whereas alterations in gonadotropin secretion in humans transitioning to menopause are preceded by the elimination of negative feedback originating from follicle depletion. Although the mechanisms differ, the resulting impact on general HPG axis activity affects both aged rodents and primates. Recent studies indicate that the increase in circulating gonadotropins may be a result of hypertrophy of kisspeptin neurons in the infundibular nucleus (Rance, 2009). These results are almost identical to rodent studies of arcuate kisspeptin neurons after ovariectomy, and may constitute a neural compensatory mechanism to ovarian failure (Rance, 2009). Further research is needed to fully elucidate the neural mechanisms underlying HPG activity during the transition to menopause. Thus, using rodent models of hypothalamic decline in reproductive aging can lend insight into human menopause, as reliable endocrine markers for predicting the full transition to anovulation (including final menses) in humans have yet to be specified (Burger, 2008).

One of the first indicators of reproductive decline in rodents involves an attenuated and delayed LH surge on the day of proestrus (Wise, 1982b). This decreased LH response occurs prior to any changes in pituitary responsiveness to GnRH or reductions in GnRH cell numbers (Wise, 1982b; Lloyd et al., 1994; Krajnak et al., 2001), although a decrease in neuronal activation is seen in GnRH neurons in middle-aged, regularly cycling rats (Lloyd et al., 1994). It is likely that the decreased and delayed LH surge seen in middle-aged animals stems from an alteration in the timed activation pattern of GnRH neurons (Lloyd et al., 1994; Rubin and King, 1994; Krajnak et al., 2001). This decline in GnRH activation may reflect alterations in the strength and timing

of the afferent inputs to this system originating from the circadian clock (Krajnak et al., 1998). Additionally, significant alterations in SCN rhythmicity are observed with advancing age and may contribute to deficits in the timing of GnRH neuronal activation at the time of the LH surge (Wise et al., 1988). Furthermore, unlike the condition in young animals, rhythms in vasoactive intestinal polypeptide (VIP) mRNA are not apparent in middle-aged female rodents (Krajnak et al., 1998). Suppression of VIP in the SCN through the administration of antisense oligonucleotides also leads to an aging-like attenuation of cyclic secretion of LH (Harney et al., 1996). Together, these findings suggest that changes in the timing and amplitude of the preovulatory LH surge may result from age-related changes in the SCN and in its neurochemical output.

Perhaps the most noticeable alteration in behavior associated with advancing age is in the quality, quantity and phase of sleep (Dijk et al., 1999). Whether such age-related changes are the result of alterations at the level of the SCN or extra-SCN clocks (e.g., clocks in the reticular activating system) remains a topic of intense interest. Several lines of evidence suggest that age-related changes in the SCN (Sutin et al., 1993; Benloucif et al., 1997; Aujard et al., 2001) are responsible for the decline in behavioral and physiological rhythms seen in aged animals (Yamazaki et al., 2002), and that these changes may contribute to reductions in fertility. For example, the amplitude of electrical activity in SCN slices taken from aged rats is dampened relative to that seen in the SCN of young rats (Watanabe et al., 1995). Furthermore, the number of SCN neurons expressing arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP), two key SCN neuropepides, decreases in aged rats (Roozendaal et al., 1987; Chee et al., 1988). Because both of these neurochemicals have been implicated in the circadian control of ovulation (Van der Beek et al., 1997; Kalsbeek and Buijs, 2002), the integrity of these neurons in the SCN may be critical for the maintenance of reproductive functioning with age.

Studies investigating age-related changes in the molecular components of the circadian clock have uncovered equivocal results. In rats, the rhythm of SCN *Per1* and *Per2* expression does not differ between young and old animals (Asai et al., 2001; Yamazaki et al., 2002). However, in mice, the day-night rhythm of SCN *Per2* is blunted in aged animals (Weinert et al., 2001). Some findings suggest that reductions in clock-gene amplitude with advanced age may be due to changes in the synchrony of the internal clock with external time. The expression of *Bmal1* in old hamsters, for example, is lower than in young hamsters during the subjective night, when *Bmal1* expression is normally at its peak (Kolker et al., 2003). These subtle changes in *Bmal1* may have marked consequences for circadian function, as *Bmal1* is the only single clock-gene deletion that causes loss of rhythmic function (Bunger et al., 2000). Aging does not seem to affect other clock genes in the SCN, as *Per1* and *Per2* rhythms do not diminish in the aged SCN (Davidson et al., 2008) and mice with *Clock* mutations do not exhibit an increase in the effects of age on circadian rhythms over wild-type littermates (Kolker et al., 2004). Therefore, the reduction in the expression of *Bmal1* in aged rats may principally account for the diminished molecular rhythms in the SCN.

As mentioned previously, the SCN is essential for ovulation in a number of rodent species and AVP may be an important output signal triggering the LH surge. Injections of AVP into estradiol-treated, SCN-lesioned rats induces an LH surge (Grace et al., 1999; Palm et al., 1999). Notably, rhythms in AVP expression are regulated by the core molecular clock through an E-BOX motif (Grace et al., 1999). Clock mutant mice have blunted AVP rhythms (Jin et al., 1999; Silver et al., 1999) that presumably impact ovulatory function negatively (Miller et al., 2006). Because AVP is regulated by the core molecular clockwork, age-related disruptions in SCN cellular function can clearly impact the output/timing of this peptide and, ultimately, ovulatory functioning. Interestingly, the diurnal expression of AVP mRNA within the SCN is maintained in aged animals (Krajnak et al., 1998), although it is possible that, with age, cells targeted by AVP, upstream of the GnRH system, become insensitive to this peptide. Neurons in the AVPV that play a critical role in the initiation of the LH surge (Terasawa et al., 1980; Le et al., 1997; Le et al., 1999) express V1a receptors (Ostrowski et al., 1994) and are synaptic targets of the SCN (Watson et al., 1995). Importantly, these cells are upregulated in the presence of estradiol in young but not middle-aged rats (Funabashi et al., 2000c). Uncovering the neural phenotype by which AVP signaling regulates the GnRH surge in both the AVPV and other target areas, such as the DMH, will provide insight into the age-related decline in circadian rhythmicity that ultimately leads to reproductive malfunction. In Chapter 3, I will investigate the role of RFamide peptides in maintaining precise GnRH pulsatility with advancing age, and whether or not the temporal disruption of GnRH during the perimenopausal period is a result of disrupted secretion of either RFRP-3 or kisspeptin.

C. RFamide Peptides in Reproductive Aging

RFamide peptide regulation may serve as the locus of circadian-mediated dysregulation of GnRH secretion that leads to age-related declines in reproductive function. No studies to date have investigated age-related alterations in RFRP-3 expression, though kisspeptin expression has been shown to change with age in multiple species. Female rhesus monkeys express a post menopause increase in *Kiss1* gene and GPR54 receptor expression (Kim et al., 2009) and reproductive senescence is preceded by a reduction in *Kiss1* mRNA in the female rat (Downs and Wise, 2009). The increase seen in Kiss1 and GPR54 expression in postmenopause rhesus monkeys is primarily due to the reduction in the negative feedback control by ovarian steroids (Kim et al., 2009), while the reduction in *Kiss1* mRNA may be the result of disrupted sensitivity to estrogen feedback or a dysregulated activation of kisspeptin by dampened circadian signaling. Characterizing the existence of circadian controlled kisspeptin activation will lend insight into the potential mechanisms of this age-related decline. The difference between non-human primates and rodents in kisspeptin expression with age is probably a result of species- specific differences in the mechanism controlling reproductive senescence. In primates, reproductive decline is primarily due to ovarian aging, whereas reproductive aging in rodent models are a result of alterations in the brain and ovary in aged animals (Yin and Gore, 2006). In humans, the number and size of kisspeptin mRNA neurons increases in postmenopausal women and in overiectomized rhesus monkeys (Rometo et al., 2007). The increase in both humans and nonhuman primates suggests that the

decreased negative feedback from declining sex steroids affects the expression of kisspeptin to a greater extent than any neurological decline in stimulatory drive to the kisspeptin system. These results are concordant with the general observations that alterations within the aging hypothalamus, rather than follicular decline, play a greater role in reproductive senescence in rodents than primates and humans.

Metabolic Status Impacts the Central Regulation of the GnRH Surge

In addition to aging-related declines in GnRH pulsatility, limited food availability represents the most ubiquitous repressor of reproduction in female mammals. Reproductive success is conferred to individuals within a species that restrict non-essential processes to periods of relative food abundance. In times of low food availability, reproduction is inhibited to favor processes critical to immediate survival, such as thermoregulation and cell metabolism. The selective advantage provided to individuals that successfully restrict energetically costly processes during periods of low available energy has led to a biological system with multiple, redundant mechanisms of metabolic detection. Furthermore, reproductive processes are highly sensitive to fluctuations in the availability of oxidizable fuels. Studies aimed at uncovering the influence of energetic status upon reproduction will help elucidate the dynamic nature of GnRH regulation.

The intimate link between reproduction and motivation to increase energy balance represents a bidirectional relationship. When energy levels are abundant, signals that trigger a decrease in foraging, hoarding and eating behaviors are associated with an increase in reproductive behaviors and processes such as spermatogenesis and ovulation. Conversely, signals indicative of a low energy balance increase immediate survival behaviors and decrease reproductive processes. The importance of this relationship is underscored by the redundant mechanisms conveying energetic status to the reproductive axis. Therefore, research into the metabolic control of reproduction has yielded more questions than answers, as no single detection system is singularly responsible for regulating the HPG axis according to energetic status. Furthermore, the various mechanisms in the brain and periphery believed to detect energy balance are intimately connected with sex steroids that regulate reproductive behavior, and the motivational state of an animal may reflect a balance between acute fluctuations in energy availability and the likelihood of successful pregnancy following reproductive behavior.

Whereas metabolic challenge attenuates reproduction in many species by inhibiting the HPG axis across multiple levels, the GnRH neurons in the hypothalamus are central to these effects (Bronson, 1989). Reproductive inhibition via food deprivation or restriction can be rescued by pulsatile administration of exogenous GnRH across multiple species, including humans (Schneider, 2004), suggesting these neurons act as gatekeepers in the shift away from reproductive processes during times of low food availability. Uncovering the mechanisms by which energy detection signals regulate GnRH activity is critical to bridging the gap between energy balance and reproduction. While energetic challenge does not alter basal GnRH gene expression, food restriction

has multiple effects on GnRH activation and release, effects that are observed in males and females of multiple species. For example, GnRH/FOS activity is markedly decreased in female Syrian hamsters that were food deprived for 48 hrs prior to the day of proestrous (Berriman et al., 1992). In male rats, GnRH synthesis is decreased following food deprivation, and metabolic challenge inhibits portal GnRH release in sheep of both sexes (Gruenewald and Matsumoto, 1993; I'Anson et al., 2000). It remains unclear how signals of metabolic fuel availability specifically regulate GnRH, either directly or indirectly, as most evidence to date indicates metabolic fuel detectors exist outside of the nuclei in which GnRH neurons reside (Schneider, 2004). Given the roles of RFamide peptides in regulating reproduction and evidence of RFamide control of feeding behavior, it is possible that kisspeptin and/or RFRP-3 act as relay signals from energy detectors to GnRH neurons to appropriately shift behavior according to metabolic status. The following sections will discuss the most likely metabolic detection signals, brain regions and modulators in the regulation of reproductive processes.

Leptin and Insulin

Leptin, a protein secreted from white adipose cells (Zhang et al., 1994) functions as a peripheral satiety signal to the hypothalamus (Ahima et al., 1996). Leptin deficient mammals, including rodents and humans, sustain hyperphagic-induced obesity, hypogonadism and infertility (Garthwaite et al., 1980; Campfield et al., 1995; Montague et al., 1997). Exogenous leptin administration can rescue this reproductive phenotype, providing a mechanism for energetic control over the reproductive axis (Barash et al., 1996; Chehab et al., 1996). Leptin levels decrease during starvation, and the negative energy balance induced by this condition leads to a reduction of LH and an inhibited reproductive phenotype across many species (Ahima et al., 1996). Conversely, animals lacking the leptin receptor (ObR), such as the infertile, ObR defiecient Zucker rats, do not respond to exogenous leptin administration from a reproductive standpoint (Fox and Olster, 2000). This suggests leptin signalling may be required for normal reproductive function. Furthermore, neurons believed to be involved with the reproductive response to fuel availability highly express the ObR, providing a mechanisms of metabolic relay to the HPG axis (Diano et al., 1998; Elmquist et al., 1998). Whereas leptin in the circulation is an indirect measure of adipose tissue levels, the secretory patterns of this hormone are capable of rapid fluctuations, suggesting leptin may be able to communicate rapid, acute fluctuations in metabolic fuel availability in addition to reflecting the long-term adiposity composition of an animal (Levy et al., 2000; Levy and Stevens, 2001).

Despite the minimum requirement of leptin to initiate reproductive processes, estrous behavior can be dissociated from circulating leptin levels such that this hormone may be necessary but not sufficient to trigger reproduction during periods of a positive energy balance. For example, 72 hours of food deprivation inhibits LH in female mice prior to any decrease in leptin circulation (Hileman et al., 2000). Additionally, food restricted animals can regain LH pulsatility and exhibit estrous behaviors prior to any upregulation in leptin levels following the refeeding period (Jones and Lubbers, 2001; Szymanski et al., 2007). It is also unclear if the effects of leptin administration on reproduction occur in the brain or as a result of increasing oxidizable fuel availability in

the periphery. The latter hypothesis is supported by the fact that while leptin may restore food deprivation induced inhibition of the HPG axis, it is incapable of restoring LH and estrous behavior in Syrian hamsters, following administration of metabolic inhibitors, such as 2DG or MP (Schneider et al., 1998; Schneider and Zhou, 1999; Nagatani et al., 2001). Furthermore, fasting-induced decreases in leptin production are not always associated with reproductive decline in Syrian hamsters (Schneider, 2004) and leptin administration does not restore estrous behavior in food deprived female Syrian hamsters (Wade et al., 1997). Therefore, whereas leptin and adipose levels are correlated with reproductive viability, a direct, causal role between circulating leptin and the induction of reproductive processes is questionable. It is more likely that leptin represents a baseline, permissive hormone that may lead to the induction of reproductive behaviors under the proper circumstances.

Like leptin, insulin is required for reproductive competence, although the specific role that insulin plays in regulating the HPG axis likely occurs via the enhancement of metabolic fuel availability. Animals unable to produce insulin are infertile without exogenous insulin treatment, and insulin levels are typically correlated with a high degree of fertility (Siegel and Wade, 1979; Griffin et al., 1994). However, food deprived female rodents will exhibit restored LH pulsatility and estrous behavior prior to any change in circulating insulin availability (Williams et al., 1996; Jones and Lubbers, 2001). Furthermore, the effects of insulin administration are contradictory, where some studies show an increase in estrous behaviors following insulin, while others indicate supraphysiological administration of insulin may actually inhibit estrous behaviors (Hileman et al., 1993; Miller et al., 1995; Cagampang et al., 1997). In diabetic rats, insulin is capable of restoring LH and estrous behavior and leads to and increase in FOS activation of GnRH neurons, suggesting that insulin may play a positive role in initiating reproduction insofar as it yields the necessary oxidizable fuels to trigger this response (Kovacs et al., 2002; Kovacs et al., 2003).

Metabolic fuel detectors in the brain

Energy detectors in the hindbrain and forebrain are critical in relaying metabolic information to the reproductive axis. Given the indirect role that leptin and insulin seem to play in regulating reproduction, these fuel detectors may act as relay stations to the HPG axis and shift behaviors according to the availability of food. The detection of oxidizable fuels, such as glucose availability, occurs within the hindbrain in the area postrema and nucleus of the solitary tract (Ritter et al., 1981) and these detectors project rostrally to hypothalamic sites that play a role in reproduction (Wade and Jones, 2004). As GnRH neurons do not express the receptors for either insulin or leptin (Finn et al., 1998), peripheral signals conveying metabolic information to the HPG axis must converge upstream of GnRH. In addition to the area postrema and nucleus of the solitary tract, fuel detectors in the forebrain may communicate this information, via two distinct neuronal populations in the ARC, and lead to behavioral responses towards feeding or various sexual and social behaviors.

Lesions to the area postrema abolish food-deprived alterations in reproductive behavior and physiology in female rats and hamsters, while administration of metabolic inhibitors that induce glucoprivation, such as 2DG, disrupt reproduction when injected into the 4th ventricle, linking these fuel detectors to reproductive processes in the hypothalamus (Schneider and Zhu, 1994; Murahashi et al., 1996; Cates and O'Byrne, 2000). Metabolic fuel information is transmitted from the hindbrain to areas involved with reproduction, such as the PVN of the hypothalamus, via NPY and catecholamines (epinepherine and norepineherine)(Chronwall, 1985; Sawchenko et al., 1985). Abolishing these contacts via null mutations or receptor antagonists in the PVN prevents food deprivation or glucoprivation-induced decreases in LH of female rats (I'Anson et al., 2003a; I'Anson et al., 2003b) suggesting the necessity of this signaling pathway in eliciting a response by the HPG axis during a negative energy balance. While NPY neurons in forebrain fuel-detection circuits may account for the unresponsive HPG phenotype in NPY KO mice, (see below) they are insufficient to inhibit reproduction under conditions of abolished hindbrain communication (Hill et al., 2003). It is likely that both fuel detection circuits, therefore, play a role in communicating metabolic information to the HPG axis.

Metabolic fuel detectors in the ARC represent likely participants in energetic regulation of reproduction. Anorexigenic hormones, proopiomelanocortin-derived peptide (POMC) and cocaine-and amphetamine-regulated transcript (CART) are activated by satiety signals, such as leptin, and inhibit feeding behavior (Pinto et al., 2004). Conversely, orexigenic hormones, agouti-related protein (AgRP) and neuropeptide Y (NPY) are inhibited by the orexigenic hormones in the hypothalamus and by leptin, leading to feeding behaviors (Pinto et al., 2004). These signals represent likely candidates to relay metabolic status to the reproductive axis, with most research emphasizing NPY specifically, thus restricting reproduction to the appropriate times during a positive energy balance.

NPY

Neuropeptide-Y is a pancreatic polypeptide that induces orexigenic behavior and physiological responses in mammals (Wade and Jones, 2004). Neurons secreting NPY are located in the ARC, locus coeruleus and nucleus of the solitary tract, all of which are in prime locations to receive peripheral metabolic signals (Chronwall, 1985). NPY secretion increases during metabolic challenge, including food deprivation and increased energy use (Brady et al., 1990; Kalra et al., 1991; Schwartz et al., 1998; Reddy et al., 1999). Furthermore, food deprivation leads to an increase in FOS immunoreactivity in multiple NPY termini, including the DMH, SON and PVN of the hypothalamus (Lambert et al., 1995; Xu et al., 1995). Food deprivation also increases NPY/FOS in the ARC of rats and hamsters (Jones et al., 2004).

As an indicator of energetic challenge, NPY is likely to suppress estrous behaviors and reproductive function. Central NPY injections decrease LH amplitude and pulsatile frequency in gonadectomized rats (McDonald et al., 1989), as well as estrous behaviors in female, steroid primed rats and hamsters (Clark et al., 1985; Corp et al.,

2001) NPY inhibits GnRH release and neuronal activity, suggesting a central mechanisms of action, rather than effects on pituitary responsiveness to GnRH. Metabolic challenges that suppress estrous behaviors also coincide with decreased NPY mRNA and immunoreactivity, including food deprivation, cold exposure and metabolic fuel inhibitors. While NPY fibers project to the vicinity of GnRH (Li et al., 1999) and GnRH neurons express the Y1R in adult rats and mice (Klenke et al., 2010), indirect regulation of the HPG axis via NPY possibly occurs upstream of these neurons (Keene et al., 2003). Specifically, dense fiber projections from NPY neurons are observed in CRH neurons in the PVN, where inhibitory signals to GnRH have been established (Sawchenko et al., 1985; Petraglia et al., 1987; Kalra et al., 1991; Broberger et al., 1999). An additional, unestablished link might involve NPY projections to the inhibitory peptide, RFRP-3. Given the dense projections from NPY secreting neurons in the ARC to the DMH (Bai et al., 1985), where RFRP-3 cell bodies reside (Kriegsfeld et al., 2006), and the impact of RFRP-3 neurons on reproductive function (Kriegsfeld et al., 2010), a novel role of this RFamide peptide my involve inhibition of the reproductive axis during a negative energy balance, which is explored in Chapter 4.

RFamide Peptides

The effects of kisspeptin and RFRP-3 on feeding behavior and the neuronal mechanisms mediating energy balance support a bidirectional relationship between RFamide peptides and energy detectors. In mice, central infusion of kisspeptin reduces fasting-induced feeding behavior, whereas RFRP-3 stimulates feeding in rats, coincident with their respective roles in reproduction and providing a mechanism for shifting behavior towards feeding or sexual behavior under the proper environmental conditions (Johnson et al., 2007; Stengel et al., 2011). Kisspeptin neurons innervate anorexigenic POMC cells and kisspeptin administration elicits a robust increase in POMC neuronal firing. These direct effects are abolished by RFRP-3 pretreatment, suggesting the interplay of these RFamide peptides may occur at the level of POMC cells, and convey reproductive status to energy detectors (Fu and van den Pol, 2010). In the ewe, tracing work indicates direct projections of RFRP-3 neurons to multiple sites involved with energy balance, including NPY, POMC orexin and melanin-concentrating hormone neurons, suggesting a potential role for RFRP-3 in energy balance modulation across species (Qi et al., 2009).

Conversely, energy status may impact reproduction via direct effects upon RFamide peptides. Body weight, nutritional status and hormonal signals associated with a fed metabolic state all impact *Kiss1* neuronal activity (Fernandez-Fernandez et al., 2006; Forbes et al., 2009). A negative energy balance also impacts kisspeptin activity, as fasting leads to a reduction in *Kiss1* mRNA in pubertal male and female rats (Castellano et al., 2005). In adult female rats, 18 hours of food deprivation similarly leads to a decrease in *Kiss1* mRNA levels (Brown et al., 2008) and 12 hours of food restriction is sufficient to reduce *Kiss1* mRNA levels in adult female mice and rats (Kalamatianos et al., 2008; Castellano et al., 2009). Together, these data suggest that reduction of kisspeptin signaling during periods of negative energy balance may act as a shifting mechanism away from reproductive processes during times of low food availability. The reproductive

phenotype of undernourished pubertal rats includes delayed vaginal opening and marked reduction in circulating gonadotropins. Interestingly, i.c.v. administration of kisspeptin-10 rescues this phenotype, as well as similar reproductive malfunctions in animal models of diabetes, and suggests kisspeptin expression is at least a partial rate limiting factor in negative energy balance effects on the HPG axis (Castellano et al., 2005; Castellano et al., 2006). Peripheral hormones conveying metabolic signals to the CNS have predictable effects on *Kiss1* mRNA, given its stimulatory role in reproduction. Central administration of the adipose hormone, leptin, rescues *Kiss1* mRNA in rodent models of hypoinsulemia and in ob/ob mice, which show a reduction in *Kiss1* mRNA expression in the arcuate (Castellano et al., 2006; Smith et al., 2006a). Conversely, ghrelin administration inhibits *Kiss1* mRNA in adult female rats, consistent with the proposed role of ghrelin in attenuating HPG function (Forbes et al., 2009).

Despite the pharmacological effects of metabolic hormones on kisspeptin expression, it is unclear if the mechanism of action is direct or through a multisynaptic connection. Immunohistochemical analysis of leptin receptor expression reveal virtually nonexistent colocalization with kisspeptin neurons in either the AVPV or ARC nuclei, pointing to an indirect mechanism of leptin in triggering this stimulatory peptide (Louis et al., 2011). Furthermore, the mechanism of ghrelin actions on kisspeptin neurons remains to be studied. Unlike kisspeptin, very little is known about the impact of metabolic status on RFRP-3 expression. Given the inhibitory actions of RFRP-3 on GnRH, one would anticipate either a negative impact of positive energy signals on RFRP-3 or an upregulation of RFRP-3 during a negative energy balance, in order to inhibit the reproductive axis during metabolic duress. Chapter 4 explores the potential of RFRP-3 as a conduit of negative energy status to the HPG axis, with an emphasis on correlating RFRP-3 activation to motivated behavior paradigms aimed at measuring energetic vs. sexual motivation.

Research Overview

Syrian hamsters represent an ideal model system in both reproductive physiology and chronobiology. The precision of the GnRH surge in female Syrain hamsters is unrivaled in mammalian species, and thus provides a predictable, tractable system to determine the upstream regulators of the preovulatory luteinizing hormone surge. Furthermore, using GnRH as an output signal allows one to understand the neural pathways underlying the regulation of endocrine rhythms by the master clock in the SCN, and how it interacts with subordinate, efferent targets in order to give rise to temporally controlled processes. The discovery and characterization of RFamide peptides represent novel avenues to pursue the undetermined regulation of GnRH by the circadian clock. Furthermore, the disruption of GnRH via internal processes (such as aging) or fluctuations in the external environment (such as suboptimal fuel availability) may occur partially by affecting RFamide peptides- providing novel avenues by which these processes impact ovulatory function.

Chapter 2

Circadian Control of Kisspeptin and a Gated GnRH Response Mediate the Preovulatory Luteinizing Hormone Surge

Introduction

Despite the established role of the circadian system in regulating ovulation across species (de la Iglesia and Schwartz, 2006; Kriegsfeld, 2006; Christian and Moenter, 2010), the precise neurochemical pathways by which a daily timing signal initiates the preovulatory luteinizing hormone (LH) surge remain to be fully elucidated. In mammals, the orchestration of circadian rhythms is controlled by a master pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore and Eichler, 1972; Stephan and Zucker, 1972). Circadian rhythms are endogenously generated (Lehman et al., 1987; Ralph et al., 1990) and synchronized to the external environment via direct neural projections from intrinsically photosensitive retinal ganglion cells to the circadian clock in the SCN (Morin and Allen, 2006). The SCN communicates to hypothalamic cell phenotypes driving reproductive function through extensive direct and indirect neural projections (Horvath, 1997; Van der Beek et al., 1997; Kriegsfeld, 2006). On the day of proestrus, in most spontaneously ovulating rodent species, the preovulatory luteinizing hormone (LH) is initiated by the SCN late in the afternoon (Legan and Karsch, 1975; Mahoney et al., 2004; Chappell, 2005; Gibson et al., 2008). Perturbations of SCN output signaling pathways or intrinsic clock activity lead to gross deficits in female rodent ovulatory function and fecundity (Nunez and Stephan, 1977; Gray et al., 1978; Wiegand and Terasawa, 1982; Miller et al., 2004).

Two SCN-derived neurochemical pathways have been implicated in the initiation of the LH surge. The first is a monosynaptic pathway whereby SCN-derived, vasoactive intestinal polypeptide (VIP) secreting neurons project directly to GnRH cells (van der Beek et al., 1993). GnRH neurons targeted by VIP express FOS around the time of the LH surge and antisense oligonucleotides directed against VIP attenuate and delay the LH surge in estradiol treated animals (van der Beek et al., 1994; Harney et al., 1996). Despite these corroborating lines of evidence, other studies indicate equivocal effects of exogenous VIP administration, with VIP inhibiting GnRH in some instances and playing an excitatory role in others (Weick and Stobie, 1992; Harney et al., 1996). Additionally, VIP cells only contact a small percentage of GnRH cells (~5-20%) across rodent species (Van der Beek et al., 1997; Kriegsfeld et al., 2002), well below the percentage activated at the time of the surge (Lee et al., 1990). Importantly, GnRH neurons do not express estrogen receptor α (ERα) (Laflamme et al., 1998; Hrabovszky et al., 2001), the estrogen receptor subtype responsible for the positive feedback effects of estradiol on the LH surge (Wintermantel et al., 2006). The fact that SCN-derived VIP projections cannot fully account for the LH surge suggests the existence of an additional mechanism(s) of circadian control that coordinate with timed VIP stimulation of the GnRH system.

The anteroventral periventricular nucleus (AVPV) is a critical neural locus for the initiation of the LH surge. Lesions of the AVPV eliminate estrous cyclicity (Terasawa et al., 1980). Furthermore, neurons within the AVPV project to GnRH cells and exhibit FOS expression coinciding with the timing of the LH surge (Le et al., 1999). The SCN projects to the AVPV providing a neural pathway for temporally controlling cell populations driving GnRH activity (Watson et al., 1995; Kriegsfeld et al., 2004). SCN cells targeting the AVPV express vasopressin (AVP) (de la Iglesia et al., 1995; Leak and Moore, 2001) and AVP injections produce surge-like LH levels in SCN-lesioned, ovariectomized, estradiol-treated rats (Palm et al., 1999). Finally, ERα-expressing neurons within the AVPV are direct targets of the SCN (de la Iglesia et al., 1995), potentially integrating circadian signals and positive feedback actions of estrogen.

Although abundant evidence indicates that the AVPV is necessary for circadian initiation of ovulation in rodents, the specific neural pathway(s) and cellular phenotype(s) involved in this process have not been fully characterized. The stimulatory neuropeptide, kisspeptin, provides an attractive target for further exploration. Kisspeptin and *Kiss1* mRNA, the gene encoding kisspeptin peptide, are expressed in the AVPV across species (Gottsch et al., 2004; Smith et al., 2005a; Mason et al., 2007) and play a significant role in positively regulating the reproductive axis (reviewed in (Oakley et al., 2009). Exogenous kisspeptin administration potently induces LH release and upregulates FOS expression in GnRH neurons (Gottsch et al., 2004; Irwig et al., 2004). A large percentage of kisspeptin-immunoreactive (ir) neurons in the AVPV express ERα and *Kiss1* mRNA is upregulated by estradiol administration in ovariectomized animals (Smith et al., 2005b). Finally, *Kiss1* cells express FOS at the time of the LH surge in naturally cycling and ovariectomized, estradiol-treated rats (Smith et al., 2006a; Adachi et al., 2007). Together, these results suggest that *Kiss1* neurons in the AVPV participate in estrogen positive feedback and are positioned to receive circadian clock input.

An additional layer of complexity in exploring the role of the circadian system in LH surge initiation is that administration of SCN neuropeptides only induces the surge within a narrow time window (Palm et al., 2001), suggesting additional temporal control at SCN target loci. The means by which this timed gating mechanism controls responsiveness of the HPG axis to SCN communication remains to be investigated. The gating of SCN information flow may be controlled either within the AVPV, at the level of GnRH neurons, or a combination of both loci. The present studies explored the role of AVP and kisspeptin signaling in the timing of the LH surge using several approaches. First, we examined whether or not kisspeptin neurons in the AVPV are targets of vasopressinergic SCN input. Next, we asked whether or not the kisspeptin system shows a daily pattern of neuronal activity consistent with a role in ovulation and whether any emergent pattern is estrogen-sensitive. Finally, we explored the possibility that this circuit is responsible for time-dependent sensitivity of the reproductive axis to SCN signaling by assessing whether: 1) kisspeptin cells within the AVPV respond in a time-dependent manner to AVP stimulation, 2) GnRH neurons display time-dependent sensitivity to kisspeptin signaling, or 3) both kisspeptin and the GnRH systems coordinate to gate the timed initiation of the LH surge. If time-dependent sensitivity to upstream circadian signaling is controlled at the level of the AVPV, then one would

expect kisspeptin cells to exhibit daily changes in sensitivity to AVP stimulation. Alternatively, if the gating of control occurs within GnRH cells, then one would expect the GnRH system to display daily sensitivity in response to both AVP and kisspeptin administration.

Materials and Methods

Animals

Adult (>60 days of age), female LVG Syrian hamsters (*Mesocricetus auratus*) (n=91) were utilized. Hamsters purchased from Charles River (Wilmington, MA) at 4-5 weeks of age were housed in translucent propylene cages (48 x 27 x 20 cm) and provided with *ad libitum* access to food and water at all times. Animals were maintained in a colony room at 23 ± 1°C with a 24 hr light:dark cycle (14:10 hr light:dark (LD)) with lights on at 07:00 and lights off at 21:00. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Berkeley.

Examination of the Circadian Pattern of Kisspeptin Activation.

To determine whether or not kisspeptin expression is coordinated with the timing of the LH surge, I examined the activational state of AVPV kisspeptin neurons over the course of the day in ovariectomized hamsters given empty implants or implants containing estradiol (Meyer-Bernstein et al., 1999). Prior to ovariectomy, estrous cyclicity was monitored by daily examination of vaginal discharge (Orsini, 1961) and only females with regular 4-day estrous cycles were retained for study. To examine the pattern of kisspeptin cell activation independent of fluctuations in peripheral sex steroids, females were bilaterally ovariectomized (n=48) under isoflurane anesthesia. To determine whether daily changes in kisspeptin expression are estrogen-dependent, animals were either treated with a SILASTIC capsule (Dow Corning Corp.; 10-mm length, 1.45-mm id, 1.93-mm od) containing powdered undiluted 17β-Estradiol (n=24) or an empty capsule (n=24). In estrogen-implanted hamsters, this treatment generates estradiol concentrations comparable to those seen on the day of proestrus and results in daily LH surges at the same time each day, ~4 h prior to onset of darkness (Norman et al., 1973). Two weeks post-ovariectomy and capsule implantation, hamsters were perfused (as described below) at either zeitgeber time (ZT) 7, 11, 13 and 16 (darkness onset at ZT 14) (n= 6/group) and brains were collected for histological analysis.

Examination of Kisspeptin Cells for SCN-Derived Input and Receptor Expression.

To determine whether or not the SCN sends AVPergic or VIPergic projections to kisspeptin cells in the AVPV, adult Syrian hamsters (n=5) were perfused on the day of proestrus at ZT 11. Brains were collected and stained for either kisspeptin and AVP (n=5) or kisspeptin and VIP using immunofluorescence (n=5) and fiber contacts onto kisspeptin cells were evaluated as described below. To ensure that any fibers found within the AVPV contacting kisspeptin cell bodies originate from the SCN, 5 additional hamsters received bilateral electrolytic SCN lesions. Under deep anesthesia induced with a ketamine cocktail (21 mg ketamine, 2.4 mg xylazine and 0.3 mg acepromazine/ml injected i.p. in a dose of 0.34 ml /100 g body mass), the head was shaved and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujanga, CA), and the hamster prepared for aseptic surgery. Lesions were aimed at the following coordinates: 0.9 mm anterior to bregma, 0.3 mm lateral to midline, and 7.8 mm below dura (74). Bilateral radio frequency lesions were made by applying 25 V for 15 seconds using a Radionics Model RFG-4A Research RF Lesion Generator (Radionics, Burlington, MA) and stainless steel electrodes insulated with Epoxylite (The Epoxylite Corp., Irvine, CA), excluding the tip (0.20mm). Lesioned brains were collected 24 hours post-surgery following perfusion as described below. Finally, to examine the co-expression of the vasopressin receptor, V1a, every 4th section was double-labeled immunohistochemically for kisspeptin peptide and V1a protein.

Examination of Kisspeptin and GnRH Activation After Timed Central Administration of Vasopressin.

Adult hamsters (n=24) were ovariectomized and implanted with SILASTIC capsules containing powdered 17β-estradiol as described above. After a one-week recovery period, a unilateral guide cannula (6mm, Plastics One, Roanoke, VA) was implanted, aimed at the lateral ventricle. Coordinates for implantation were 0.6mm lateral and 1.5mm posterior to bregma and 4.5 mm ventral from the surface of dura mater. To maintain patency, dummy cannulas were attached to guide cannulae after surgery. After a one-week recovery period, cannula placements were confirmed by assessing drinking behavior in response to Angiotensin II injections (10ng in 5µl sterile saline). Because AVP fibers, but not VIP fibers (see Results), targeted kisspeptin cells, we focused on the role of this peptide in stimulating the LH surge. AVP (n=12) (2ng/5µl) or saline vehicle (n=12) was administered early in the day at ZT 1 (when AVP is ineffective at stimulating the GnRH system) and in the afternoon at ZT 11 (when AVP stimulates the LH surge). Hamsters were perfused 1 hour after injection and brains were collected and double-labeled for Kisspeptin/FOS and GnRH/FOS using immunofluorescence as described below.

Examination of GnRH Activation After Timed Kisspeptin Administration.

To determine whether or not the GnRH system responds in a time-sensitive manner to kisspeptin, and whether or not the GnRH system requires the presence of estradiol for the response to exogenous kisspeptin, hamsters were ovariectomized and treated with empty (n=36) SILASTIC capsules or capsules containing estradiol (n=36). One week after surgery, kisspeptin (kisspeptin-10 (mouse); Phoenix Pharmaceuticals) was injected (i.p. (2nmol or 4nmol)) at ZT 1 or ZT 11 and hamsters perfused 1 hour after injection. Brains were collected and double-labeled for GnRH/FOS as described below.

Perfusion and Histology

For brain collection, hamsters were deeply anesthetized with sodium pentobarbital (200mg/kg) and perfused transcardially with ~150 ml of 0.9% saline, followed by 300-400 ml of 4% paraformaldehyde in 0.1 M PBS, pH 7.3. Double-label immunofluorescence was performed on every fourth, 40-µm coronal section. Sections were incubated for 48 hours at 4ºC with a rabbit polyclonal anti-kisspeptin-10 antiserum (1:2000; generated by Jens Mikkelsen), previously shown to bind with high affinity to kisspeptin neurons in the AVPV and arcuate nuclei and exhibit minimal cross-reactivity to related RFamide peptides (Mikkelsen and Simonneaux, 2009; Desroziers et al., 2010), or rabbit anti-FOS (1:50,000; Santa Cruz) and NGS diluted at 1:1000 with 0.1% PBT for 48 hours. After incubation in the first primary antibody, brains were incubated for 1 hour in biotinylated goat anti-rabbit (1:300; Vector Laboratories, Burlingame, CA) and then in ABC for 1 hour. For kisspeptin or FOS, the signal was amplified with biotinylated tyramide solution (0.6%) for 30 min as previously described (Kriegsfeld et al., 2006). Cells were then labeled by using Cy-2 conjugated streptavidin (1:200; Jackson) as the fluorophore. After labeling the primary antibody, sections were incubated in either a rabbit anti-GnRH antiserum (LR-5; 1:20,000; generous gift from Dr. R. Benoit), rabbit anti-FOS antibody (1:5,000; Santa Cruz), a guinea pig anti-AVP antibody (1:10,000; Santa Cruz), a rabbit anti-V1a (1:500; Santa Cruz) or a guinea pig anti-VIP antibody (1:500; Santa Cruz) with 0.1% PBT for 48 h (Kriegsfeld et al., 2006). The second primary antibody was labeled with CY-3 donkey anti-rabbit (GnRH, V1a, FOS; 1:200; Jackson) or CY-3 anti-guinea pig (AVP, VIP; 1:200; Jackson) as the secondary antibody/fluorophore.

Several control procedures were implemented to ensure the specificity of immunohistochemical labeling. First, all antibodies were preadsorbed with their respective ligands for 24 h prior to tissue application. This procedure eliminated staining in all cases. Because kisspeptin is an RFamide peptide with a C-terminus structure similar to that of RFamide-related peptide (RFRP), the kisspeptin antibody was preadsorbed with RFRP peptide to examine potential cross-reactivity. This procedure did not result in a change in the pattern or intensity of kisspeptin labeling, indicating that the antibody does not exhibit cross reactivity with this peptide.

Light microscopy

Sections were examined using the standard wavelengths for CY-2 (488 nm) and CY-3 (568 nm) using a Zeiss Z1 microscope. Every 4th section through the anteroventral periventricular nucleus (AVPV; AVP/kisspeptin, VIP/kisspeptin, or kisspeptin/FOS) and the medial preoptic area (MPOA; GnRH/FOS) were assessed. For light microscopy, kisspeptin cells identified as having AVP or VIP contacts or expressing FOS, or GnRH cells expressing FOS were digitally captured at 400x (fiber contacts) or 200x (FOS) in 8 bit greyscale using a cooled CCD camera (Zeiss). Each label was captured as a single image without moving the position of the stage or plane of focus between captures. Images were superimposed digitally. Brain areas were examined by two independent observers using Photoshop software in order to view CY-2 and CY-3 channels independently. Variation between observers was 1.4% for single cell analysis and 3.7% for double-label analyses. Kisspeptin or GnRH cells with a clear nucleus were quantified using single-channel analysis. Cells were considered to be double-labeled if FOS was expressed in the cell nucleus, but not beyond the borders of each pre-defined nuclear area. All kisspeptin cells identified as having putative AVP or VIP contacts were examined using confocal analyses (see below). For FOS expression, only those kisspeptin and GnRH cells with a visible nucleus in which FOS expression was localized to the nucleus were counted as double-labeled cells.

Confocal microscopy

Cells were observed under a Zeiss Axiovert 100TV fluorescence microscope (Carl Zeiss, Thornwood, NY) with a Zeiss LSM 510 laser scanning confocal attachment. The sections were excited with an Argon-Krypton laser using the standard excitation wavelengths for CY-2 and CY-3. Stacked images were collected as 0.5 μm (fiber assessment) or 1.0 μm (FOS expression) multitract optical sections. Using the LSM 3.95 software (Zeiss), red and green images of the sections were superimposed. Kisspeptin or GnRH cells in a given brain region were examined through their entirety. To examine SCN contacts, kisspeptin cells with putative AVP or VIP contacts were scanned though the extent of each cell in 0.5 μm increments at 400x. Only those cells in which the AVP- or VIP-labeled fiber contacted a kisspeptin cell in the same 0.5 μm scan were counted as close contacts. Cells characterized as double-labeled for FOS/kisspeptin or FOS/GnRH at the conventional microscopy level were confirmed in the same manner to ensure that FOS was expressed within the cells rather than in overlapping cells in the same light microscopic field of view. Likewise, cells classified as single-labeled were assessed to ensure that the conventional microscopy strategy did not result in false negatives. At least 10% of cells quantified using conventional microscopy were assessed in confocal scans for FOS co-labeling. Regions of the brain with putative double-label identified at the light level were scanned in 1.0 μm steps at 400×.

Statistics

Data were analyzed using SigmaStat software for all studies. Data for kisspeptin cell counts, FOS expression in kisspeptin cells and GnRH cells were analyzed using 2 x 4 (Hormonal Condition x Time of Day) analyses of variance (ANOVA) for those studies assessing the change in activation over the course of the day. Total cell counts and FOS expression in GnRH and kisspeptin cells were analyzed using 2 x 2 (Treatment x Time of Day) AVOVA for studies following injection of AVP. Data for total cell counts and FOS expression in GnRH cells were analyzed using 2 x 2 x 2 ANOVAs (Hormonal Condition x Treatment x Time of Day) for studies following injection of kisspeptin. Group differences were evaluated using Tukey HSD tests. Differences were considered significant if $p<0.05$.

Results

The Daily Activational State of AVPV Kisspeptin Cells is Coordinated with the LH Surge and Estradiol-Dependent.

Daily fluctuations in kisspeptin neuronal cell activity were assessed at time points before, during, and after the time of the LH surge (ZT 7, 11, 13, 16) in ovariectomized hamsters provided with either empty **(Figure 2.1A-D)** or estradiol-filled **(Figure 2.1E-H)** capsules. Syrian hamsters held in a 14:10 LD cycle express LH concentrations on proestrous that peak 4 hours before onset of darkness, with an initial increase in LH around 6 hrs before darkness and cessation of the surge ~2 hours before lights out (Stetson, 1978; Gibson et al., 2008). In hamsters provided with empty capsules and those with capsules containing estradiol, the percentage of kisspeptin cells expressing FOS exhibits a significant daily pattern consistent with a circadian-controlled mechanism participating in LH surge initiation **(Figure 2.1I)**. Neuronal activation was low in the early afternoon (ZT 7), increased markedly around the time of the LH surge (ZT 11 and ZT 13), and decreased 2 hours after lights out (ZT 16). OVX hamsters exhibited a significantly lower percentage of kisspeptin cells expressing FOS than OVX+E2 treated females at each time-point measured ($p<0.05$ in each case), with the exception of (ZT 7) **(Figure 2.1I)**. The total number of kisspeptin-ir cells was not impacted by time of day or hormonal condition ($p>0.05$ in each case; **Figure 2.1J)**.

Kisspeptin Cells in the AVPV are Contacted by SCN-Derived AVP-ir, But Not VIP-ir, Fibers.

AVPV kisspeptin cells received extensive contacts (37.7%± 6.3%) from AVP-ir fibers **(Figure 2.2C)**. All contacts at the light level were confirmed to be in the same 0.5 μm plane by confocal microscopy (**Figure 2.2F**). Cells not exhibiting contacts at the light microscopic level did not have contacts at the confocal level. Conversely, in both light and confocal analyses, kisspeptin-ir neurons in the AVPV did not receive any contacts

from VIPergic fibers (**Figure 2.2D**), despite prominent VIP-ir fiber staining in relevant brain loci (MPOA, SCN, subparaventricular zone; **Figure 2.2A**). Given that AVP-ir, but not VIP-ir, fibers contact AVPV kisspeptin cells, we sought to determine whether or not this cell population expresses the main AVP receptor subtype, V1a. 42%±7.4% of kisspeptin-ir cells exhibited V1a-ir labeling (**Figure 2.3**). To assess whether or not AVP-ir contacts on kisspeptin cell bodies in the AVPV originate from the SCN, electrolytic lesions were directed at the SCN and double-label immunocytochemistry was performed for kisspeptin and AVP. Hamsters with lesions sparing the SCN served as controls. AVP-ir fiber appositions on kisspeptin cell bodies were eliminated in SCN-lesioned hamsters (**Figure 2.2G, H**). Importantly, SCN lesions eliminating AVP-ir contacts on kisspeptin cells spared the supraoptic nucleus (SON) and paraventricular nucleus (PVN)**(Figure 2.2G**).

Central Vasopressin Administration Reveals Indiscriminate Activation of Kisspeptin Neurons Concomitant with Gated Activation of the GnRH System.

Kisspeptin neuronal activation was assessed following intracerebroventricular injections of saline or AVP at ZT 1 and ZT 11 in SCN-intact, OVX+E2 females **(Figure 2.4)**. At ZT 1, the percentage of kisspeptin-ir cells expressing FOS was significantly increased over saline controls (p<0.05) (**Figure 2.4B).** At ZT 11, kisspeptin cells were maximally activated, with no differences observed between AVP-treated and saline controls **(**p>0.05; **Figure 2.4B)**. In contrast, AVP infusions did not increase GnRH cell activation at ZT 1 relative to saline controls (**Figure 2.4B**) (p>0.05). At ZT 11, both GnRH and kisspeptin cells were maximally activated, with no differences between saline or AVP-treated females (p>0.05 in both cases) (**Figure 2.4B**). Total GnRH-ir and kisspeptin-ir cell counts did not differ significantly across treatments or time periods (p>0.05 in all cases)(**Figure 2.4C, D)**, indicating differences in FOS-ir are representative of proportional changes in activation states in all cases.

The GnRH System Exhibits Time-Dependent Sensitivity to Kisspeptin Stimulation.

In order to examine whether or not the GnRH system is differentially sensitive to kisspeptin treatment at times when the LH surge cannot (ZT 1) or can (ZT 11) be induced, GnRH neuronal activation was assessed after injections of saline or kisspeptin (**Figure 2.5**). The estrogen dependence of any differences was examined in OVX females treated with an empty or estradiol-filled capsule. In all cases, excluding the 4nmol dose of kisspeptin in the medial septum/diagonal band of broca (MS/DBB) of OVX hamsters and the POA of OVX+E2 hamsters, kisspeptin was more effective at activating the GnRH system at ZT 11 than at ZT 1 (p<0.05 in each case**; Figure 2.5A, B)**. In both brain loci, and both time points, estrogen increased the percentage of GnRH neurons expressing FOS in vehicle-treated controls and 2nmol kisspeptin-treated hamsters compared to OVX females bearing empty capsules (p<0.05 in all cases). The presence of estrogen also increased the percentage of GnRH neurons expressing FOS at the 4nmol

dose of kisspeptin relative to OVX females ($p<0.05$). In OVX+E2 hamsters probed at ZT11, there were no differences among groups in either brain region, suggesting that the GnRH systems is maximally activated at this time due to endogenous stimulation of the GnRH in the presence of estradiol ($p>0.05$ in all cases). Finally, the total number of GnRH cells was not affected by time, kisspeptin treatment, or estradiol ($p>0.05$ in all cases)**(Figure 2.5C, D)**.

Discussion

The present findings reveal a hierarchical organization of the hamster ovulatory circuitry involving interactions among the circadian system, a sex steroid integration center, and a final common pathway gating information flow. First, we find that AVPergic cells originating in the SCN target kisspeptin cells in the AVPV, a subpopulation that express the vasopressin receptor subtype, V1a. The AVPV population of kisspeptin neurons exhibits a daily pattern of activation with peak activity coincident with the timing of the GnRH/LH surge (Gibson et al., 2008). The daily pattern of kisspeptin cellular activity is partially dependent on the presence of estradiol; ovariectomy markedly attenuates, but does not abolish, this daily activation. We also found that GnRH neurons act as gatekeepers, restricting the preovulatory LH surge to the late afternoon, at least in part, through daily changes in responsiveness to kisspeptin. More specifically, central administration of AVP increases kisspeptin cellular activation at both time points examined, whereas GnRH neurons are only sensitive to AVP administration during the afternoon, suggesting insensitivity to AVP-induced kisspeptin at this time. Finally, pharmacological studies of ovariectomized and OVX/estrogen-primed hamsters confirm daily changes in GnRH cell responsiveness to kisspeptin that are dependent on the presence of estradiol. Together, these findings point to kisspeptin neurons in the AVPV as an integration center for estradiol and circadian signals necessary for the generation of the LH surge and suggest that daily changes in GnRH neuron sensitivity to kisspeptin signaling further ensures appropriate timing of the surge (**Figure 2.6**).

In spontaneously ovulating rodents (e.g., rats, mice, hamsters), the SCN initiates the LH surge on the day of proestrus when estrogen concentrations are elevated (de la Iglesia and Schwartz, 2006). That estrogen is permissive for circadian stimulation of the GnRH system is somewhat paradoxical as estrogen negatively regulates GnRH secretion throughout the remainder of the cycle (Petersen et al., 2003). We have previously shown that this feat is accomplished, at least in part, by the removal of inhibitory influences of the mammalian ortholog of avian gonadotropin hormone, RFamide-related peptide-3 (RFRP-3), at the time of the surge (Gibson et al., 2008). Historically, it was believed that direct VIPergic SCN projections targeting GnRH neurons were responsible for the positive arm of the ovulatory circuit (van der Beek et al., 1994; Horvath et al., 1998). However, given the paucity of estrogen receptor expression in GnRH cells (Laflamme et al., 1998; Hrabovszky et al., 2001), researchers began searching for estrogen responsive

targets of the SCN upstream of the GnRH system. Kisspeptin cells in the AVPV represented a likely candidate as this brain tissue expresses FOS coincident with the surge (Le et al., 1999), contains estrogen receptors (Herbison, 2008), and receives SCN input (Watson et al., 1995; Kriegsfeld et al., 2004). Likewise, kisspeptin cells are upregulated by estrogen and express FOS at the time of the LH surge (Smith et al., 2005a; Smith et al., 2006b), suggesting an important role in LH surge initiation. Whether or not AVPV kisspeptin cells represent an integration point for circadian/sex steroid signaling and might participate in the gating of circadian communication has not been explored.

Because AVPergic cells in the SCN project to the AVPV across species (de la Iglesia et al., 1995; Leak and Moore, 2001), and AVP can only induce the LH surge during a limited time window in estradiol-implanted rats (Palm et al., 2001), we asked whether or not this population of kisspeptin cells might receive AVPergic SCN input and gate responsiveness to this peptide. We find that kisspeptin cells receive AVPergic SCN input and express V1a receptors, providing a direct means of communication from the circadian clock to this cell population. Recent findings in mice indicate that SCN-derived AVP cells project to the kisspeptin system (Vida et al., 2010), suggesting a common mechanism of control across rodent species. We also found that i.c.v. injections of AVP increase kisspeptin cellular activity. Importantly, although AVP can only initiate the LH surge during the afternoon in estrogen-treated animals (Palm et al., 2001), administration of this peptide enhances kisspeptin cellular activity in the morning (**Figure 2.4**). In contrast, although kisspeptin activity was increased by AVP administration in the morning, GnRH activity was not. As expected, given endogenous AVPergic signaling during the afternoon, both GnRH and kisspeptin activity are maximal in both vehicle and AVP-treated hamsters. This finding suggested that circadian information is not gated at kisspeptin cells, but downstream of this cell population. Because GnRH neurons are the major target of kisspeptin (Mikkelsen and Simonneaux, 2009; Desroziers et al., 2010), this cell population represented a possible gating locus mediating the timing of ovulation.

Using immortalized GT1-7 cells, our laboratory documented that GnRH neurons exhibit time-dependent sensitivity to upstream stimulatory signals that initiate the LH surge, including kisspeptin and VIP (Zhao and Kriegsfeld, 2009a). GnRH cells express the same clock genes driving circadian function at the cellular level (Chappell et al., 2003; Zhao and Kriegsfeld, 2009a; Hickok and Tischkau, 2010), providing a potential time-keeping mechanism necessary to appropriately phase daily changes in sensitivity to upstream signaling. By administering kisspeptin both in the morning and afternoon to OVX hamsters with and without concurrent estrogen treatment, several findings emerged that enhance our understanding of the mechanisms that time ovulation (**Figure 2.5**). In the morning (ZT 1), GnRH neurons are considerably less responsive to kisspeptin administration than the afternoon (ZT 11), where treatment reliably enhances GnRH cellular activity. This attenuated responsiveness is unlikely due to ineffective doses of kisspeptin, as previous studies indicate that the GnRH system responds robustly to doses as low as 1nmol in rats and 1fmol in mice (Navarro et al., 2005a; Smith et al., 2005a). The presence of estradiol generally enhanced GnRH cellular activity in kisspeptin and vehicle-treated hamsters. These findings suggest that the gating of GnRH activation may

occur via circadian-mediated differences in kisspeptin receptor expression or high inhibitory tone during the morning. Notably, a reliable dose-response to kisspeptin is observed in the POA population of GnRH neurons of estradiol-implanted hamsters at ZT 1 (**Figure 2.5B).** During the afternoon, GnRH cellular activity is at a maximum in OVX hamsters treated with estradiol regardless of treatment in both brain regions, likely due to high endogenous release of AVP and kisspeptin at this time (**Figures 2.5 & 2.6**), thereby masking a dose-response. It is noteworthy that OVX hamsters (figure 5B, left) exhibit a dose response to kisspeptin at ZT 11, further suggesting that the endogenous activation of GnRH in estrogen-implanted hamsters accounts for the 'ceiling' effect in estradiol-implanted hamsters.

If the LH surge is initiated solely by the integration of estradiol with circadian signaling at AVPV kisspeptin neurons, all downstream effects should reflect the extent of kisspeptin release, and kisspeptin treatment should maximize GnRH cellular activity at any time of day, even in the absence of estrogen. The fact that the addition of estradiol potentiates kisspeptin-stimulated GnRH cellular activity suggests additional, estrogen-dependent mechanisms of stimulatory control. Estrogen receptors have been localized to the SCN of humans (Kruijver and Swaab, 2002), mice (Mitra et al., 2003) and rats (Corp et al., 2001), suggesting that AVP or VIP neurons might be directly responsive to this sex steroid. Likewise, in addition to the requirement of estradiol to maximize GnRH cellular activity, the GnRH response to kisspeptin stimulation is time-dependent, suggesting that the surge is not simply elicited when the GnRH system is stimulated with kisspeptin, but that the GnRH system gates daily responsiveness to this peptide. These findings agree with previous findings of daily changes in the sensitivity of the GnRH system to kisspeptin administration using GT1-7 cells (Zhao and Kriegsfeld, 2009a), and studies in which central infusion of kisspeptin i.c.v. (Roa et al., 2008a) or into the mPOA (Neal-Perry et al., 2009) fail to advance the onset of the LH surge in either naturally cycling or OVX and estradiol/progesterone-primed female rats.

Recent findings in female mice provide converging support for the present results implicating circadian control of kisspeptin in ovulation. *Kiss1/c-fos* mRNA is maximally co-expressed to ~40% around the time of the GnRH surge when mice are held in constant conditions (Robertson et al., 2009), indicating circadian control of kisspeptin cellular activity. Our immunohistochemical studies indicate a maximum co-expression of kisspeptin/FOS of 32% around the time of the LH surge, slightly lower than the maximal *Kiss1/c*-fos mRNA co-expression previously reported. The disparity between these findings and the present findings may be the result of post-transcriptional *Kiss1* regulation leading to a lower number of cells expressing the mature peptide. Additionally, it is possible our quantification strategy was more conservative, restricting quantification to only those FOS-labeled cells with a clear nucleus. In this same report, ovariectomy without estradiol replacement abolished this daily pattern. The latter finding is at odds with the present result indicating that the rhythm of kisspeptin/FOS is grossly attenuated, but maintained, in OVX hamsters not treated with estradiol. This partial dependence on estrogen is consistent with the daily pattern of LH secretion that persists, albeit with a lower amplitude than estradiol-implanted animals, in ovariectomized Syrian hamsters (Stetson, 1978). Whether this disparity is due to species differences or a

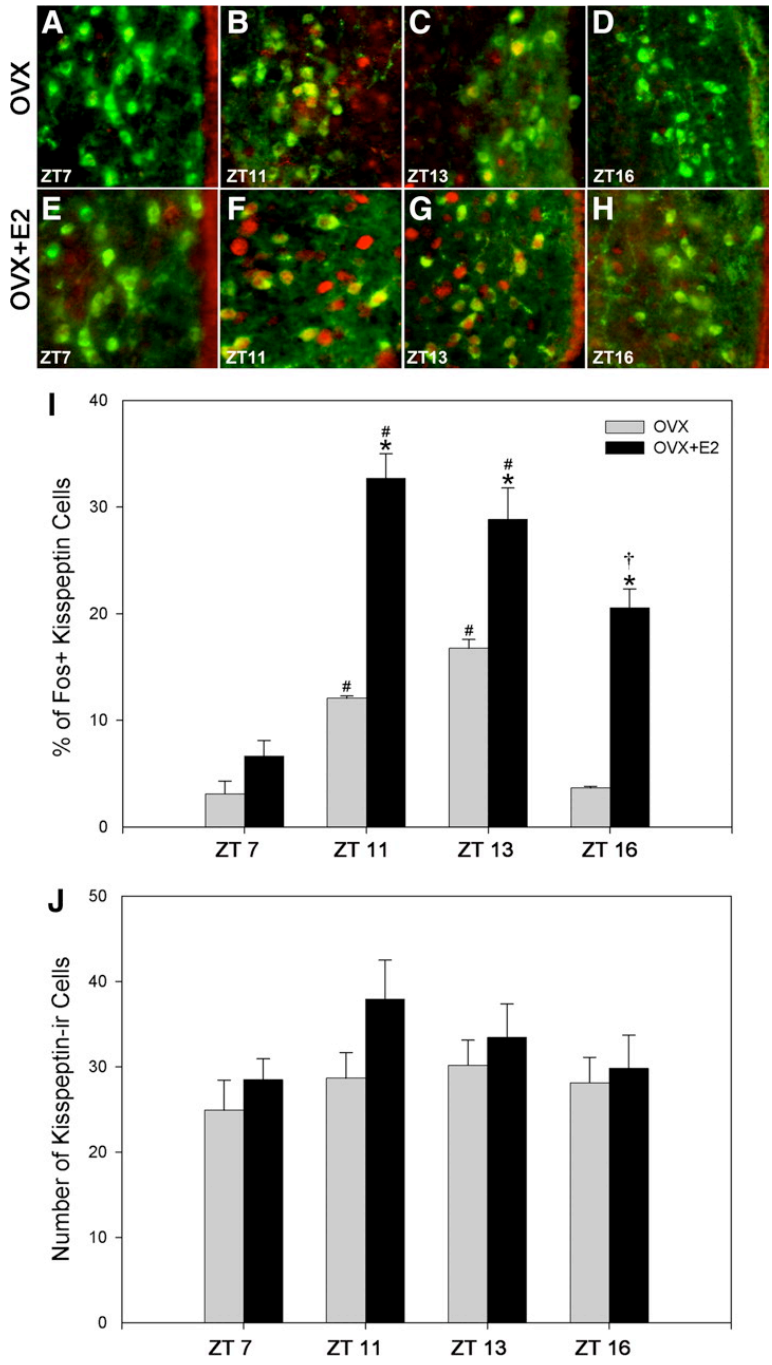
discrepancy in transcriptional/translational differences in the daily pattern of *Kiss1*/kisspeptin represents an interesting question for further exploration.

It is likely that other neural loci upstream of the GnRH systems are targets of the SCN involved in the timing of the LH surge. Dual-phenotype neurons expressing GABA/glutamate within the AVPV, for example, have been implicated in the control of GnRH activity and are regulated by estradiol feedback (Leranth et al., 1985; Jarry et al., 1995; Christian et al., 2009). Likewise, GnRH neurons respond to GABA differentially across the day, with excitatory responses in the afternoon (Petersen et al., 2003; Christian and Moenter, 2007). Though no direct connections have been reported between the SCN and GABA neurons within the AVPV, the diurnal shift in GABA release (Christian and Moenter, 2007) and the expression of V1a receptors in GABA neurons within the AVPV (Kalamatianos et al., 2004) point to a potential circadian mechanism regulating this cell population. Furthermore, kisspeptin upregulates GABA transmission in the AVPV during estrogen negative (but not positive) feedback, further suggesting a local control within the AVPV (Pielecka-Fortuna and Moenter, 2010). In one previous study, unilateral AVPV administration of a V1aR antagonist did not alter the timing or amplitude of the LH surge in rats (Palm et al., 2001). However, because the V1aR antagonist was injected unilaterally, and the position of the dialysis probe was variable, it is unclear to what extent the full population of kisspeptin cells was impacted. Other studies of rats indicate that bilateral suppression of V1aR attenuates the LH surge in proestrous rats (Funabashi et al., 1999). Additionally, in co-cultures of POA and SCN, the GnRH surge is coordinated with the rhythm in AVP, but not VIP (Funabashi et al., 2000c), providing further evidence for an important role of AVP in surge generation.

The present studies reveal a novel, circadian-controlled neurochemical pathway participating in the LH surge. Kisspeptin neurons in the AVPV are targets of AVPergic SCN fibers and express V1a receptors, providing evidence for a direct connection between the master circadian clock and kisspeptin neurons in the AVPV. Kisspeptin neurons exhibit a daily activation pattern coincident with the timing of the GnRH/LH surge that is dependent on the presence of estradiol, further implicating these neurons as integrators of circadian and estrogenic signals necessary for the preovulatory LH surge. AVPV kisspeptin cells are indiscriminately activated by SCN peptidergic signaling, whereas the GnRH system displays time-dependent responsiveness to AVP and kisspeptin stimulation. Together, these findings point to a novel mechanisms of ovulatory control whereby circadian and estrogenic signals converge on AVPV kisspeptin cells and kisspeptin signaling is gated by daily changes in GnRH cell sensitivity to this peptide.

Legend for **Figure 2.1.** (following page)

AVPV kisspeptin cellular activity follows a daily pattern of expression coincident with the LH surge. The percentage of AVPV kisspeptin-ir cells expressing FOS increases in the afternoon and peaks at ZT 11, around the time of the LH surge, and decreases thereafter. The daily pattern of expression is robust in OVX+E2 hamsters, with the magnitude of this daily pattern at each time point significantly attenuated in the absence of estradiol, excluding ZT 7. (A-D) Low-power photomicrographs of kisspeptin-ir cells expressing FOS in OVX hamsters at ZT 7 (A), around the time of the GnRH surge at ZT 11 (B) and ZT 13 (C), and two hours after lights out at ZT 16 (D). (E-F) Low-power photomicrographs of kisspeptin-ir cells expressing FOS in OVX+E2 hamsters at ZT 7 (E), around the time of the GnRH surge at ZT 11 (F) and ZT 13 (G), and two hours after lights out at ZT 16 (H). (I) Mean (±SEM) percentage of kisspeptin-ir cells expressing FOS at various time points in OVX and OVX+E2 hamsters. (J) Mean (±SEM) number of kisspeptin-ir cells at various time points in OVX and OVX+E2 females. #, Significantly greater than kisspeptin cells expressing FOS at ZT 7 and ZT 16 within the same hormonal treatment, $P < 0.05$. *, Significantly greater than kisspeptin cells expressing FOS in OVX hamsters within the same time point, $P < 0.05$. †, Significantly greater than kisspeptin cells expressing FOS at ZT 7 within the same hormonal treatment.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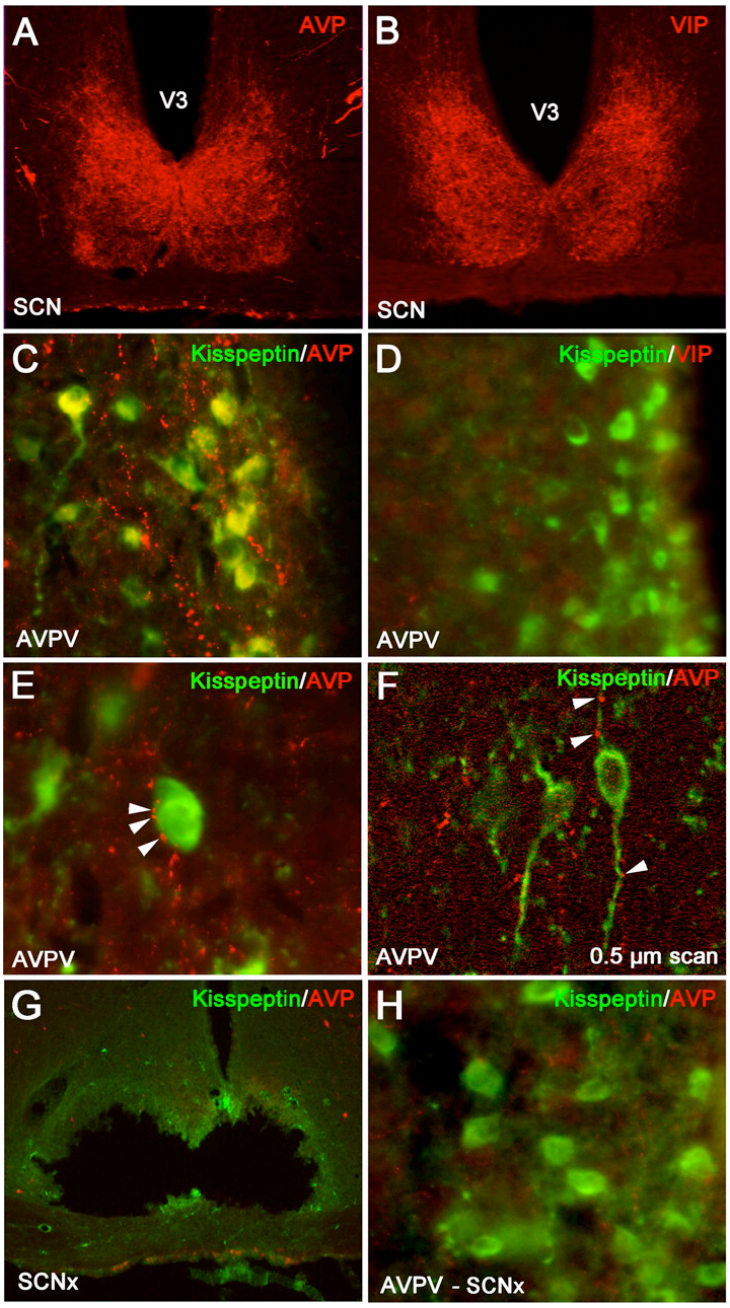

Figure 2.2

Kisspeptin-ir cells in the AVPV receive SCN-derived fiber contacts expressing AVP-ir, but not VIP-ir. (A, C) Low-power photomicrographs of AVP-ir in the SCN (A) and in the AVPV (C), where kisspeptin cell bodies receive extensive AVP-ir fiber contacts. B;D, Low-power photomicrographs of VIP-ir in the SCN (B) and in the AVPV (D),where VIP-ir is virtually non-existent around kisspeptin-ir cell bodies. (E), High-power photomicrograph showing several presumptive AVP-ir terminal boutons on a kisspeptin-ir cell body. Arrows are indicative of close contacts. (F), Confocal image (0.5 µm scan taken at 400X) confirming AVP-ir contacts upon kisspeptin-ir cell body and processes. Arrows are indicative of presumptive boutons. (G,H) Low-power photomicrographs of AVP-ir in an SCN-lesioned hamster, at the level of the SCN (G), where AVP-ir is maintained in the PVN and SON, and in the AVPV (H), where AVP-ir contacts upon kisspeptin-ir cell bodies is virtually eliminated after SCN lesions, confirming the AVP-ir fibers contacting kisspeptin-ir cell bodies originates from the SCN in (C), and see *Results.*

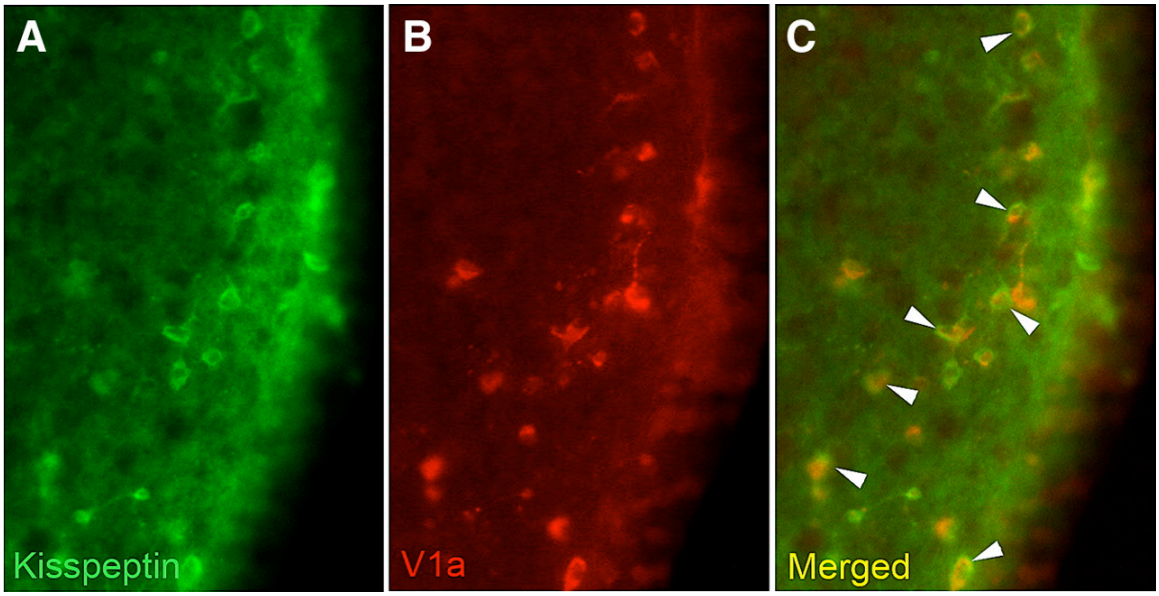

Figure 2.3

Kisspeptin cells in the AVPV express the V1a receptor. Low-power photomicrographs of kisspeptin-ir cells in the AVPV (A), V1a-ir cells in the AVPV (B), and the merged image showing overlap between kisspeptin-ir and V1a-ir (C).

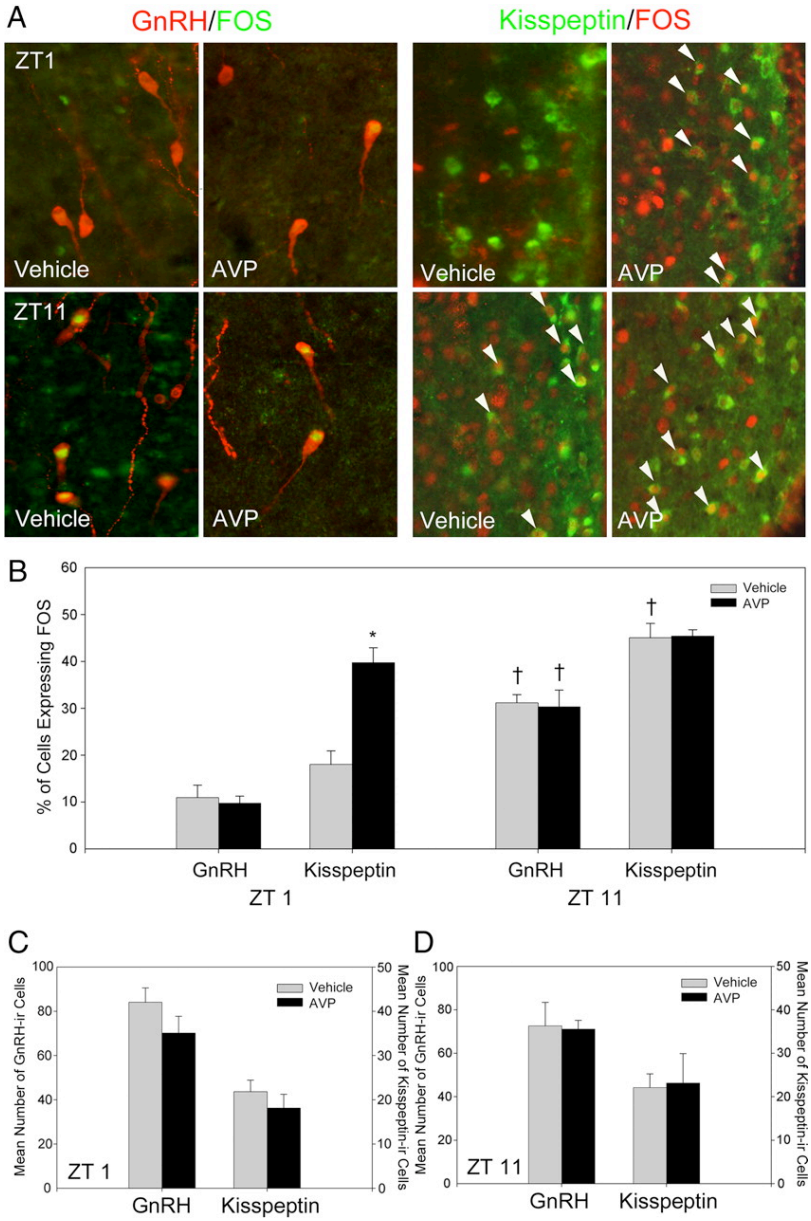

Figure 2.4

Central vasopressin administration reveals indiscriminate activation of kisspeptin neurons concomitant with gated activation of the GnRH system. I.C.V. administration of AVP robustly activates kisspeptin-ir cells in the morning (ZT 1) while GnRH-ir cells remain inactive at this time point. In the afternoon (ZT 11), both kisspeptin-ir and GnRH-ir cells express high levels of FOS activation after saline or AVP injection. (A, left) Low-power photomicrographs of GnRH-ir cells expressing FOS after i.c.v. saline (vehicle), or AVP administration at ZT 1 or ZT 11. (A, right) Low-power photomicrographs of kisspeptin-ir cells in the AVPV expressing FOS after i.c.v. saline (vehicle), or AVP administration at ZT 1 or ZT 11. (B) Mean (±SEM) percentage of GnRH-ir and kisspeptin-ir cells expressing FOS after saline or AVP at ZT 1 or ZT 11. (C, D) Mean (±SEM) total number of GnRH-ir and kisspeptin-ir cells after saline or AVP at ZT 1 (C) or ZT 11 (D). *, Significantly greater than the percentage of kisspeptin cells expressing FOS in hamsters treated with vehicle at ZT 1, $P<0.05$. †, Significantly greater than females given the same pharmacological treatment at ZT 1, $P<0.05$.

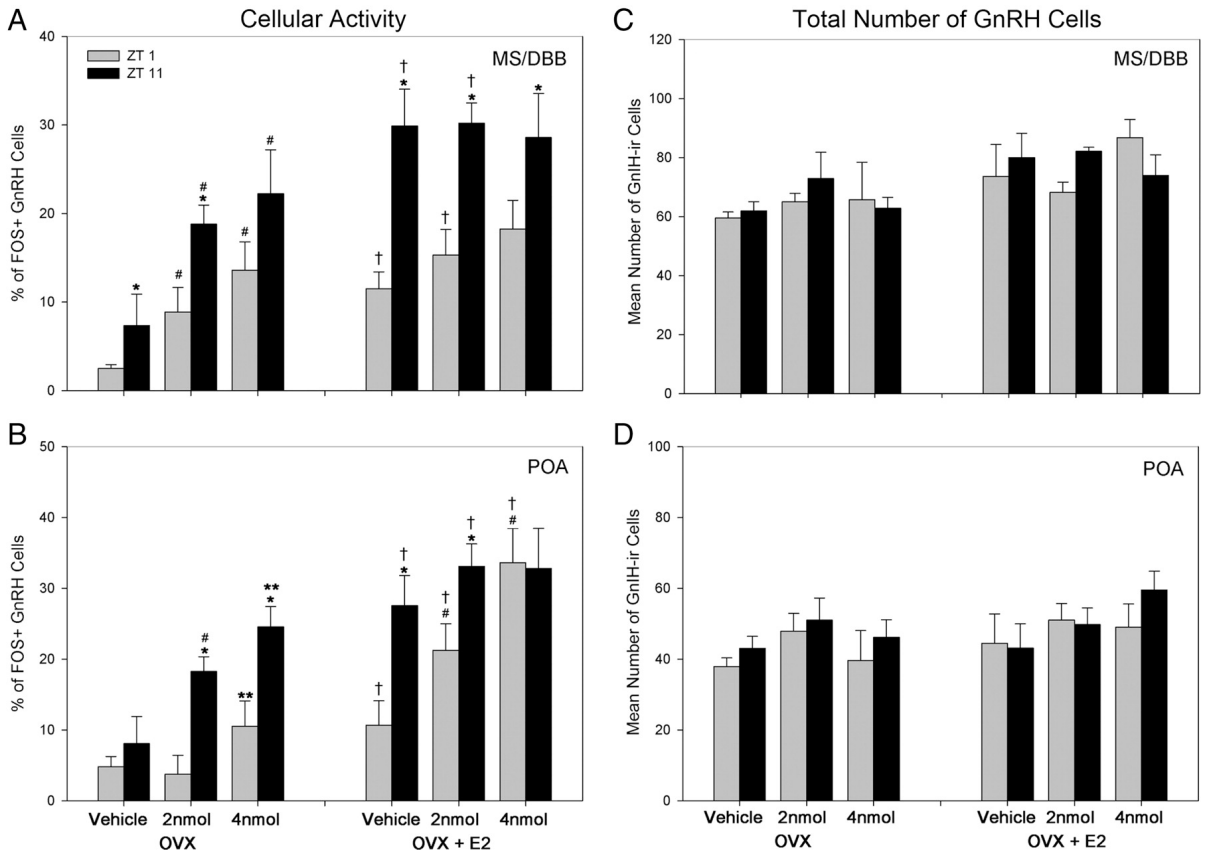

Figure 2.5.

The activation of GnRH following kisspeptin administration is time-dependent, shows regional differences and is enhanced by the presence of estradiol. (A,B) The percentage of GnRH-ir cells expressing FOS following vehicle, 2nmol or 4nmol kisspeptin administration at ZT 1 or ZT 11 in OVX and OVX+E2 hamsters. (A) Mean (±SEM) percentage of MS/DBB GnRH-ir cells expressing FOS after kisspeptin administration at ZT 1 or ZT 11 in OVX (left) and OVX+E2 (right) hamsters. (B) Mean (±SEM) percentage of POA GnRH-ir cells expressing FOS after kisspeptin administration at ZT 1 or ZT 11 in OVX (left) and OVX+E2 (right) hamsters. GnRH activation after kisspeptin administration is time dependent in OVX hamsters and is time dependent after 2nmol kisspeptin in OVX+E2 hamsters. (C) Mean (±SEM) total GnRH-ir cells in the MS/DBB after kisspeptin administration. (D) Mean (±SEM) total GnRH-ir cells in the POA after kisspeptin administration. No differences were seen between cell counts, indicating the differences in FOS-ir represent proportional changes in GnRH activation levels. *, Significantly greater than hamsters provided with the same pharmacological treatment, at the same time point, and the same hormonal condition $P<0.05$. #, Significantly greater than vehicle controls at the same time point and hormonal condition. **, Significantly greater than vehicle controls and 2nmol kisspeptin-treated hamsters at the same time point and same hormonal condition. †, Significantly greater than OVX hamsters at the same time point and pharmacological treatment. All differences are significant at $P<0.05$.

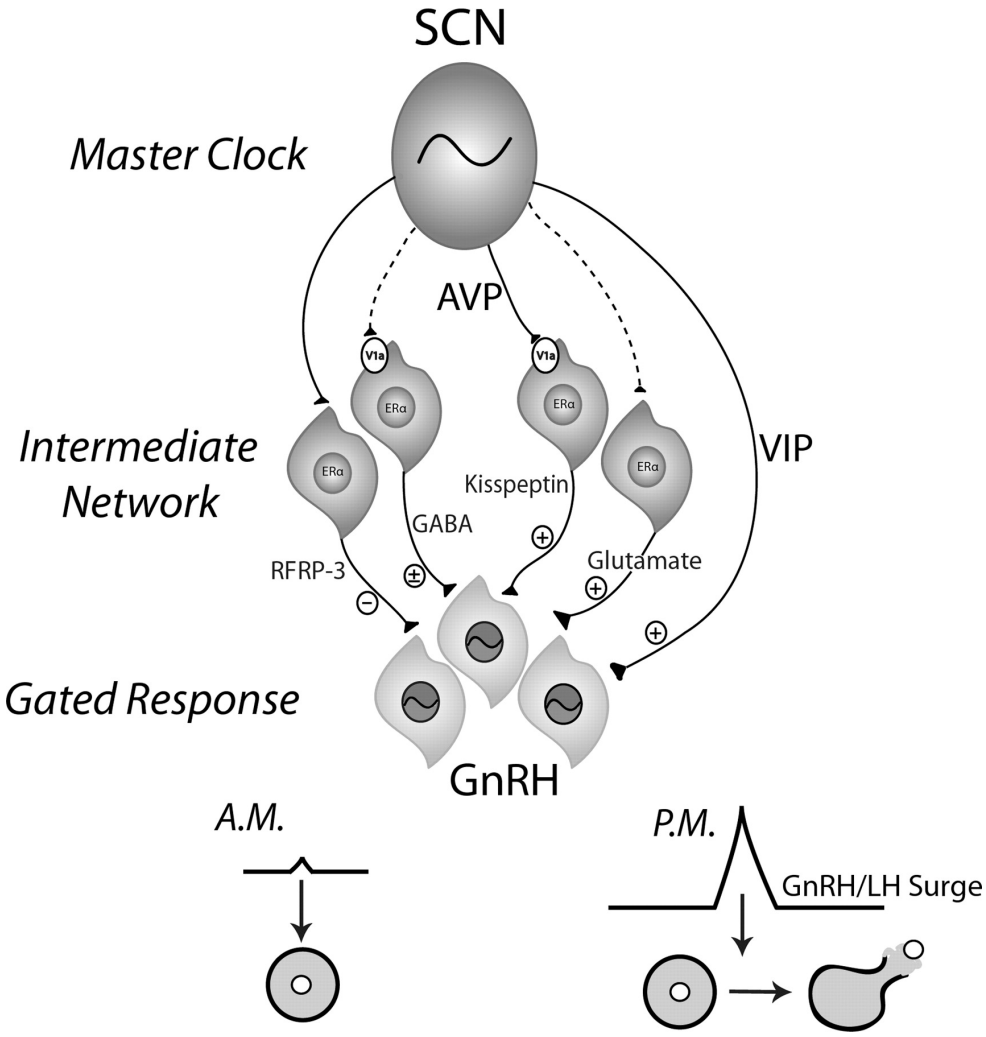

Figure 2.6

Model for the Circadian Control of the GnRH/LH Surge. Proposed model by which several well-characterized, circadian-controlled neurochemicals participate in ovulation. The present studies and pervious work indicate that the SCN sends efferent projections to kisspeptin neurons in the AVPV that express the V1a receptor and RFRP-3 (i.e., gonadotropin-inhibitory hormone) neurons in the dorsomedial hypothalamus (DMH). GABAergic and glutamatergic neurons within the AVPV likely receive SCN input, but these pathways have not been explored (indicated by dashed lines). Together, these intermediate signals represent integration sites of circadian and estrogenic input, as all neuronal phenotypes have been shown to express estrogen receptors. GnRH neurons, in turn, receive input from these inhibitory and stimulatory afferents, as well as direct VIPergic input from the SCN. In turn, the consequent response of the GnRH system will depend on the sum total of positive and negative influence and on time of day, the latter due to an inherent daily timing mechanism in GnRH cells.

Chapter 3

Alterations in RFAmide Peptide Rhythmicity with Advanced Age

Introduction

In most laboratory rodents (e.g., rats, Syrian hamsters, and mice), an intact circadian system is essential for females' reproductive success (Kriegsfeld and Silver, 2006). Lesions of the SCN result in arhythmicity in all behavioral and physiological parameters, including endocrine function (Moore and Eichler, 1972; Stephan and Zucker, 1972; Wiegand and Terasawa, 1982). With regard to endocrine function specifically, SCN lesions or mutation of 'clock' genes driving rhythms at the cellular level result in marked deficits in ovulation and fecundity in most rodents (Nunez and Stephan, 1977; Wiegand and Terasawa, 1982; Miller et al., 2004). In mice, rats, and hamsters the circadian clock is essential for normal ovulation, whereas ovulation and sexual behavior in primates is under less stringent temporal control (Yamaji et al., 1971; Knobil, 1974).

One of the first indicators of reproductive decline involves an attenuated and delayed LH surge on the day of proestrous (Wise, 1982a, b). Serum LH levels peak 1hour later in middle -aged animals versus young animals and have a significantly blunted surge. This decreased LH response occurs prior to any changes in the pituitary's responsiveness to GnRH or any decline in GnRH cell numbers. It is likely that the decreased and delayed LH surge seen in middle- aged animals stems from a decrease in the activation pattern of GnRH neurons, as a decline in GnRH neurons expressing cFos during proestrous has been observed in middle aged animals (Lloyd et al., 1994). Middle aged, intact animals also lack the increase of GnRH mRNA on proestrous that is seen in young animals (Yin and Gore, 2006). This decline in GnRH activation may reflect alterations in the strength and timing of the afferent inputs to this system originating from the circadian clock in the SCN of the hypothalamus.

The timing of the LH surge is tightly regulated by the circadian system in Syrian hamsters. In this species, the LH surge occurs four hours prior to the onset of locomotor activity, with a cessation of the surge occurring two hours later (Stetson, 1978). GnRH neurons exhibit robust increases in immediate-early gene expression one hour after the peak in the LH surge (Lee et al., 1990; Lee et al., 1992; Hoffman et al., 1993). The overall rhythm of the SCN is generally altered with age, as glucose utilization rhythm, a marker of neural activity, becomes dampened in response to the light-dark cycle (Wise et al., 1988). Furthermore, vasoactive intestinal polypeptide (VIP), one of the most abundant neuropeptides in the SCN, projects directly to GnRH neurons (Horvath et al., 1998), which significantly increase following puberty in female rats (Kriegsfeld et al., 2002) and has a rhythmic mRNA expression that disappears by the time female rodents reach middle age (Krajnak et al., 1998). Additionally, the number of SCN neurons expressing arginine vasopressin and vasoactive intestinal polypeptide is decreased in

middle-aged rats (Roozendaal et al., 1987; Chee et al., 1988). Since both of these neurotransmitters have been implicated in the circadian control of ovulation (Van der Beek et al., 1997; Kalsbeek and Buijs, 2002), and SCN tissue transplants do not restore circadian endocrine rhythms necessary for the LH surge in SCN lesioned Syrian hamsters (Meyer-Bernstein et al., 1999), the integrity of these neurons in the SCN may be the rate limiting factors in reproductive functioning. Insight into the mechanisms by which AVP and VIP control the timing of the GnRH surge will give insight into how the central oscillator controls ovulation, and becomes disrupted with age.

The regulation of RFamide peptides by the circadian clock represents a novel mechanism of temporal control of the GnRH surge. Therefore, desynchronized input to these peptides may contribute to the reproductive decline observed in middle-aged rodents. As described previously, the cellular activity of the inhibitory peptide, RFRP, appears to be orchestrated by the SCN in ways that remove negative input to the GnRH system during the LH surge (Gibson et al., 2008). Likewise, the kisspeptin system represents a key integration point for estrogenic and circadian signals necessary to stimulate the LH surge, and the timed sensitivity of GnRH to this peptide likely gates the temporal precision of the GnRH surge (Williams et al., 2011). Together, these findings suggest that the SCN acts to temporally balance the contribution of negative and positive inputs to the GnRH system, thereby permitting the LH surge and ultimately ovulation. Because the precision in the coordination of these neuropeptides is important for ovulatory control in young animals, I examined whether changes in the expression or timing of RFRP and kisspeptin are associated with reproductive decline in middle-aged Syrian hamsters.

Materials and Methods

Animals

Three cohorts (3 months, 12 months/regular and 12 months/irregular) of female Syrian hamsters (*Mesocricetus auratus*), purchased from Charles River were used. Animals were maintained on a light:dark (14:10; lights off at ZT 14) schedule during the course of the experiment. The presence of post-estrous discharge every fourth day was used to assess estrous cyclicity in all animals. Within the cohort of young hamsters, only females with regular 4-day estrous cycles were retained for the study. Within the middle-aged group, hamsters exhibiting at least two, regular 4-day estrous cycles were considered to be 'regularly cycling.' Hamsters within the middle-aged group that exhibited any irregularities within a 12 day period were considered to be 'irregularly cycling.' To examine the cyclical pattern of kisspeptin and RFRP-3 cell activation independent of fluctuations in peripheral sex steroids, females were bilaterally ovariectomized (n=48) under isoflurane anesthesia, and treated with a SILASTIC capsule (Dow Corning Corp.; 10-mm length, 1.45-mm id, 1.93-mm od) containing powdered undiluted 17β-Estradiol (n=9/group).

Perfusion and Histology

For brain collection, hamsters were deeply anesthetized with sodium pentobarbital (200mg/kg) and perfused transcardially with ~150 ml of 0.9% saline, followed by 300-400 ml of 4% paraformaldehyde in 0.1 M PBS, pH 7.3. Brain and serum samples were collected at one of four time points in relation to the LH surge (ZT2, 6, 10, or 12), with the surge onset occurring at ZT10. Serum samples were assayed for LH as previously described (Greives et al., 2007; Gibson et al., 2008). Brains were post-fixed for 2-3 hours and transferred to 30% sucrose for 24 hours. Double-label immunofluorescence was performed on every fourth, 40-µm coronal section.

For simultaneous visualization of RFRP and FOS, every fourth 40-µm coronal section from the mediobasal hypothalamus was washed in 0.1M PBS, incubated in 0.5% H_2O_2, and incubated in normal goat serum (1:50; Jackson) in 0.1% Triton X-100 (PBT) for 1 hour. Sections were then incubated for 48 hours at 4ºC with a rabbit anti-FOS Ab diluted at 1: 50,000 (Santa Cruz, Santa Cruz, CA) and NGS diluted at 1:1000 with 0.1% PBT for 48 hours. After incubation in anti-FOS, brains were incubated for 1 hour in biotinylated goat anti-rabbit (1:300; Vector Laboratories, Burlingame, CA) and then in ABC for 1 hour. The FOS signal was amplified with biotinylated tyramide solution (0.6%) for 30 min as previously described (Kriegsfeld et al., 2006) Cells were then labeled by using Cy-2 conjugated streptavidin (1:200; Jackson) as the fluorophore. This protocol allowed for the amplification of the highly diluted anti-FOS required for double-labeling with two antibodies generated in the same species (rabbit). After labeling for FOS, sections were incubated in anti-RFRP antibody (1;10,000; PAC 123/124) with 0.1% PBT for 48 h (Kriegsfeld et al., 2006). RFRP cells were labeled with CY-3 donkey anti-rabbit (1:200; Jackson) as the secondary/fluorophore.

For simultaneous visualization of kisspeptin and Fos, every 4^{th} 40 µm section from the AVPV was washed in 0.1M PBS, incubated in 0.5% H_2O_2, and incubated in normal goat serum (1:50; Jackson) in 0.1% Triton X-100 (PBT) for 1 hour. Sections were incubated for 48 hours at 4ºC with a rabbit polyclonal anti-kisspeptin-10 antiserum (1:2000; generated by Jens Mikkelsen), previously shown to bind with high affinity to kisspeptin neurons in the AVPV and arcuate nuclei and exhibit minimal cross-reactivity to related RFamide peptides (Mikkelsen and Simonneaux, 2009; Desroziers et al., 2010) and NGS diluted at 1:1000 with 0.1% PBT for 48 hours. After incubation in the first primary antibody, brains were incubated for 1 hour in biotinylated goat anti-rabbit (1:300; Vector Laboratories, Burlingame, CA) and then in ABC for 1 hour. For kisspeptin, the signal was amplified with biotinylated tyramide solution (0.6%) for 30 min as previously described (Kriegsfeld et al., 2006). Cells were then labeled by using Cy-2 conjugated streptavidin (1:200; Jackson) as the fluorophore. This protocol allowed for the amplification of the highly diluted anti-kisspeptin required for double-labeling with two antibodies generated in the same species (rabbit). After labeling for kisspeptin, sections were incubated in rabbit anti-FOS (1:5,000; Santa Cruz) with 0.1% PBT for 48 h. FOS cells were labeled with CY-3 donkey anti-rabbit (1:200; Jackson) as the secondary/fluorophore.

Light microscopy

Sections were examined using the standard wavelengths for CY-2 (488 nm) and CY-3 (568 nm) using a Zeiss Z1 microscope. Every 4th section through the anteroventral periventricular nucleus (AVPV; kisspeptin/FOS) and the dorsomedial hypothalamus (DMH; RFRP-3/FOS) were assessed. For light microscopy, kisspeptin cells identified as expressing FOS, or GnRH cells expressing FOS were digitally captured at 200x in 8 bit greyscale using a cooled CCD camera (Zeiss). Each label was captured as a single image without moving the position of the stage or plane of focus between captures. Images were superimposed digitally. Brain areas were examined by two independent observers using Photoshop software in order to view CY-2 and CY-3 channels independently. Kisspeptin or RFRP-3 cells with a clear nucleus were quantified using single-channel analysis. Cells were considered to be double-labeled if FOS was expressed in the cell nucleus, but not beyond the borders of each pre-defined nuclear area. For FOS expression, only those kisspeptin and RFRP-3 cells with a visible nucleus in which FOS expression was localized to the nucleus were counted as double-labeled cells.

Statistics

Data were analyzed using SigmaStat software for all studies. Data for kisspeptin cell counts, RFRP-3 cell counts and FOS expression in kisspeptin cells and RFRP-3 cells were analyzed using 3 x 4 (Age x Time of Day) analyses of variance (ANOVA) for those studies assessing the change in activation over the course of the day. Group differences were evaluated using Tukey HSD tests. Differences were considered significant if $p<0.05$.

Results

The Preovulatory Luteinizing Hormone Surge is Blunted in Middle-Aged Hamsters.

Although the timing of the LH surge was unaffected in middle-aged females, the amplitude was significantly lower in middle-aged relative to young animals ($p<0.05$; **Figure 3.1**). No significant differences were observed between irregular and regular cycling middle-aged hamsters and these sub-groups were collapsed. The LH surge in middle-aged hamsters was ~ 50% lower than that of the young counterparts (1ng/ml vs. 2 ng/ml).

The Circadian Pattern of RFRP-3 Activity Reduced around the LH surge and is Unaffected by Age

In both young and middle-aged animals, the total number of RFRP-3-ir cells was greatest during the LH surge (ZT10; $p<0.05$ in both cases) and the percentage of cells

expressing FOS was lowest during the LH surge in both age groups ($p<0.05$ in both cases; **Figure 3.2**). Additionally, the number of RFRP cells labeled and the percentage of RFRP cells expressing FOS was unaffected by age ($p>0.05$ in all cases; **Figure 3.2**).

The Circadian Pattern of RFRP-3 Activation is altered in Irregularly Cycling, Middle-Aged Animals

In young and regularly cycling middle-aged animals, RFRP activity is significantly reduced during the onset of the LH surge (ZT 10) ($p<0.05$ vs. all other time points; **Figure 3.3**). In irregularly cycling middle-aged females, this reduction in activity occurs prior to the onset of the LH surge (ZT 6), and is significantly lower than RFRP-3/FOS activation at this time point in both young and middle-aged, regularly cycling hamsters ($p<0.05$ in all cases; **Figure 3.3**). The total cell count is not significantly different across these groups (data not shown), and does not account for the shift in RFRP-3/FOS in middle-aged/irregular animals.

The Daily Pattern of Kisspeptin Activity is Associated with the LH Surge and the Pattern of Kisspeptin Immunoreactivity is Affected by Age

Numbers of AVPV kisspeptin cells were reduced during the LH surge in young animals ($p<0.05$) but not in middle-aged ($p>0.05$) ones (**Figure 3.4**). In both age groups, the percentage of cells expressing kisspeptin was increased at ZT10/ZT12 relative to time-points prior to the LH surge ($p<0.05$ in all cases; **Figure 3.4**). There were no age differences in the percentage of cells expressing FOS at any time-point ($p>0.05$ in all cases; **Figure 3.4**).

Discussion

One of the hallmarks of reproductive senescence in aged populations is disruption of the reproductive axis, including a delay in the timing and a reduction in the amplitude of the LH surge. Presumably, these deficits result from a dysregulation of the circadian system, although the specific mechanisms leading from temporal desynchronization in the circadian clock to the resulting decline in GnRH pulsatility remain uncertain. Based on our previous work indicating the circadian control of two RFamide peptides, RFRP-3 and kisspeptin (Gibson et al., 2008; Williams et al., 2011), I sought to determine whether or not alterations in the pattern of RFamide activation is associated with the transition to reproductive acyclicity.

Predictably, middle-aged rats exhibited a blunted LH surge compared to young animals, although no shifts in the timing of the surge were apparent in either age group.

The precision in the timing of LH in both groups is mirrored by the reduction in RFRP-3 activation patterns around the time of the LH surge in both groups, indicating the SCN remains capable of attenuating this inhibitory peptide to allow for proper stimulation of the GnRH surge in the late afternoon. It is unclear whether or not more pronounced changes in the RFRP-3 system would have been observed in aged hamsters had the animals been left intact. However, as middle-aged females were cycling irregularly, it was impossible to select appropriate time-points for comparisons without ovariectomy and replacement of estradiol. Given that RFRP-3 neurons express ERα in multiple species (Kriegsfeld et al., 2006), and estradiol downregulates RFRP-3 mRNA in mice (Molnar et al., 2011), it is plausable that irregular levels of estradiol feedback in middle-aged animals would have impacted the secretory patterns of this peptide, thus contributing to reproductive decline in intact models. Conversely, the ability of Syrian hamsters to exhibit daily LH surges following OVX without estradiol replacement, may preclude this species for exhibiting estradiol-mediated disruptions in the timing of the GnRH surge with advanced age. It is critical to note that the timing of RFRP-3 activation is shifted in middle aged/irregularly cycling hamsters compared to young and middle aged/regularly cycling hamsters. In this cohort, the decline in RFRP-3 activation occurs earlier than in other groups, beginning at ZT 6. The early reduction in inhibitory input to GnRH may reflect the initial decline in SCN synchronization of the GnRH surge observed in animals exhibiting early signs of estrous acyclicity.

Somewhat surprisingly, the levels of kisspeptin activation patterns were unaltered between young and middle-aged animals. In both groups, the percentage of kisspeptin activation in the AVPV increased around the time of the LH surge, indicative of the circadian drive of this stimulatory peptide to trigger the GnRH surge established here and in previous work (Robertson et al., 2009; Williams et al., 2011). The total number of kisspeptin-immunoreactive cells were significantly different between age groups around the time of the LH surge. The total number of kisspeptin-ir cells was lower in the young cohort than the middle-aged group. That the number of kisspeptin immunoreactive cells at this time point was higher in the middle-aged group without a difference in FOS activation may be indicative of improper levels of kisspeptin release at this time point. The higher level of 'stored' peptide,' therefore, may reduce stimulatory tone to GnRH, thus leading to a blunted LH surge. As with RFRP, it is possible that changes in kisspeptin might have been observed had animals been examined under conditions of endogenous estrogen exposure, particularly given that the activity of kisspeptin is sensitive to estradiol (Smith et al., 2005b; Roa et al., 2008b). Recent work supports this contention, as estradiol administration yields a reduction in the number of kisspeptin-ir cells of middle-aged vs. young female rats (Lederman et al., 2010). Again, the relative level of kisspeptin immunoreactivity as a function of estrogen feedback, vs. circadian control may differ between species. For example, OVX mice exhibit a non-rhythmic expression of *Kiss1* mRNA (Robertson et al., 2009), whereas the circadian pattern of kisspeptin activation in Syrian hamsters is maintained following ovariectomy (Williams et al., 2011).

Given the relatively small differences in kisspeptin and RFRP expression in young and middle-aged animals, it is unlikely that alterations in these systems account

entirely for the reduction in the amplitude of LH surge in older animals. Both in young and middle-aged animals, it appears that the circadian system is equally capable of removing the influence of RFRP inhibition at the time of the surge. In young animals, there is a reduction in kisspeptin cell numbers at the time of the surge, possibly due to increased transport and release in this age group relative to middle-aged animals. Likewise, we have recently shown that the GnRH system is differentially responsive to kisspeptin stimulation across the day, presumably due to local regulation by clock genes (Zhao and Kriegsfeld, 2009b). Thus, it is also possible that middle-aged animals lose the ability to time the responsiveness of GnRH to kisspeptin to coincide with times of RFRP disinhibition and concomitant stimulation by estradiol.

In agreement with results from studies of the effects of estradiol on kisspeptin neurons in the arcuate of Syrian hamsters (Smith et al., 2005a; Franceschini et al., 2006; Roa et al., 2008b) but see (Lederman et al., 2010), postmenopausal rhesus monkeys exhibit an increase in *Kiss1* gene expression and cell size relative to reproductively competent animals. The authors speculated that this increase is due to decreased negative feedback control by ovarian steroids (Rometo et al., 2007; Kim et al., 2009). In humans, the number and size of *Kiss1* mRNA neurons increases in postmenopausal women (Rometo et al., 2007). In primates, reproductive decline is primarily due to ovarian aging, whereas reproductive aging in rodents may be the result of alterations both in the brain and ovary (Yin and Gore, 2006). Results in Syrian hamsters indicate that kisspeptin expression is higher during the LH surge in middle-aged animals relative to young animals. Whether this increased expression pattern represents a reduction in release with continued mRNA turnover, or an increase in mRNA transcription/translation, requires further examination. However, because estrogen concentrations were clamped at equivalent values both in young and middle-aged animals, these changes are not the result of alterations in estrogen negative feedback as might occur in human/non-human primates. Instead, these findings suggest that kisspeptin regulation may change independently of decreasing ovarian steroids.

The maintained circadian rhythm of kisspeptin activity in middle-aged animals may be a reflection of the SCN-derived control of this peptide. The results in Chapter 2 provide evidence for a vasopressin-derived pathway from the SCN to kisspeptin neurons, which has been replicated in mice (Vida et al., 2010). Given that previous studies have shown no age related decline in *Avp* mRNA expression with age, but significant attenuation of *Vip* mRNA rhythms in aged rats (Krajnak et al., 1998), the specific neuronal phenotype regulating kisspeptin activity may maintain a rhythmic stimulatory drive to this peptide with advanced age. Conversely, the decline in *Vip* mRNA, combined with evidence for monosynaptic projections to GnRH may be indicative of a VIPergic mediated decline in GnRH pulsatility (Kalsbeek et al., 1993). In middle-aged animals, although significant suppression of the amplitude of the surge in LH is observed, there is little evidence that alterations in the RFRP system contribute to this deficit in Syrian hamsters. In contrast, changes in the positive drive by the kisspeptin system, independent of gonadal steroids, may impact reproductive aging. Additional exploration of the role of the circadian timing system in the neural circuits regulating the LH surge and ovulatory function is necessary to further understand how aging might impact timing mechanisms

contributing to reproductive senescence. Examining differences in GnRH sensitivity to upstream signals, for example, may provide new avenues for determining the underlying mechanisms behind the decline in GnRH pulsatility with advanced age in rodents.

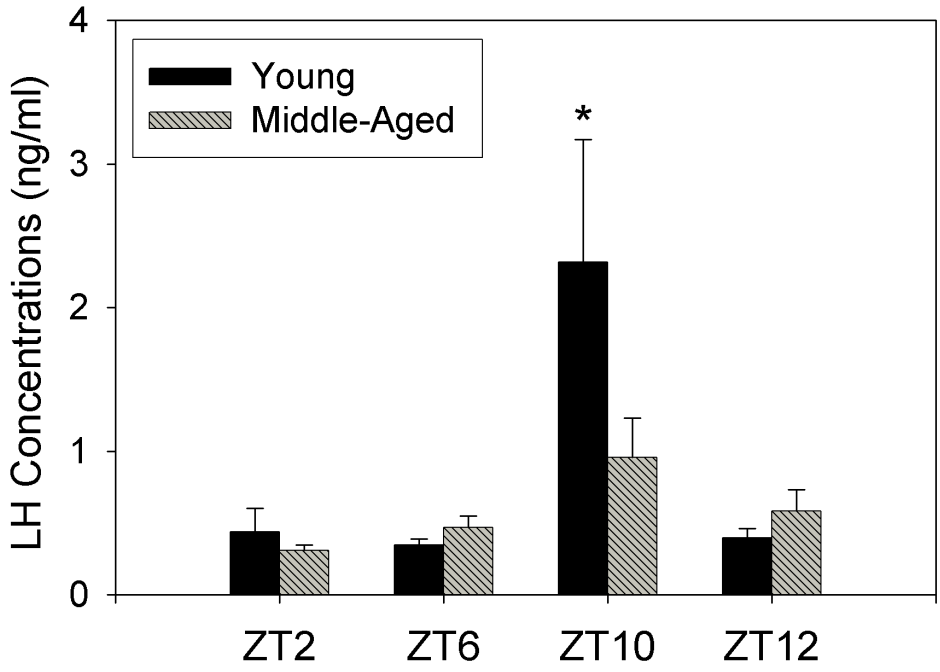

Figure 3.1

The preovulatory LH surge is blunted in middle-aged hamsters. LH concentrations are shown relative to zeitgeber (light:dark cycle) time, with ZT0=lights on and ZT14=lights off. Serum LH concentrations were measured in duplicate in a single RIA with reagents obtained from the National Institutes of Health (Bethesda, MD). The antiserum was rLH-S-11, and the standard was rLH-RP3. The sensitivity was 0.01 ng/tube, and the intraassay coefficient of variation was 2.8% for the low pool and 8.4% for the high pool. The antisera were highly specific for the hormones measured, with low cross-reactivity with other hormones. * Significantly greater than all other groups, $p<0.05$.

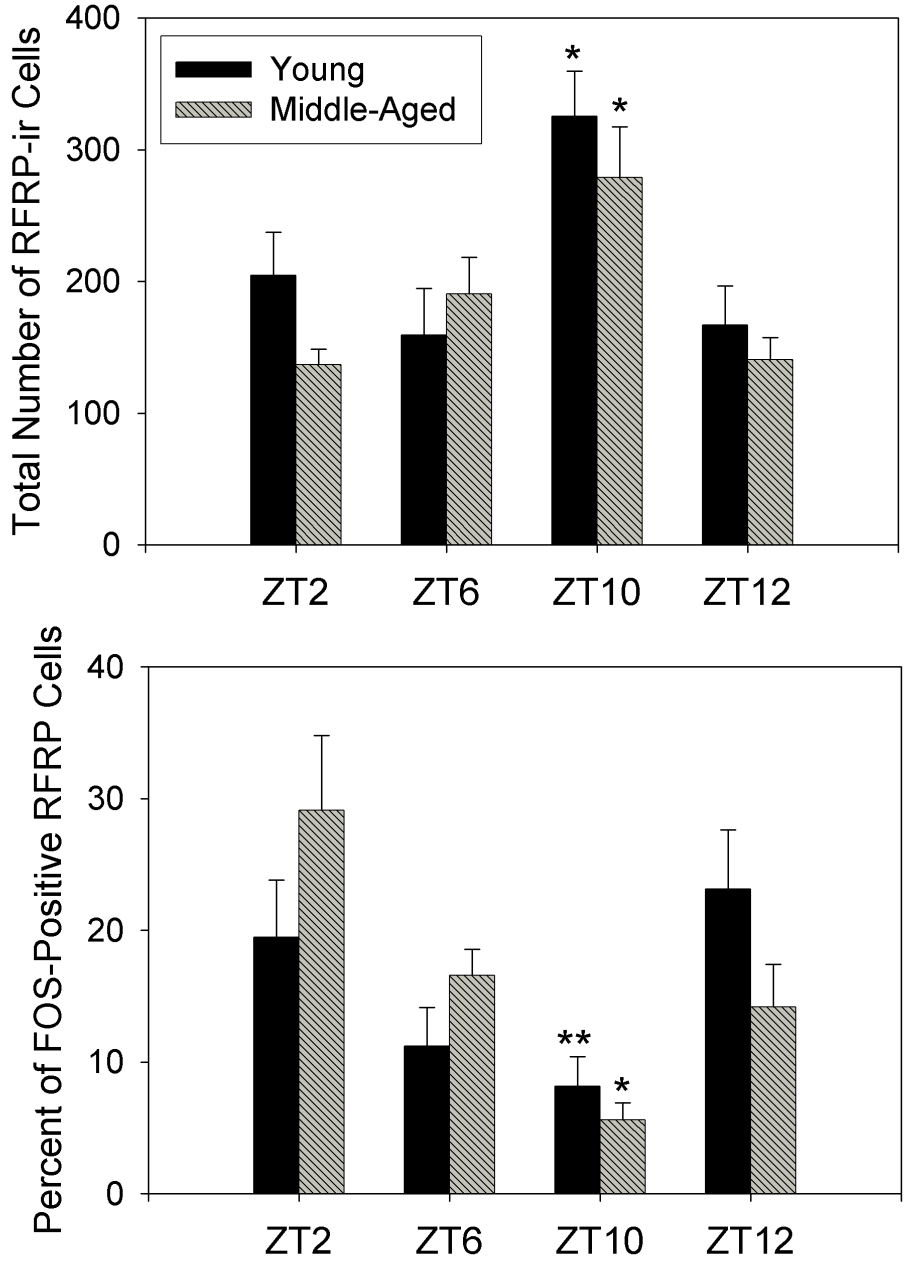

Figure 3.2

Number of, and percent of FOS-positive, RFRP-ir cells are associated with the timing of the LH surge. The mean (±SEM) number of RFRP-ir cells and percent (±SEM) of FOS-positive RFRP-ir cells do not differ in young and middle-aged animals at any time point ($p>0.05$ in all cases). Data are shown relative to zeitgeber (light:dark cycle) time, with ZT0=lights on and ZT14=lights off. RFRP cells and FOS were labeled immunohistochemically as previously described (see Methods). * Significantly greater (top) or less (bottom) than all other time-points in the same age group $p<0.05$. ** Significantly less than all other time points from the same age group, excluding ZT6, $p<0.05$.

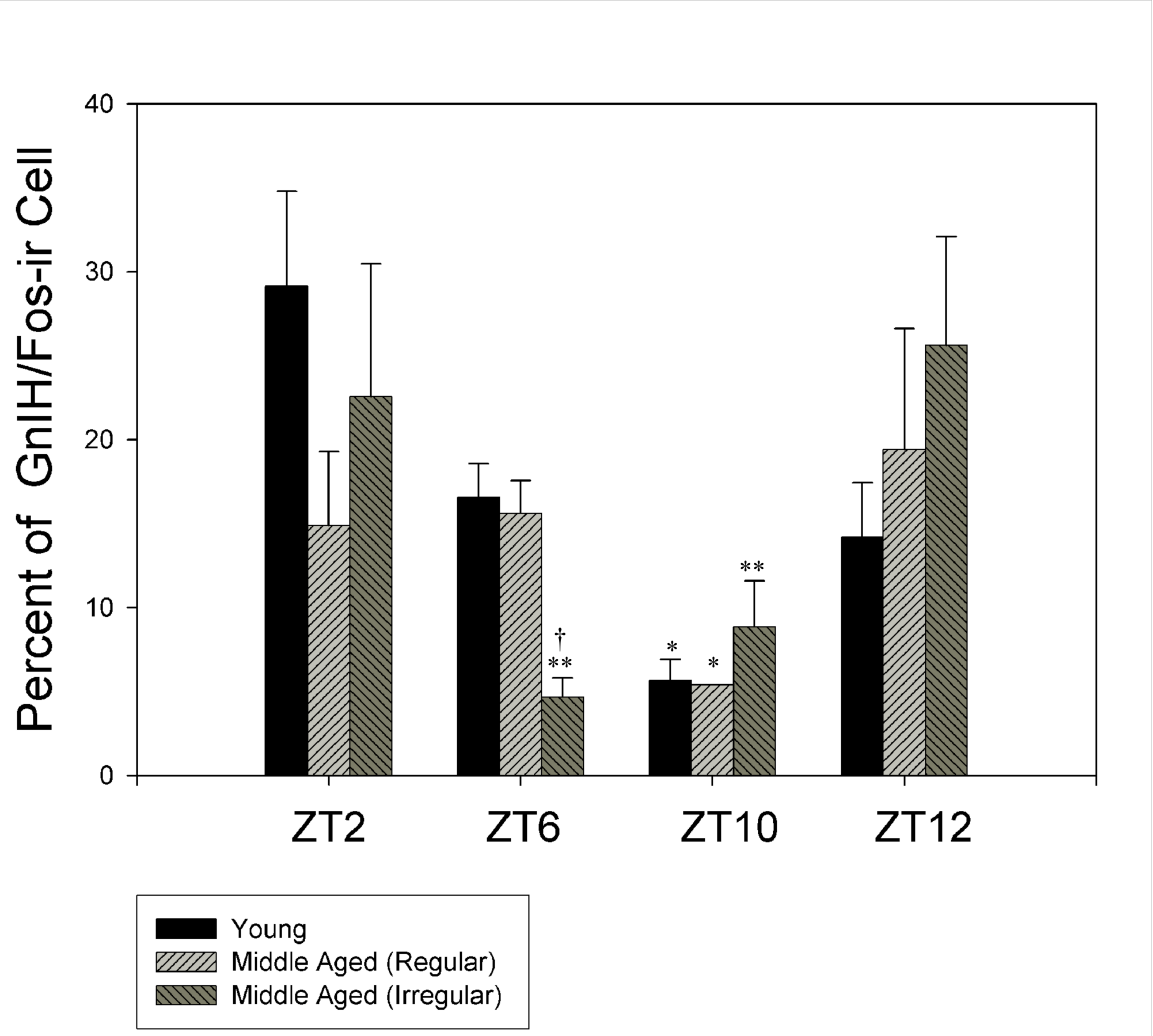

Figure 3.3

Percentage of FOS-positive, RFRP-ir cells Exhibits Temporal Disruptions in Middle-Aged, Acyclic Hamsters. The percent (±SEM) of FOS-positive RFRP-ir cells in young and regularly cycling middle-aged animals is reduced during the onset of the LH surge (ZT 10). In irregularly cycling middle-aged females, this reduction in activity occurs prior to the onset of the LH surge (ZT 6), and is significantly lower than both young and middle-aged, regularly cycling animals at this time point ($p<0.05$). *, Significantly less than all other time points within the same age group, $p<0.05$. **, Significantly less than ZT 2 and ZT 12 within the same age group, $p<0.05$. †, Significantly less than all other age groups within the same time point, $p<0.05$.

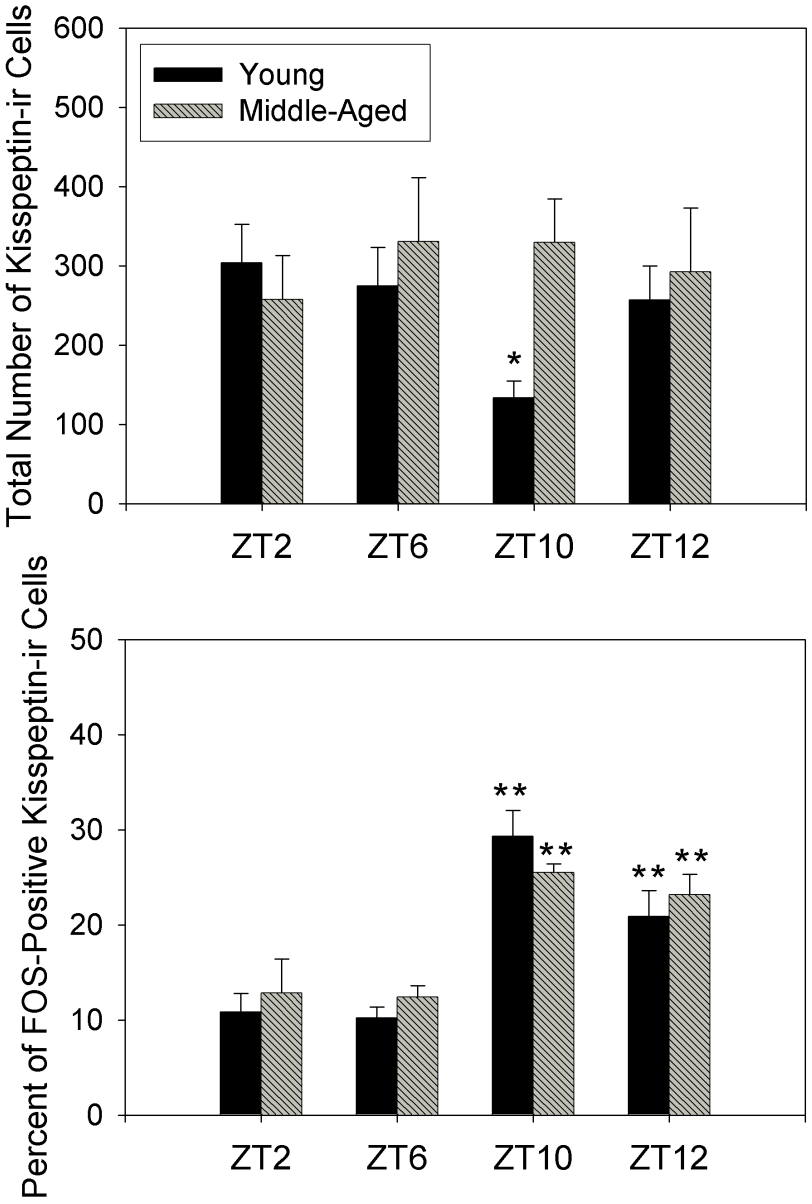

Figure 3.4

Number of, and percent of FOS-positive, kisspeptin-ir cells are associated with the timing of the LH surge. The mean (±SEM) number of kisspeptin-ir cells was significantly different between age groups at ZT 10, where the number of kisspeptin-ir cells was significantly lower in young animals compared to middle-aged animals ($p<0.05$). The percent (±SEM) of FOS-positive kisspeptin-ir cells did not differ between young and middle-aged animals at any time point ($p>0.05$ in all cases). Data are shown relative to zeitgeber (light:dark cycle) time, with ZT0=lights on and ZT14=lights off. Kisspeptin cells and FOS were labeled immunohistochemically as previously described (See Methods). *, Significantly less than all other groups, $p<0.05$. **, Significantly greater than values at ZT2 and ZT6, $p<0.05$.

Chapter 4

Energetic and Reproductive Status impact RF-Amide Related Peptide-3 Immunoreactivity in Female Syrian Hamsters

Introduction

The availability of metabolic fuels represents the single most important environmental factor regulating mammalian reproduction. The high energetic costs of species propagation, especially pregnancy and lactation, require extended periods of relative food abundance that can be difficult to predict in natural environments. Individual survival requires invariable physiological processes, including cellular maintenance and blood flow that must be maintained regardless of energetic status. Conversely, reproductive processes can be inhibited during periods of negative energy balance without threatening individual survival. Therefore, signals indicative of a metabolic challenge originating from low food intake or excessive energy use (i.e., thermoregulation or exercise) have a profound impact upon the HPG axis. Accordingly, rats and hamsters exhibit reductions in estrous behaviors when subjected to food deprivation, low ambient temperatures that require excessive thermoregulation, or following administration of metabolic fuel inhibitors (Dickerman et al., 1993; Li et al., 1994; Early et al., 1999; Jones et al., 2002; Jones and Wade, 2002). Seasonal breeders principally rely on changes in photoperiod to successfully predict periods of relative food abundance and coordinate the immense energetic requirements of gestation and lactation with the optimal availability of oxidizable fuels (Bronson, 1989). Additionally, short-term food availability may impact reproduction as well; food deprivation inhibits reproduction in seasonally breeding sheep and hamsters subjected to photoperiods that would otherwise indicate optimal breeding conditions (Schneider, 2004). The inhibition of reproductive processes occurs via the inhibition of GnRH release from the hypothalamus, leading to a blunted LH surge and the inhibition of follicular growth. Additionally, undernutrition inhibits sexual motivation, an effect that may occur independently of estrogen feedback. For example, proceptive behaviors are inhibited in food deprived female Syrain hamsters without affeting estrous cyclicity or plasma luteinizing hormone concentrations (Schneider et al., 2007).

In contrast to extreme food shortages, periods of unpredictable food availability reveal the impact of the ovulatory cycle on motivation to engage in feeding vs. sexual behavior. Following mild food restriction, seasonal breeders, such as Syrian hamsters, will elect to engage in estrous behaviors over food hoarding when pregnancy is likely (i.e., during proestrous), while appetitive aspects of feeding behavior, such as hoarding, are more common during other stages of the estrous cycle (Klingerman et al., 2010). This dynamic shift in motivation reveals a rapid response of the HPG axis to changes in sex steroids, even in the face of uncertain metabolic availability. Despite the fact that the

reproductive axis monitors, and is exquisitely controlled by energetic status, the neural circuitry and neurochemical systems interpreting and relaying energetic information to the reproductive axis are not well understood. Furthermore, it is unclear how metabolic status and estrous cyclicity interact to determine shifts in ingestive or sexual motivation, independent of any changes in consummatory behavior. The inhibitory peptide, RFamide-related peptide-3 (RFRP-3), is in a unique position to receive information regarding food availability and signal the reproductive axis. Given the important modulatory role of RFRP-3 on reproductive axis function, the present experiments characterized the state of this neuropeptidergic system following a metabolic challenge in Syrian hamsters and to determine the neuronal phenotype connecting metabolic fuel sensors to this inhibitory peptide. Furthermore, whether or not shifts in RFRP-3 activation patterns following various periods of food-restriction are correlated with alterations in motivation to engage in appetitive behaviors was explored, a finding that would uncover a novel role for RFRP-3 in mediating proceptive sexual behavior under differential conditions of energetic status.

Materials and Methods

Animals and housing

All subjects were adult (age 3-4 months), female Syrian hamsters obtained from Charles River Breeding Laboratories (Wilmington, MA). Upon arrival to the facility, hamsters were housed singly in opaque, Nalgene cages (31 × 19 × 18-cm) in a room maintained at 23 ± 1°C with a 14L:10D cycle. Hamsters were fed Harlan Rodent Chow 2016 and water was available at all times. All procedures followed the National Institutes of Health Guide for the Care of Use of Laboratory Animals, the United States Department of Agriculture, and a protocol approved by the Lehigh University Institutional Animal Care and Use Committee.

Food Deprivation Paradigm

Adult female Syrian Hamsters (n=7/group) were fed ad lib, food restricted for 24 hrs (85% normal body weight) or food deprived for 36 hours, beginning on diestrus I. In the latter group, half were considered lean (<95g) prior to the deprivation period while half were considered fat (>120g) allowing for the dissociation between baseline adiposity and acute metabolic challenge in the response of RFRP-3.

Preference Testing

Hamsters were given access to a preference apparatus to examine behaviors associated with the motivation for food and sex described previously (Schneider et al., 2007; Klingerman et al., 2010). Each apparatus consisted of a home cage for the subject female connected to a box with an adult male hamster (male box) and another box containing a food source (food box). Home cages were made from opaque, Nalgene cages (31 × 19 × 18-cm) lined with fine wood shavings with a specialized door that was kept closed when the animals were not being trained or tested. The door to the home cage led to a vertical tube (134 cm in length) that was aimed upward and connected to the male box and food box in a T-configuration (both tubes 40-50 cm in length). The food box contained a weighed amount (150 ± 5 g) of hoardable pellets made from standard laboratory chow (Harlan Rodent Chow 2016) that was broken into 2 cm pieces to encourage pouching and to enable hamsters to fit readily through the tubes. The male box was made from a clear, Plexiglas cage (27 × 20 × 15 cm) and a wire barrier that allowed hamsters to interact, but prevented mating. The male and food boxes did not contain food or water.

Training to the preference apparatus

Females were acclimated to the home cage for 1 week prior to testing, which reduced any tendencies to sleep, move bedding, or hoard food into any other compartments. During this time, females were trained to expected food in the food box and a male in the male box. At the onset of the dark period on days 1 and 2 of the estrous cycle, females were trained to the food box and allowed to keep the food they hoarded into their home cage. On days 3 and 4, females were trained to the male box. On day 3, females were allowed to enter into the male box with an unrestrained male (females cannot become pregnant on this day) for 5 min or until fighting occurred, after which the male was placed behind a wire barrier to prevent injury to either animal. On day 4, females were again allowed to directly interact with a male in the male box for 5 min and receive ectopic mounts without intromissions or ejaculations. Previous experiments have shown that female hamsters that receive similar training reliably hoard food or interact with a male during testing (Schneider et al., 2007; Klingerman et al., 2010).

Testing in the preference apparatus

Testing began at the onset of the dark period (1200 h) on day 3 and was conducted under dim red light illumination. The door to the home cage was opened and females were allowed access to the male and food boxes for a total of 90 min. During the first 15 min, vaginal marking (VM), flank marking (FM), hoarding and eating as well as location (male, food, or home cage) were recorded. After 15 min of observation, the experimenter stopped recording and the test continued for an additional 75 min (90 min total); the females continued to have access to the male and food boxes. After the 90 min test was complete, the female was returned to the home cage and the door to the home cage was closed. Weight of food in the home cage and food box was measured and

recorded to determine the amount of food hoarded and eaten when the female has to choose between allocating her time towards mate solicitation, foraging, or eating.

Females were first tested in the preference apparatus to measure baseline behaviors including food hoarding and male preference. After baseline testing, 48 hamsters were randomly placed into 1 of 6 groups that did not differ in body weight (115-175 g): food-restricted for 4 d, food-restricted for 8 d, food-restricted for 12 d, food-restricted for 12 d and re-fed for 4 d, food-restricted for 12 d and re-fed for 8 d, or ad libitum.

Blood collection and animal perfusion

After female hamsters were tested in the preference apparatus on day 3 of the estrous cycle, they were sacrificed for examination of differences in RFamide-related peptide expression and plasma luteinizing hormone levels among the groups on day 4. Four-hours before sacrifice, all hamsters were fed 1 small food pellet for 15 minutes. After 15 minutes, any food remaining was removed. All hamsters were sacrificed before the onset of the dark period (1200 hrs). Three ml of blood was removed by cardiac puncture and centrifuged at 3000 rpm and 5°C for 20 min. Plasma was collected and frozen at -20°C until analysis. Animals were perfused intercardially into the left ventricle with 200 ml of chilled KPBS (pH 7.4) and 4% paraformaldehyde in KPBS.

Brains were removed and post-fixed for 24 hr at 4°C in 4% paraformaldehyde. They were then stored at 4°C in 20% sucrose and 0.001% thimerosol until sectioning. All brains were sectioned within 30 days using a freezing microtome set at 40µm. Hypothalamic brain sections were placed into cryoprotectant (polyvinyl pyrollidone, PVP) and kept at -20°C until immunohistochemistry could be performed.

RFRP-3 immunohistochemistry

Tissue was collected and every 4th 40 µm section was double-labeled using fluorescence immunohistochemistry. FOS (1:50,000, Jackson)) or NPY (1:10,000, Santa Cruz)) was amplified with biotinylated tyramine (0.6%) for 30 min at room temperature prior to incubation in CY-2 conjugated streptavidin (1:200; Jackson) for 1 h. Following labeling for FOS or NPY, sections were labeled using an antibody directed against RFRP specifically for Syrian hamsters (1;10,000; PAC 1365), with CY-3 donkey anti-rabbit (1:200) as the secondary antibody/fluorophore. Light and confocal analysis were conducted as described in chapters 2 and 3.

Statistical analysis

Food Deprivation: Data were analyzed using a 1-way analysis of variance (ANOVA) to compare the effects of food deprivation on differences in RFRP immunoreactivity and RFRP-3/ FOS activation. Group differences were evaluated using Tukey HSD tests. Differences were considered significant if $p<0.05$.

Preference Testing: Data were analyzed using 1-way analysis of variance (ANOVA) to compare the effects of various lengths of food restriction or ad libitum feeding on food hoarding, scent marking, male preference, food intake and RFRP-3. When main effects were significant, data were analyzed using Duncan's Multiple Range test for posthoc comparisons. Simple regression tests determined correlations among the variables tested. Differences were considered statistically significant if $p< 0.05$.

Results

Energetic Challenge Increases RFRP-3/FOS Activation and Decreases RFRP-3-ir

The percentage of RFRP-3-ir neurons expressing the neuronal activation marker, FOS, as well as total RFRP-3 immunoreactivity was assessed in female Syrian hamsters fed ad lib, following food restriction to 85% normal body weight, food deprived for 48 hours with a lean body weight (<95g) or a fat body weight (>120g). The percentage of RFRP-3-ir cells expressing the neuronal activation marker, FOS was significantly lower in lean, food deprived hamsters over fed controls. Food restricted and fat, food deprived hamsters did not express RFRP-3/FOS levels that were significantly different than any other groups. The total number of RFRP-3-ir cells was significantly lower in in food deprived, fat hamsters and in food deprived, lean hamsters compared to fed controls (**Figure 4.1**; $p<0.05$ in each case).

Mild Food Restriction Increases RFRP-3/FOS Activation and Decreases RFRP-3-ir

The percentage of RFRP-3-ir neurons expressing the neuronal activation marker, FOS, as well as total RFRP-3 immunoreactivity was assessed in female Syrian hamsters fed ad lib, following food restriction to 85% body weight for 4, 8, or 12 days, and refed for four or eight days. All tissue was collected on proestrus, which was not attenuated by this food restriction paradigm. The percentage of RFRP-3-ir cells expressing the neuronal activation marker, FOS, increased significantly following 8 and 12 days of food restriction over ad lib, 4 days food restriction and following 8 days of refeeding. RFRP-3/FOS remained tonically high following 4 days of refeeding over ad lib and 8 days re fed groups. The total number of RFRP-3-ir cells significantly decreased following 8 and 12 days of food restriction compared to all other groups. (**Figure 4.2**; $p<0.05$ in each case).

Shifts in RFRP-3 Activity Mimic Changes in Appetitve Behaviors for Food or Sex in a T-Maze reference Paradigm

Food hoarding significantly increased following 8 and 12 days of food restriction and persisted following 4 days of refeeding, compared to ad lib, 4 days of food restriction and following 8 days of refeeding. Total food intake per 90 minute session did not significantly change across groups, suggesting a dissociation between appetitive and consumatory asects of feeding behavior following food restriction. The number of vaginal scent marks/90 minutes, an index of appetitive sex behavior, was significantly elevated in the ad lib and 4 day food restricted group compared to all other groups tested, even after 4 and 8 days of refeeding (**Figure 4.3**; $p<0.05$). A non-significant trend of time spent with a tethered male/90 minutes indicated a n.s., incremental decrease in time spent with the male from ad lib through 4 days of refeeding, while an upward trend of time spent with a male conspecific was observed following 8 days of refeeding (**Figure 4.3)**.

RFRP-3-ir Cells in the DMH Receive NPY-ir Neuronal Input

DMH RFRP-3-ir cells received extensive contacts (43.6±5.2%) from NPY-ir fibers. All contacts at the light level were confirmed to be in the same 0.5 μm plane by confocal microscopy. Cells not exhibiting contacts at the light microscopic level did not have contacts at the confocal level **(Figure 4.4)**.

Discussion

Despite the well characterized relationship between energetic status and reproduction, the specific central mechanisms by which a negative energy balance supresses the reproductive axis remain to be fully characterized. Furthermore, in some species, including Syrian hamsters, motivation to engage in appetitive components of ingestive and sex behavior, such as food hoarding and proceptive behaviors, are impacted by reproductive and metabolic status, without influencing ingestive behavior (Silverman and Zucker, 1976; DiBattista, 1987; Schneider et al., 1988; Buckley and Schneider, 2003). Likewise, it is unclear how reproductive and metabolic status are integrated in the CNS to tip the scale of motivation towards either ingestive or sexual behaviors. It was expected that as animals are subjected to varying periods of food deprivation, RFRP-3 expression would increase in order to inhibit the reproductive axis and shift motivation towards appetitve behaviors for food over sex.

The results indicate that neuropeptide Y (NPY), a fuel detector in the brain that increases during periods of food deprivation, projects to RFRP-3 neurons, and may bridge the gap between energy detection and reproduction. Further studies are neccesary to determine whether or not this afferent pathway originates from hindbrain or forebrain

fuel detectors, as both neural loci express NPY and project to the DMH (Chronwall, 1985; Shiotani et al., 1986). Changes in RFRP-3 activation in animals subjected to mild food restriction or chronic food deprivation was examined. The results show an increase in RFRP-3 activation following chronic food deprivation of 36 hours, but only when the animal was characterized as a lean body type prior to the deprivation period, indicating that RFRP-3 activation depends upon initial stored energy availability as well as the level of food deprivation. These studies are the first to explore the impact of metabolic status on RFRP-3 activation levels, as well as characterize projections from metabolic fuel sensors to this inhibitory peptide. Additionally, these results identified a novel potential role for RFRP-3 in determining motivated behaviors following periods of mild energetic challenge. Food restriction for 8 or 12 days increased RFRP-3 activity, which persisted following 4 days of refeeding and was correlated with an increase in food hoarding as well as a decrease in vaginal scent marking during that time period. These results suggest RFRP-3 may be involved in dictating whether or not a female hamster is motivated for food or sex in a choice paradigm, even during conditions under which consummatory aspects of behavior, such as food consumption, remain unaltered. RFRP-3 activity may be sensitive to decreases in body mass, as these hamsters lost 20g following 12 days of food deprivation and did not recover body mass following the refeeding period. As in the first experiment, where RFRP-3 activity increased when lean hamsters were food deprived for 36 hours, this inhibitory peptide may act as an 'emergency brake' on the reproductive axis if body mass decreases below a critical threshold level.

Previous work has indicated that RFRP-3 administration is capable of inducing feeding behavior in addition to inhibiting sex behaviors in rodents and chicks (Tachibana et al., 2005; Johnson et al., 2007). These studies have focused on consummatory aspects of behavior, such as ejaculatory behavior in male rats and amount of food consumed in both species studied. Furthermore, RFRP-3 neurons have been implicated in modulating neuronal firing of anorexigenic POMC neurons, providing a mechanism of feeding regulation by this peptide (Fu and van den Pol, 2010). A similar prefence paradigm, in which female Syrian hamsters are subjected to varying degrees of food restriction across the estrous cycle or following OVX + estradiol and progesterone treatment, indicate that a negative energy balance allows for observing the role that sex steroids play in shifting appetitive behaviors from food to sex. Proestrous or OVX+E&P females significantly increase time spent with a male conspecifc and decrease food hoarding behavior when compared to all other days of the estrous cycle. The estradiol-mediated shift in appetitve behavior occurred without any differences in lordosis or amount of food consumed (Klingerman et al., 2010).

These results provide evidence that metabolic and reproductive status are integrated in the CNS to alter motivational decision making. The results presented here imply RFRP-3 as the potential site of integration of these signals and may direct behavior accordingly. Given that an increase in RFRP-3 activation occurred following mutliple days of food restriction and was correlated with appetitve shifts from sex to food, this peptide may inhibit sexual motivation and thus proceptive behaviors during a negative energy balance. Given that all testing occurred during proestrous, the impact of estradiol on RFRP-3 under these conditions would provide further evidence of the connection

between sex hormones and fuel detectors within this neuronal phenotype. Furthermore, future studies utilizing the RFRP-3 receptor antagonist, RF-9 (Pineda et al., 2010), will be beneficial to determine if blocking this inhibitory peptide shifts appetitive behavior back towards sex despite a negative energy balance.

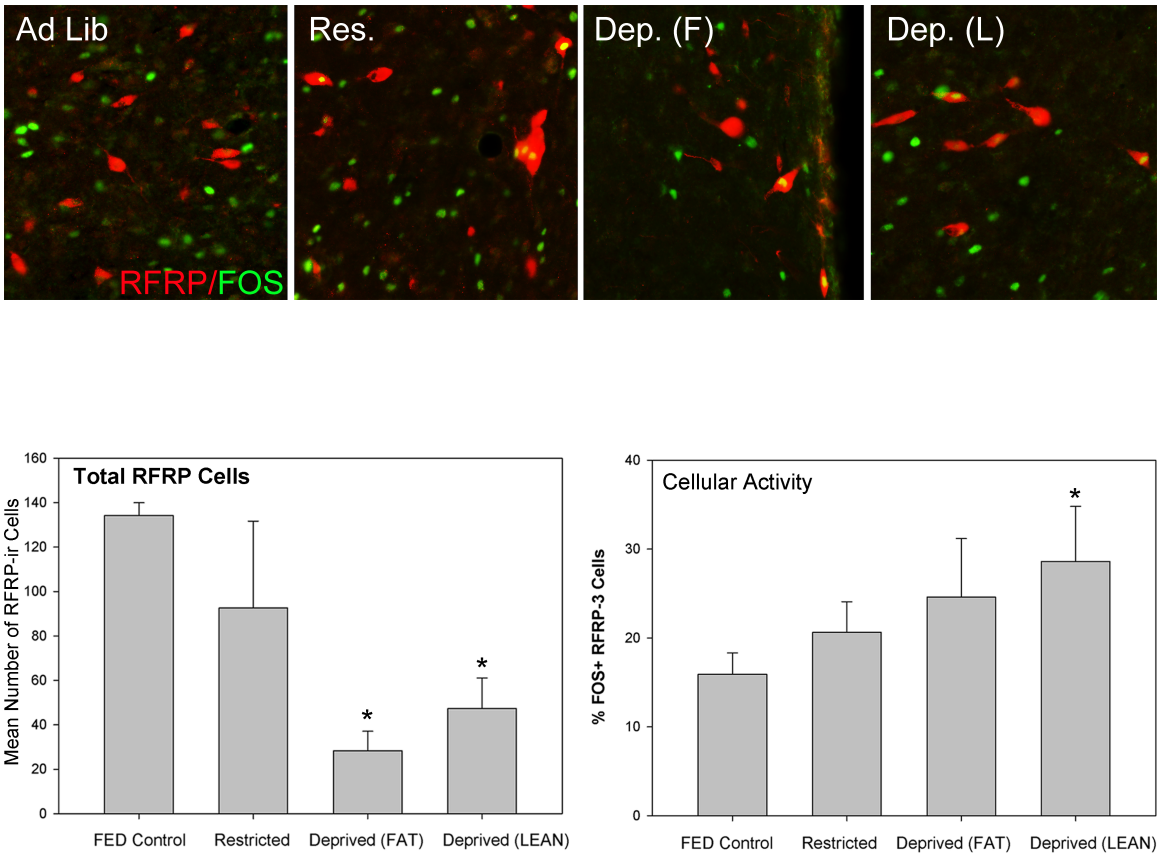

Figure 4.1

Energetic challenge increases RFRP-3 cell activation and decreases RFRP-ir cell numbers. RFRP-3 neuronal activation increases when lean Syrian hamsters (85% normal body weight) are food deprived for 36 hours. Deprivation decreases RFRP-3 cell numbers regardless of body mass. (Top) Representative photomicrographs of RFRP-3-ir neurons showing FOS activation after various energetic challenges. (Bottom) Quantification of RFRP cell numbers and RFRP/FOS across various energetic challenges. *, Significantly different than control ($p<0.05$)

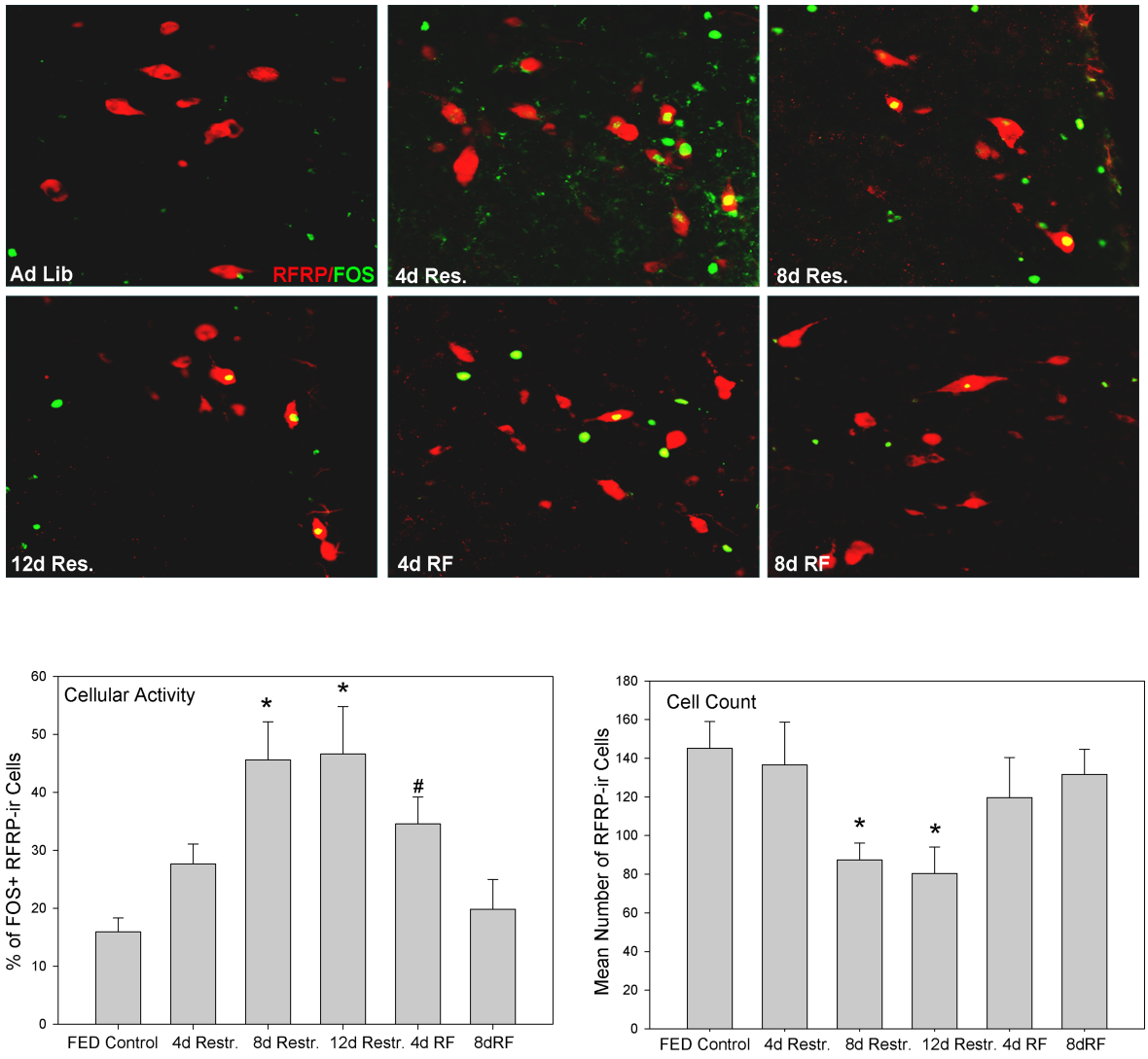

Figure 4.2

RFRP-3 activation mimics shifts in food-restriction induced appetitive behavior. RFRP-3 cell activation increases with 8 and 12 days food restriction and persists after 4 days refeeding. This pattern mimics hoarding behavior seen in Figure 4.3 (and see Results). (Top) Photomicrographs of RFRP/FOS-ir following food restriction and refeeding. (Bottom) Quantification of RFRP cell numbers and RFRP/FOS following periods of mild energetic challenge and refeeding. All testing occurred on proestrous. *, Significantly different than ad lib, 4d res. and 8d RF (p<0.05). #, Significantly different than ad lib and 8d RF (p<0.05).

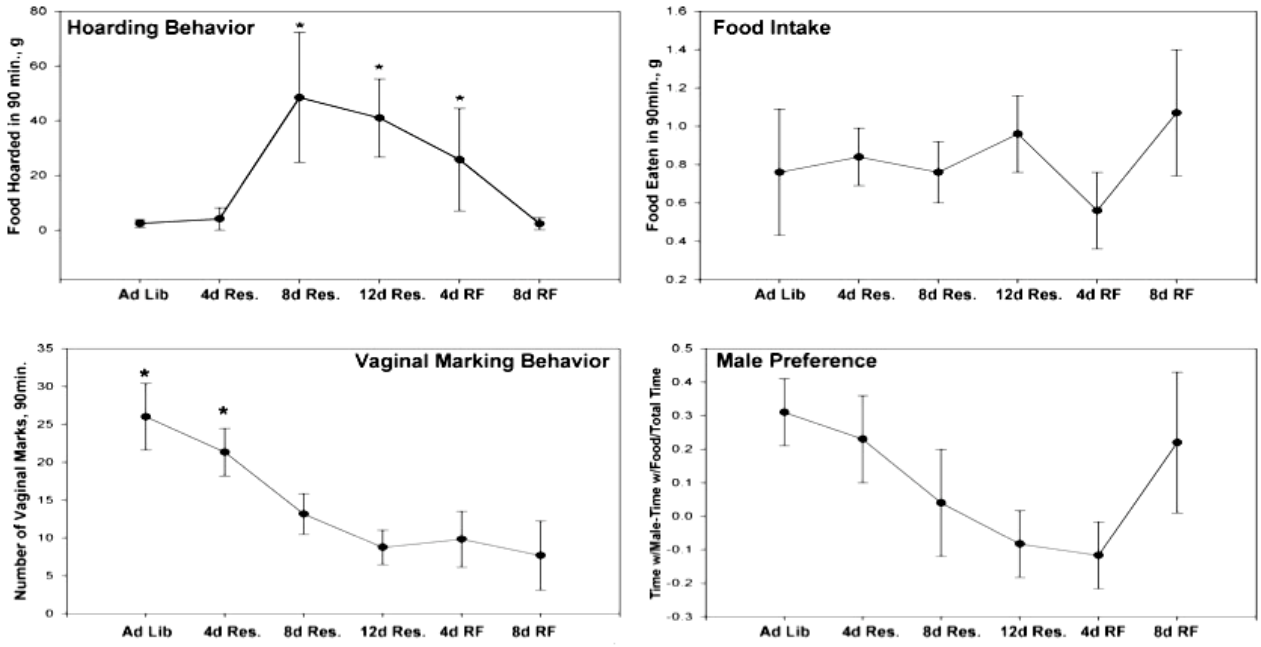

Figure 4.3

Food restriction shifts appetitive behaviors in proestrous female Syrian hamsters. Food restriction impacts appetitive behaviors without impacting consummatory behavior. Subtle changes in energy availability shift various aspects of appetitive behavior, such as vaginal marking and food hoarding without altering consummatory behaviors such as overall food intake or lordosis. These differences are correlated by shifts in RFRP-3 seen in figure 4.2 (and see Results). *, Significantly different than all other groups not denoted by an asterisk, (p<0.05).

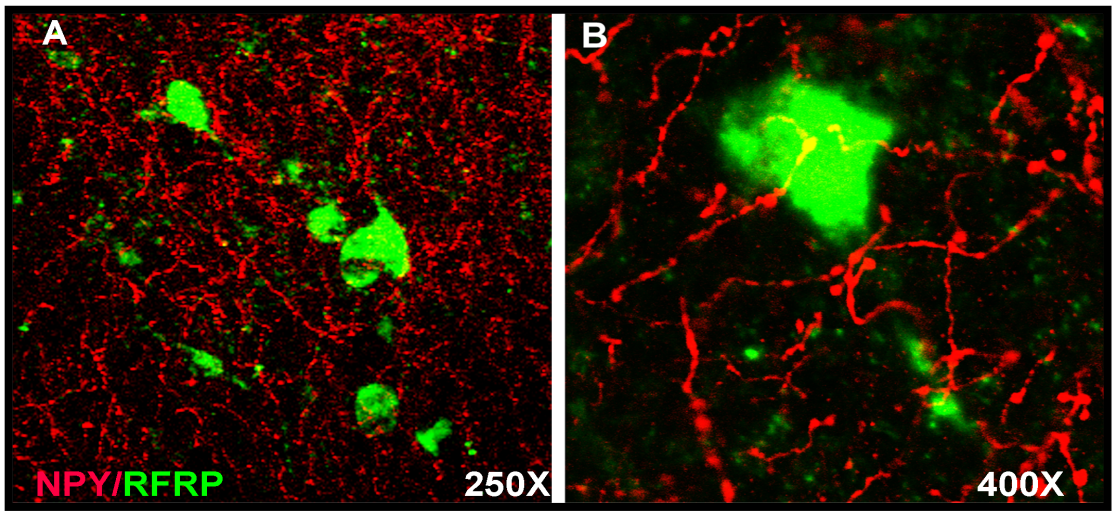

Figure 4.4

RFRP-3-ir neurons in the DMH receive projections from NPY-ir neuronal fibers. NPY fibers project to RFRP-3-ir cell bodies (43.6±5.2%) in the DMH at the confocal level. (A) A cluster of RFRP-3 neurons receives extensive NPY projections at 250x. (B) A single RFRP-ir neuron with presumptive NPY boutons at 400x.

Chapter 5

Conclusions

Successful reproduction depends upon a highly orchestrated cascade of events during optimal environmental conditions in order to appropriately time ovulation. The onset of the preovulatory GnRH surge is triggered by the master circadian clock in the SCN and the decline in circadian function with age leads to a blunted GnRH release that ultimately results in reproductive senescence. Additionally, energetic status influences this system and a negative metabolic state may interfere with the GnRH surge as well as sexual behavior. Here, I have explored the roles of two RFamide peptides, kisspeptin and RFRP-3 in the circadian control of ovulation and have examined the impact of aging and metabolic state on the expression of these peptides as they relate to the onset of the GnRH surge. These results provide insight into the mechanisms underlying the dynamic regulation of GnRH by multiple inputs.

Despite extensive evidence that the GnRH surge is triggered by the SCN, the specific pathways underlying the circadian control of ovulation remain to be fully characterized. In chapter 2, whether or not kisspeptin is part of the essential neural circuit linking the SCN to the gonadotropin-releasing hormone (GnRH) system to stimulate ovulation in Syrian hamsters was examined. The findings indicate that kisspeptin neurons exhibit an estrogen-dependent, daily pattern of cellular activity consistent with a role in the circadian control of the LH surge and that the SCN targets kisspeptin neurons via vasopressinergic (AVP), but not vasoactive intestinal polypeptide (VIP)-ergic, projections. Because AVP administration can only stimulate the LH surge during a restricted time of day, the possibility that the response to AVP is gated at the level of kisspeptin and/or GnRH neurons was examined. Kisspeptin and GnRH activation were assessed after intracerebroventricular (i.c.v.) administration of AVP during the morning (when AVP is incapable of initiating the LH surge) and the afternoon (when AVP injections stimulate the LH surge). Kisspeptin but not GnRH cellular activity was upregulated after morning injections of AVP, suggesting that time-dependent sensitivity to SCN signaling is gated within GnRH but not kisspeptin, neurons. In support of this possibility, the GnRH system exhibits pronounced daily changes in sensitivity to kisspeptin stimulation, with maximal sensitivity in the afternoon. Together, these studies reveal a previously unexplored mechanism of ovulatory control with interactions among the circadian system, kisspeptin signaling, and a gating mechanism at the level of the GnRH system.

In chapter 3, whether or not alterations in the RFamide-related peptide and kisspeptin systems contribute to reproductive senescence and the cessation of ovulatory function associated with advanced age was explored. Using young and middle-aged (some regularly cycling while other were cycling irregularly) animals, the circadian

regulation of RFRP-3 and kisspeptin activation and correlated these patterns with the presence or absence of the GnRH/LH surge in these groups was characterized. The findings revealed that young and regularly cycling middle-aged animals exhibit expected increases in kisspeptin activity and decreases in RFRP activity around the time of the LH surge, suggesting an undeterred regulation of RFamide peptides by the circadian clock. However, age-related changes in the RFRP system are associated with reproductive decline. Irregularly cycling middle-aged females exhibit an advanced decrease in RFRP/FOS activity (ZT 6) compared to regularly cycling young animals. Furthermore, aged animals show a prolonged decrease in RFRP activation (ZT 12). Somewhat surprisingly, changes in kisspeptin peptide expression are not apparent in irregularly cycling females, suggesting that pronounced alterations in the kisspeptin system are not responsible for reproductive decline.

In chapter 4, a novel role of RFRP-3 as a conduit of metabolic challenge to the reproductive axis and associated activity with metabolic-induced alterations in RFRP-3 with shifts in motivated behaviors between sex and food was examined. Given the important modulatory role of RFRP-3 on reproductive axis function, the experiments sought to characterize the state of this neuropeptidergic system following a metabolic challenge. The findings revealed that the percentage of RFRP-3 cells expressing FOS increases with food restriction and this effect was amplified following chronic food deprivation. Furthermore, the extent of RFRP-3/FOS activation depended on the initial body fat content prior to the deprivation period. These findings are consistent with the fact that the degree of hypothalamo-pituitary-gonadal (HPG) axis inhibition is directly proportional to the magnitude of food deprivation. Furthermore, the results uncovered a mechanism by which negative energy-balance detectors may inhibit the reproductive axis, as NPY fibers project to RFRP-3 neurons in the DMH. Mild energy restriction, insufficient to greatly alter body weight or impact ingestive behaviors (including amount of food consumed), decreases RFRP-3/FOS activation patterns, suggesting an influence of acute metabolic changes on this inhibitory peptide. Interestingly, these changes were correlated with shifts in appetitive behavior from sex (time spent with a male conspecific and vaginal scent marking) to food (food hoarding behavior). These results suggest a potential effect of RFRP-3 on motivated behaviors, providing the basis for a novel, extra-GnRH role of this peptide.

In summary, these results provide a complex portrayal of the myriad processes that regulate, and are regulated by, the RFamide peptides kisspeptin and RFRP-3. The circadian regulation of the GnRH surge involves a multifaceted regulation of kisspeptin signaling and offers a novel approach to understanding the nature of the preovulatory luteinizing hormone surge. Additionally, RFamides may contribute to the transition into reproductive senescence and interact with previously unexplored neuronal systems, including the regulation of motivated behavior.

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