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The Regulation of Seasonal Reproduction by RFamide Peptides

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The Regulation of Seasonal Reproduction by RFamide Peptides

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Committee in charge:

Professor Lance J. Kriegsfeld, Chair
Professor Irving Zucker
Professor George E. Bentley

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Abstract

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Animals inhabiting temperate environments have evolved mechanisms to interpret environmental cues and adjust their behavior and physiology in order to maximize survival and reproductive success. Reproduction is an energetically costly process that is inhibited in the winter in seasonal breeders to conserve energy when the chances of successful reproduction are minimal. Seasonally breeding rodents serve as an ideal model system to investigate the neural control of the reproductive axis in a controlled laboratory setting, as surgical and pharmacological methods of reproductive axis inhibition are not required. The aim of the present series of experiments was to elucidate how the brain processes information about the length of the day in order to communicate time of year to the reproductive axis to coordinate reproductive function and behavior. Specifically, the studies herein aimed to investigate the role that two novel neuropeptides, kisspeptin and gonadotropin-inhibiting hormone (GnIH or RFRP in mammals) serve in processing and communicating day length information to the reproductive axis in several species of seasonally breeding rodents. The results uncover important roles for these two neuropeptides in the interpretation of photoperiodic signals for regulating the process of gonadal regression in response to the short day lengths typical of winter in these species. Additionally, by exploiting natural genetic variability within populations of seasonal breeders, these studies suggest that the phenomenon of nonresponsiveness and phenotypic variation in reproductive photoresponse may result, at least in part, from variation in the morphological features and seasonal response of the RFRP system. As a whole, this body of work provides important insight into the complex neural mechanisms that have evolved for integrating environmental information in order to coordinate reproductive physiology with a changing external environment.

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CHAPTER 1

General Introduction

Overview and scope of dissertation

Rhythmic phenomena are ubiquitous features of the physical and biological world. Two salient environmental periodicities are the perpetual rhythms of day and night and the progression of the seasons, both of which impose a potent selective force profoundly influencing the evolution of life on Earth (Paranjpe and Sharma 2005; Sharma 2003). These daily and annual rhythms predict changing environmental conditions and define the temporal structure that signals, among other conditions, appropriate food availability/foraging times and potential for predation. The fundamental influence of these rhythms is powerfully evident in the temporal organization in behavior and physiology of most extant organisms (Rusak and Zucker 1975).

Reproduction is an energetically expensive endeavor that, while essential for evolutionary success, is not necessary for immediate survival. Physiological and behavioral trade-offs are inevitable outcomes for organisms that inhabit environments that pose frequent threats to survival. Seasonal cycles of reproduction in mammals inhabiting temperate climates are quintessential examples of environmental rhythms dictating the behavioral patterns and the physiology of organisms. Natural selection has favored the survival and reproductive fitness of those organisms that optimally time their reproductive effort so that birth of offspring coincides with times of abundant resources, thus maximizing the likelihood of survival (Bronson 1989). The fitness benefit of a successful winter reproductive event rarely outweighs the severe cost; therefore, fertility and attendant reproductive behaviors are curtailed in order to conserve energetic resources during the phases of the year when energetic challenges are maximal and survival must take precedence at the expense of non-essential physiological processes.

The common thread running through the body of work described herein is the simple but axiomatic fact that most mammals in their natural habitats face recurrent and predictable challenges that demand a shift in energetic resource allocation from reproduction to survival. Whereas the phenomenology of seasonal breeding patterns in nature is an intrinsically fascinating topic in itself, the proximate neural and hormonal mechanisms that enable the precise temporal control of reproductive function are the specific focus of my research. In explicit terms, all of the experiments described within this dissertation are aimed at answering the following question: How is information about photoperiod and other environmental variables processed by the central nervous system and communicated to the reproductive axis in order to synchronize an organism's internal physiology with rhythms of the external environment?

Seasonally breeding rodents offer a powerful tool to investigate questions of ecological significance in a controlled laboratory setting. My specific efforts have focused upon two novel neuropeptides of the highly conserved RFamide related peptide family, kisspeptin and gonadotropin inhibitory hormone (GnIH), that have been shown to exert potent stimulatory and inhibitory effects on the reproductive axis, respectively. My primary objective was to

characterize the roles that these two novel neuropeptides serve in the photoperiodic control of reproduction in seasonally breeding rodents. Through this research, a better understanding of the organization and operation of the neuroendocrine axis can be gained, as the neuroendocrine events mediating reproduction are remarkably conserved across the animal kingdom, including primates and humans (Belsham and Lovejoy 2005). A greater understanding of upstream neuropeptidergic modulators of GnRH may offer insight into the mechanisms of puberty initiation as well as the etiology and treatment of various fertility disorders and other reproductive related pathologies in human populations.

Seasonal Cycles of Reproduction (Phenomenology)

At a fundamental level, all of life on Earth depends on the solar energy radiated by the Sun that strikes the surface of the planet. Plants and animals utilize this energy to fuel the biochemical reactions that allow for survival, growth, and reproductive processes. Photosynthetic reactions enable plants to capture sunlight and directly extract energy through synthesis of food molecules (Nelson 2005). Animals, on the contrary, must forage, hunt, or otherwise navigate their environments in order to procure energy in the form of food. Herbivores obtain nutrients and extract energy to fuel biochemical reactions from the released chemical bond energy in oxidized plant material. Carnivores assimilate energy from the consumption of other animals and omnivores can acquire it from either source. Ultimately, energy from the sun cycles through the biosphere and, in the process, permits organisms to survive, maintain and modify the structure of their bodies, preserve internal order, and eventually reproduce and pass on the genetic instructions for building the next generation of organisms (Holliday 2001).

Life, of the form that has evolved on Earth, could not exist without the energy supplied by the Sun. Likewise, the rotation of the Earth and its particular astronomical relationship to the Sun produces the daily and seasonal rhythms that account for the pronounced climatic and habitat variation that exists across the planet (Paul et al. 2008). These factors have shaped the selective environment that has determined the behavioral repertoires and physiological systems that permit animals to survive and reproduce under the specific conditions to which they are exposed. The seasonal cycles of reproduction displayed by many non-tropical dwelling mammals are particularly salient examples of how animal behavior must be interpreted within the framework of the environment in which it evolved.

The majority of animals living in temperate or boreal climates experience a seasonal fluctuation between states of reproductive competence and reproductive quiescence (Bronson, 1989; Paul et al., 2008; Kriegsfeld and Bittman, 2009). Any environment that undergoes cycles of climate change, vegetative growth, and resource availability is more conducive for supporting life during periods of abundance. As a result, animals inhabiting these climates have evolved systems for temporal control in order to restrict energetically expensive processes to those phases of the annual cycle when resources are plentiful. The pressure to survive has resulted in physiological and metabolic systems that dynamically allocate energetic resources between essential processes that sustain life, such as cellular maintenance and immune function, and those non-essential processes that permit growth and reproduction (Bronson 1989; Demas 2004; Demas and Nelson 1998).

Many of the seasonal adaptations that are implemented by seasonal breeders are quite pronounced. Significant modifications of physiological state do not occur instantaneously; therefore, in order to capitalize on their utility, energy-conserving adaptations are initiated before environmental conditions have maximally deteriorated. The selective advantage gained by those individuals that anticipate and prepare in advance of predictable environmental challenges has favored the evolution of timing mechanisms, endogenous biological clocks that endow their possessors with the ability to assess time of year and prepare in advance for predictable climatic changes (Nelson et al. 1990; Prendergast 2002). Adaptations for energy conservation, such as gonadal involution, requiring weeks to fully implement, can be initiated in advance of inclement conditions so that the organisms utilizing these timing mechanisms are physiologically prepared for the challenge of winter.

Many aspects of the environment fluctuate along with the seasons. Food quality and abundance, temperature, humidity, and rainfall patterns are all potential environmental signals that animals could use to time their reproductive effort. These specific factors are commonly referred to as "ultimate" causes of seasonality as their availability has direct adaptive value (Baker 1938; Nelson et al. 1990). In contrast to these ultimate factors are the "proximate" factors that are highly correlated with the cycles of ultimate factors and can be used to forecast the arrival of favorable conditions. The most highly reliable proximate cue is day length, or photoperiod, the duration of the light portion of the daily cycle (Goldman 2001). Photoperiod is generally considered the most precise and "noise-free" cue for determining the phase of the annual cycle as the length of days and nights are relatively invariant features of the environment that have changed minimally during evolutionary history (Sharma 2003). Therefore, the annual cycle of day length has likely been an important environmental signal directing the evolution of diverse life forms throughout evolutionary history. It is speculated that timekeeping capabilities arose early in evolutionary history because of their adaptive value as a mechanism to efficiently anticipate periodic events in the external environment, a trait valuable even for single celled organisms that need to restrict specific cellular events to times when ultraviolent light exposure would be minimal (Paranjpe and Sharma 2005; Pittendrigh 1993). Most significantly for the present work, photoperiod is the cue that is most widely employed by organisms that limit reproductive activity to certain phases of the annual cycle.

While seasonal reproduction tends to be the rule among diverse mammalian species, the specific proximate and ultimate factors mediating this process varies among mammals. One principal distinction among types of seasonal breeders concerns whether they engage in reproductive behaviors during fall or spring months. The former are termed "short-day" breeders as their reproductive systems are stimulated by decreasing day lengths of autumn and the latter are referred to as "long-day" breeders because their reproductive system is inhibited by declining day lengths and is active during the relatively long days of spring and summer months (Paul et al. 2008; Prendergast 2002). Ecological and life history factors of the specific species employing the strategy provide explanations for why reproductive activity occurs during a specific phase of the year. Larger mammals have longer gestation periods, a fact that demands that reproductive activity and conception coincide with unfavorable conditions to ensure that offspring births and the energetically demanding process of lactation overlap with peak resource availability (Bronson, 1989). Sheep have been an invaluable model system for study of "short day" seasonal breeding mechanisms in the laboratory setting (Bittman 1984). Their large size and docile nature

permit frequent blood sampling and, therefore, a more precise understanding of the effects of day length on secretion of HPG axis hormones (Bittman and Karsch 1984; Bittman et al. 1985). Most rodents are long day breeders, as these animals have short gestation periods that permit breeding and birth within the same season.

Syrian (*Mesocricetus auratus*) and Siberian hamsters (*Phodopus sungorus*) are the most commonly employed species for studying seasonal reproduction in a laboratory setting. As theses two hamster species figure prominently in several studies herein, their annual cycle of reproductive function is presented here in greater detail for instructive purposes. Both hamsters are highly sensitive to photoperiod and in the field, Siberian hamsters breed, reproduce, and raise their offspring during the increasing day lengths following the vernal equinox and the long days of summer. Unfortunately, the reproductive behavior of Syrian hamsters in their natural habitat has been little studied, but their highly sensitive response to photoperiod in the lab would suggest a similar annual pattern of reproduction to the Siberian hamster, although field studies are necessary to confirm this conjecture. When day lengths begin growing shorter at the conclusion of summer and beginning of autumn, a cascade of neural and endocrine events is initiated that culminates in the reproductive system entering a temporary state of dormancy. Reproductive organs regress and the hormones mediating reproductive function decline markedly from their baseline levels (Berndtson and Desjardins 1974; Gaston and Menaker 1967). At this stage of the annual cycle hamsters are said to be in a "photoinhibited" state. This condition of reproductive quiescence persists for approximately 20 weeks, after which the reproductive system "spontaneously" reactivates, initiating another cascade of events, which permits the animal to achieve full reproductive condition in time for the start of the breeding season (Reiter 1972, 1974). During this stage of reactivation, the hamster is termed "photorefractory" as day lengths remain short yet are no longer inhibitory to the reproductive axis. Classic studies that utilized static photoperiods and abrupt transitions between long and short day conditions established the dogma that hamsters in the refractory state must experience 8-12 weeks of long day conditions (or short duration melatonin signals) in order to regain sensitivity to the inhibitory effects of short days (or long duration melatonin signals) (Paul et al. 2008; Prendergast 2002). However, more recent studies exploring the phenomenon of refractoriness, some of which aimed at ecological relevance by employing simulated natural photoperiods (SNP), have determined that as few as six weeks of long day melatonin exposure are sufficient to break refractoriness in Siberian hamsters (Butler et al. 2007; Butler 2010; Kauffman et al. 2003). In the laboratory, a photosensitive hamster will not undergo another cycle of regression if it is held in constant long day lengths (Hoffman and Reiter 1965; Vitale et al. 1985). Essentially, declining day lengths (or their endocrine correlates, as discussed below) signal to the animal that winter is impending and energy conserving adaptations must be initiated to adopt the appropriate physiological state for successfully coping with winter. An interval timer mechanism is initiated that governs the time since the initiation of short days and determines when the hamster will become refractory to short days and begin preparation for the start of the breeding season (Paul et al., 2008; Prendergast et al., 2002).

Melatonin and Circadian Rhythms

As described in the previous section, seasonal breeders, such as Syrian and Siberian hamsters, initiate seasonal adaptations, such as modifications in reproductive activity, in advance of declining climatic conditions through the use of a seasonal timekeeping mechanism. This predictive capacity depends on the use of the proximate environmental cue of photoperiod to precisely assess the time of year. These timekeeping mechanisms effectively function as an internal calendar, whereby day length is encoded into a hormonal signal that internalizes the temporal state of the external world to be communicated to the brain and body. Downstream effectors whose output must be modified in accordance with the changing seasons process this internal message. Reproduction is the focus of my research; however, many other systems fluctuate on a seasonal basis, such as metabolic, immune, and behavioral systems.

The understanding of seasonal timekeeping mechanisms requires some familiarity with circadian rhythms as these two phenomena are inextricably connected. In mammals, discrimination of day length depends on the circadian clock that resides in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is a bilateral nucleus at the base of the hypothalamus, situated directly above the optic chiasm. This brain region serves as the master circadian pacemaker in all mammals and is responsible for regulating a myriad of behavioral and physiological rhythms that fluctuate with a 24-hour period (Moore and Eichler 1972; Stephan and Zucker 1972). Activity of the SCN synchronizes to the external light cycle through inputs from specialized retinal ganglion cells containing the photopigment melanopsin (Berson et al. 2002; Hattar et al. 2002; Provencio et al. 1998; Provencio et al. 2000). The entire organism can be conceived as a multi-oscillatory network of rhythmic cells, organs, and systems that are synchronized by the master pacemaker rhythm of the SCN (Mendoza and Challet 2009). Circadian rhythms throughout the brain and body are maintained by outputs from the circadian clock in the form of direct neural projections, communication with the autonomic nervous system, or rhythmically secreted humoral factors (Kalsbeek et al. 2006; Meyer-Bernstein et al. 1999; Silver et al. 1996). The SCN output that has been most well characterized and is the most relevant to the discussion of seasonal rhythms is the pathway between the SCN and the pineal gland, which governs the nightly rhythm of melatonin secretion (Card 2000; Larsen et al. 1998).

Melatonin is an indoleamine hormone that is produced and secreted from the pineal gland exclusively during the night and in direct proportion to the length of the night (Chattoraj et al. 2009; Foulkes et al. 1997). Based on this pattern of secretion, melatonin serves as the internal representation of day length in all vertebrates. A circuitous neural pathway leads from the SCN to the pineal and controls the rhythmic secretion of melatonin. Noradrenergic efferents of the superior cervical ganglion (SCG) synapse upon pinealocytes and regulate the activity of rate limiting enzymes for melatonin synthesis, which increases markedly at night (Ganguly et al. 2002; Klein 2004). As a result of this circadian control, the melatonin signal faithfully encodes the duration of the night. This photoperiodic message can subsequently act on any cell or tissue that expresses the cognate melatonin receptor, of which the most important is the MT1 receptor (Prendergast 2010). Through actions on this receptor subtype, physiological systems modify their output by responding to melatonin signals of a duration that predicts the impending seasonal transition.

Neural Control of Reproduction

The hypothalamus is a vital brain region that controls many essential functions necessary for survival and reproduction (Pfaff 2004). Located at the base of the brain, the hypothalamus is a critical integration center for information from many other brain regions including prominent connections with many limbic structures. The division between the nervous and endocrine systems is blurred significantly at the junction between the hypothalamus and the pituitary gland, as hormones and other neurochemicals produced in various hypothalamic nuclei act on the pituitary to produce widespread physiological effects throughout the body. Within the purview of hypothalamic regulation are fundamental homeostatic and physiological processes, such as: (1) osmoregulation, (2) control of body temperature, (3) energy balance and metabolism, (4) emergency and stress responses, and most importantly for the purpose the present series of studies, (5) reproductive behavior and physiology (Pfaff 2004).

Neuroendocrine control systems are commonly organized into axes that consist of a hierarchical interaction between the hypothalamus, pituitary gland, and target organ. At the hypothalamic level, neurosecretory cells synthesize hormones or other chemical messengers that are released from terminals in the median eminence into a dedicated portal system to act on specific cells of the anterior pituitary. These pituitary cells are stimulated to synthesize and release other hormones that travel through the general circulation to act on a specific target organ or system (Herbison 1998; Maffucci and Gore 2009). Hormones from each of the tiers in the hierarchy exert negative feedback effects to regulate the activity of the overall system around an optimal set point determined by the internal needs of the organism and the state of the external environment. The hypothalamic-pituitary-gonadal (HPG) axis is the specific neuroendocrine control system responsible for maintaining all aspects of reproductive function. Ultimately, activation of the HPG axis is what is modulated by seasonal cues to ensure that reproductive behaviors coincide with conditions optimal for offspring and parent survival.

The master molecule regulating reproductive function across species is the peptide, gonadotropin-releasing hormone (GnRH). Peptides belonging to the GnRH family are essential regulators of reproduction in all vertebrates (Clarke and Pompolo 2005; Wray 2002). In mammals, GnRH neurons are diffusely distributed within the preoptic area, anterior hypothalamus and mediobasal hypothalamus and constitute the apex of the HPG axis (Silverman 1994). GnRH neurons are active in an episodic manner and communicate with the pituitary as discrete pulses of hormone into the pituitary portal circulation. The frequency of pulsatile release is the critical parameter determining pituitary gonadotropin secretion and, ultimately, the reproductive state of the organism (Karsch 1987; Terasawa 2001). GnRH acts via cell surface receptors on a specific class of cells, the gonadotropes, of the anterior pituitary. The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are synthesized and released in response to the GnRH stimulus. The gonadotropins travel through the bloodstream to target cells in the gonads where the dual functions of gametogenesis and steroidogenesis are regulated.

The sex steroid hormones, androgens in males or estrogens in females, are produced in the gonads and have diverse effects on behavior and physiology, in addition to mediating the negative feedback control of the HPG axis. An important unanswered question in the field of reproductive endocrinology is the location and mechanism by which sex steroids produce their

negative feedback effects. Classic steroid hormone actions are mediated by intracellular receptors that serve as ligand-activated transcription factors (Flanagan-Cato and Fluharty 1997). Therefore, steroids exert their effects by altering cellular function at the level of gene transcription and translation; however, some behavioral evidence supports a more rapid mechanism of steroid action, likely mediated by membrane initiated intracellular signaling pathways (Falkenstein et al. 2000; Terasawa et al. 2009). Sex steroid receptor distribution has been mapped throughout the brains of a variety of mammals and while they are observed in the proximity of GnRH cell bodies, most evidence suggests that steroid receptors, particularly the ones implicated in negative feedback control (e.g. ERα), are not colocalized in GnRH producing neurons (Herbison and Theodosis 1992; Huang and Harlan 1993).

As noted above, the temporal dimension of hormone secretion is a critical parameter for determining the downstream physiological effect of hormone action. This fact is particularly true with respect to reproductive axis control. The hypothalamic network of GnRH neurons responsible for pulsatile stimulation of the pituitary is termed the "GnRH pulse generator." The frequency of GnRH pulses determines the specific action on pituitary gonadotropin release (Krsmanovic et al. 2009; Marshall et al. 1993; Marshall and Griffin 1993; Terasawa 2001). Many of the properties and mechanisms of synchronous action of this scattered network of cells remain unspecified, but evidence suggests that the rhythmic activity is endogenously generated but highly susceptible to outside modulatory inputs (Herbison 1998, 2006; Terasawa 2001). Moreover, it is widely recognized that reproductive function is very susceptible to a myriad of internal and external environmental factors (i.e. stress, nutrition, social cues, photoperiod). However, the mechanisms by which these signals modulate reproductive axis activity at the neural level have not been fully elucidated. The present work focused on examining the neural and endocrine mechanisms by which the brain processes and relays reproductively relevant environmental information to the GnRH neuronal network.

In pursuit of this goal, I investigated the role of two recently discovered neuropeptides that have been shown to exert significant positive and negative regulatory control of reproductive function. These peptides, kisspeptin and gonadotropin inhibitory hormone (GnIH), belong to a family of structurally related RFamide peptides, that have been recognized for the role they play in neuroendocrine regulation (Kriegsfeld 2006). The working hypothesis for the present series of studies is that kisspeptin and GnIH act in a coordinated fashion to integrate relevant environmental information and precisely control reproductive behavior and physiology (Kriegsfeld 2006). The model employed to test this hypothesis is two species of seasonally breeding hamsters, the Syrian (*Mesocricetus auratus*) and Siberian (*Phodopus sungorus*).

Neural Control of Seasonal Breeding

The profound shifts in reproductive activity that accompany the changing seasons are critically dependent on changes in activity of the GnRH neural network. As described previously, melatonin from the pineal is indispensable for the initiation of seasonal adaptations. Destruction of the pineal produces an animal that is effectively "blind" to day length and unable to make the necessary physiological adjustments, such as changes in reproductive state or body fat stores, that are appropriate for a specific phase of the annual cycle (Goldman 2001; Hoffman

and Reiter 1965; Vitale et al. 1985). Melatonin faithfully encodes day length via its exclusive secretion during the dark phase. Therefore, the interaction of the seasonal timekeeper and the reproductive axis must occur either directly or indirectly through actions on target tissues expressing melatonin receptors.

The neural sites of action where the melatonin signal may be decoded have been localized and characterized in mammals using the radiolabeled melatonin analog, 2-[^{125}I]iodomelatonin (IMEL) (Weaver et al., 1989; Bartness et al., 1993). One notable discovery from this effort is the significant species differences in high affinity MEL binding sites. The one structure to bind melatonin robustly across all mammal species studied is the pars tuberalis of the pituitary. Research has shown this site to be critical for the photoperiodic control of prolactin secretion (Morgan and Williams 1996). In Siberian hamsters, the SCN is a key target for the interpretation of the melatonin signal, as animals with SCN lesions are unable to respond to a long day melatonin signal with inhibition of the reproductive system (Bartness et al, 1991). While the SCN also binds MEL in Syrian hamsters, the critical locus for decoding the melatonin message has been shown to be in, or involves a circuit that includes, the dorsomedial hypothalamus (Maywood et al, 1996; Lewis et al., 2002). Syrian hamsters with lesions of the DMH do not respond to long infusions of melatonin with gonadal collapse. Interestingly, the prolactin response to the short day signal is unaffected by DMH lesions, supporting the notion that multiple parallel pathways that are trait specific may differentially process photoperiodic information (Freeman and Zucker 2001; Teubner et al. 2008).

Two principal mechanisms have been described to account for the marked changes in reproductive activity that occurs in response to photoperiod in seasonal breeders: steroid-dependent and steroid independent. As already noted, the negative feedback effects of steroids are a mechanism of critical importance in the regulation of the reproductive axis. During the non-breeding season (winter in long-day breeders, summer in short-day breeders), gonadal steroids become markedly more effective at inhibiting gonadotropin secretion. The potency of the negative feedback effects of sex steroids is magnified in long day breeding rodents when day lengths start declining. This phenomenon has been demonstrated through castration and replacement of a constant level of sex steroid to animals exposed to stimulatory or inhibitory day lengths (Ellis and Turek 1980a; Tamarkin et al. 1976; Turek et al. 1975). During inhibitory day lengths, minimal concentrations of gonadal steroids are able to suppress gonadotropin secretion from the pituitary. The same concentration is ineffective at restraining gonadotropin secretion during stimulatory photoperiods. This effect has been demonstrated with melatonin injections to hamster in long day photoperiods, where artificially increasing the duration of the melatonin signal increases the potency of the negative feedback effects of sex steroids (Sisk and Turek, 1982). It has been suggested that the "steroid-dependent" effects of seasonal changes in gonadotropin secretion are the most physiologically relevant mechanism by which reproductive capacity is controlled (Kriegsfeld & Bittman, 2009).

The second mechanism of photoperiodic control of seasonal breeding does not depend on the feedback effects of gonadal steroids. These steroid-independent effects are observed when castrated animals are not provided with sex hormones. The typical response to castration is a vast increase in gonadotropin release due to the loss of the feedback agent. This castration response has been shown to vary between animals held in long and short photoperiods, demonstrating that changes in photoperiod can have a direct effect on the reproductive axis that

does not rely on changes in gonadal steroid sensitivity (Ellis and Turek 1980b). These steroid-independent effects likely rely on changes in "neural drive" to the GnRH pulse generating mechanism. Seasonal variation in the frequency of GnRH pulse release into the pituitary portal vasculature has been observed in sheep, with stimulatory photoperiods increasing the frequency of LH pulses (Bittman et al. 1985; Goodman et al. 1982). The data suggest that photoperiod acts in a complex manner to adjust reproductive activity in seasonal breeders, both by neural circuits that process the melatonin message directly and by altering the normal homeostatic feedback control of the reproductive axis, namely the negative feedback action of sex steroids.

Photoperiodic Nonresponsiveness

In both laboratory and field settings, a small proportion of every population of seasonal breeders opts to forgo the energy conservation strategy and maintains full activity of the reproductive axis (Prendergast and Freeman, 1999 Prendergast et al., 2001). This subset of the population has been given the label "reproductive nonresponders" because the day length cues that signal to most of the population that winter is approaching are either ignored or not processed by these individuals. The underlying neural mechanisms of the nonresponsive phenomenon are incompletely understood, however, this subset of the population provides a useful tool for studying the effects of photoperiod on the neural systems controlling reproduction, independent of actual changes in the activity of the reproductive axis. These non-responding individuals have been an integral part of several studies presented herein.

Genetic and environmental factors have been implicated in the underlying control of non-responsiveness in various rodent species. The ability to respond to photoperiod with reproductive inhibition has proven to be a selectable trait in controlled directional selection studies. In only a few generations it is possible to produce lines of white footed mice or Siberian hamsters where the majority of the individuals exclusively respond or do not respond to photoperiod (Goldman et al. 2000; Heideman and Bronson 1991; Lynch et al. 1989). These studies strongly suggest a genetic basis for nonresponsiveness, which may be present as an evolutionarily stable strategy in nature whereby the nonresponsive individuals achieve greater fitness in relatively mild winters (Prendergast et al. 2001). It is not clear what underlying genetic mechanism is being selected in these studies but evidence from white-footed mice suggest that the number of GnRH cells in the brain is much greater in the line that fails to inhibit reproduction in the winter, even when animals are housed in long day lengths (Avigdor et al. 2005).

Non-responsiveness may also be the result of a deficit in the information processing pathway transmitting day length information from the eye to the SCN, between the SCN and the pineal, or at the level of target tissue response to the melatonin message. Pre-pineal and post-pineal deficits have been reported in different species, suggesting that there are multiple pathways to achieving non-responsiveness. In deer mice (*Peromyscus maniculatus*), a post-pineal mechanism has been implicated as a long-duration melatonin signal is secreted but fails to initiate the appropriate seasonal changes in reproductive activity (Blank and Freeman 1991). In contrast, the mechanism of Siberian hamster non-responsiveness involves a failure of the photoperiod signal transduction mechanism at the level of the SCN (Margraf et al. 1991). In

short days, the melatonin signal that is generated resembles that of a long-day animal, presumably accounting for the failure of these individuals to initiate a short day response (Puchalski and Lynch, 1988). Components of the reproductive axis downstream of the circadian clock appear to be intact, as nonresponders provided with an exogenous administration of long duration melatonin are capable of responding with reproductive regression (Puchalski and Lynch, 1988).

RFamide peptides and the neuroendocrine control of reproduction

More recent developments in reproductive biology have led to the identification of two neuropeptides, kisspeptin (also known as metastin) and gonadotropin inhibitory hormone (GnIH; also known as RFRP in mammals) that act as key regulators of the reproductive axis. Kisspeptin and GnIH belong to an evolutionarily diverse and highly conserved family of peptides whose existence and functional importance have been characterized in an impressive array of vertebrate and invertebrate species (Walker et al. 2010). The RFamide family of peptides is structurally defined by the arginine-phenylalanine-amide motif (aka RFamide) of the C-terminus, which is conserved in all members of this diverse family and confers biological activity of individual peptides. All of the signaling molecules comprising the RFamide family share this portion of their amino acid sequence. FMRFamide was the first member of this family to be characterized when Price and Greenberg isolated and sequenced this tetrapeptide from the ganglia of the clam *Macrocallista nimbosa* and reported that it exerted cardioexcitatory effects (Price and Greenberg, 1977). LPLRFamide was the earliest member of the family indentified in vertebrates when it was sequenced from the chicken brain (Dockray et al., 1983). It is now believed that the mammalian genome includes at least five genes encoding members of the RFamide family of peptides. An emerging theme in the study of RFamide peptides, suggested by their central and peripheral expression patterns and functional analysis, is the prominent role that these molecules play in the regulation of various neuroendocrine and physiological processes. For example, these peptides have been implicated in analgesia and pain modulation, feeding behavior, cardiovascular function, water balance, and most importantly for the present research, the neuroendocrine control of reproduction (Kriegsfeld 2006).

The biggest advance and significant step forward in linking RFamide peptides with the control of reproduction occurred when Tsutsui and colleagues isolated a novel dodecapeptide of the RFamide family from quail brain that was demonstrated to potently and dose-dependently inhibit gonadotropin secretion from cultured pituitaries (Tsutsui et al. 2000). This peptide was given the name gonadotropin inhibitory hormone (GnIH) based on this biological effect and offered evidence of a long suspected inhibitory complement to gonadotropin releasing hormone. Initially, GnIH research was primarily restricted to avian species. A succession of studies followed the initial characterization of GnIH that probed the mechanism of action this peptide, and solidified its role as a prominent inhibitory regulator of the reproductive axis in birds. Immunohistochemical and *in situ* hybridization determined that GnIH cell bodies were localized to the paraventricular nucleus of the hypothalamus (PVN) with widespread fiber projections throughout the brain including to GnRH cell bodies and the median eminence (Bentley et al. 2003; Ukena et al. 2003). A behavioral role was supported when central GnIH administration to

female white-crowned sparrows reduced copulation solicitation displays, an indicator of sexual readiness (Bentley et al. 2006).

Kisspeptins, the translated protein products of the *Kiss1* gene, are members of the RFamide family of peptides, to which GnIH also belongs. Initially, the peptides that are now known as kisspeptins in the reproductive endocrinology literature were termed metastin for their tumor suppressive actions (Lee et al. 1996; Lee and Welch 1997). The integral reproductive role for these peptides was first recognized in 2003 when individuals with hypogonadotropic hypogonadism (HH) were shown to have a mutation in the G protein coupled receptor 54 (GPR54), which is the cognate receptor necessary for kisspeptin signaling (de Roux et al., 2003; Seminara et al., 2003). These findings generated significant interest among reproductive biologists directed towards elucidating the specific mechanistic role of kisspeptins in reproductive axis control. Hundreds of published research reports and reviews in a period of less than eight years attest to the considerable interest kisspeptin has aroused amongst researchers focused on all facets of reproductive biology.

The initial studies on hypogonadotropic hypogonadism suggested that kisspeptin may act upstream of the GnRH neural network controlling fertility, as HH is characterized by a lack of ability on the part of the hypothalamus to drive sufficient pituitary gonadotropin secretion to initiate puberty and maintain an active reproductive system. Following these initial studies, it was demonstrated that LH and FSH levels increased markedly and dose dependently following kisspeptin administration and this effect was consistent across multiple species (Gottsch et al., 2004; Navarro et al., 2004; Navarro et al., 2005; Shahab et al., 2005; Thompson et al., 2004). The level of the reproductive axis at which kisspeptin exerts its effect appears to be the GnRH system as suggested by the increase in FOS protein expression in GnRH cell bodies following administration of kisspeptin (Irwig et al., 2004; Shahab et al., 2005). FOS is the protein product of the immediate early gene *c-Fos*, which serves as a convenient marker of cellular activity in response to any external signal impinging on a particular cell type of interest. The antagonism of kisspeptin's effect on gonadotropin stimulation by administration of the GnRH receptor antagonist acyline, provides further support for a direct modulatory role of kisspeptin upon GnRH, rather than a hypophysiotropic effect (Gottsch et al., 2004; Irwig et al., 2004; Shahab et al., 2005).

Dissertation Plan

In the following chapters I explore the role of these two novel neuropeptides, GnIH and kisspeptin, in the control of seasonal reproduction in Syrian hamsters, Siberian hamsters, and white-footed mice. My goal was to understand how brain function has been shaped throughout evolution by the selective forces present in the environments in which they evolved. To that end, I primarily made use of seasonally breeding rodents as a mechanism to investigate how the neuroendocrine axis controlling reproduction has evolved to process ecologically relevant signals that impact reproductive behavior and physiology.

The first stage involved characterizing the novel inhibitory regulator of the reproductive axis, GnIH, in mammals, as the role of this neuropeptide had only been firmly established in avian species. Chapter 2 describes a series of experiments directed towards this goal. As

mentioned previously, RFamide peptides are a highly conserved family of peptides that are present in mammals. However, it was unknown whether GnIH was present in mammalian brain and served a similar functional role to that seen in avian species.

After establishing that GnIH was present in the mammalian brain and also functions as an inhibitory modulator of reproductive function, Chapter 3 describes a series of experiments aimed at determining a role for the mammalian homolog of GnIH, RFRP, in seasonal reproduction in Syrian hamsters. RFRP cell bodies are localized to a key region of the hypothalamus for mediating the response to photoperiodic cues, the DMH, in this species. My goal was to determine if RFRP was the specific cell phenotype providing the inhibitory drive to the reproductive axis under winter photoperiods in this species. This study also introduced the nonresponder as a tool for probing the way that individuals from the same population differentially interpret the same environmental signal.

Chapter 4 further explores the role of RFRP in seasonal reproduction and the role of this neuropeptide in photoperiodic nonresponsiveness. However, this experiment used the white-footed mouse (*Peromyscus leucopus*). Again, my goal to capitalize on the advantage presented by a species that shows significant variability in its reproductive photoresponse in the wild to understand how individuals of a population perceive day length cues in a diametrically opposed manner, by exploring the pattern of RFamide peptide expression following manipulation of photoperiod.

Chapters 5 and 6 focus on kisspeptin and the role it plays in the seasonal response to photoperiod in Siberian hamsters. It was not necessary to characterize a role for kisspeptin in mammals as the reproductive role for kisspeptin was identified in mammals and much interest and research had already been conducted on this peptide. However, when these studies were carried out, the role of kisspeptin in seasonal changes in reproductive function had not been explored.

CHAPTER 2

Identification and Characterization of a Gonadotropin-Inhibitory System in the Brains of Mammals

Introduction

The final common pathway in the neural regulation of reproduction is the gonadotropin-releasing hormone (GnRH) neuronal system. Neurons that synthesize and secrete GnRH occupy a midventral continuum from the diagonal band of Broca to the mediobasal hypothalamus (Silverman 1994). GnRH neurons regulating gonadotropin secretion project to the median eminence to control synthesis and secretion of the pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) (Jennes and Stumpf 1980; Sawyer 1975; Silverman et al. 1990).

In both males and females, gonadal steroids act through negative feedback to maintain the reproductive axis within the favorable operating limits necessary for fertility and successful mating. Negative feedback control of the GnRH system could be accomplished by sex hormones acting on either cognate receptors expressed in GnRH neurons or on gonadal-steroid-responsive systems upstream of GnRH. Although early evidence before this millennium suggested only the latter (Gore and Roberts 1997; Herbison 1998; Herbison et al. 1996; Herbison and Theodosis 1992; Huang and Harlan 1993; Lehman and Karsch 1993; Shivers et al. 1983), more recent evidence suggests that both of these mechanisms act in concert to regulate precisely the reproductive axis in females (Abraham et al. 2003; Butler et al. 1999; Hrabovszky et al. 2000; Petersen et al. 2003; Skynner et al. 1999), whereas the mechanisms regulating negative feedback in males remain obscure (Herbison et al. 1996; Huang and Harlan 1993).

Given the importance of reproductive axis regulation in normal fertility, it is essential to gain an understanding of the precise neural mechanisms responsible for its regulation. An RFamide (Arg-Phe-NH_2 in the C terminus) peptide that inhibits gonadotropin secretion *in vitro* and *in vivo*, and named gonadotropin-inhibitory hormone (GnIH), has recently been identified in avian brain (Bentley et al. 2003; Osugi et al. 2004; Satake et al. 2001; Tsutsui et al. 2000; Ukena et al. 2003). In birds, regulation of reproduction is controlled, in part, by specialized adaptations such as deep hypothalamic photoreceptors that contact and potentially regulate GnRH (Saldanha et al. 2001; Silver et al. 1988). To date, a hypothalamic neuropeptide negatively regulating gonadotropins at the level of the pituitary has not been identified in mammals. The goal of the present studies was to explore the possibility that GnIH occurs in mammals and to characterize the distribution and potential function of GnIH, its relationship to the reproductive system, and its possible role as a key neuropeptide involved in sex steroid negative feedback regulation in mammals.

Materials and Methods

Animals.

Thirty-five (>60 days) female and five male LVG hamsters (*Mesocricetus auratus*), five female Sprague–Dawley rats (*Rattus norvegicus*), and five female C57BL/6 mice (*Mus musculus*) were

used in the present experiment. All animals were purchased from Charles River Breeding Laboratories. Animals were housed in translucent propylene cages (48 × 27 × 20 cm for hamsters and rats; 29 × 19 × 12.5 cm for mice), provided with *ad libitum* access to food and water for the duration of the study, and cared for in accordance with the Columbia University Institutional Animal Care and Use Committee and Animal Welfare.

Double-Label Immunofluorescence.

Animals were anesthetized deeply with sodium pentobarbital (200 mg/kg) and perfused intracardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.3). For visualization of gonadotropin-inhibitory hormone (GnIH), sections were washed in PBS, incubated in 0.5% H_2O_2, and incubated in normal goat serum in 0.1% Triton X-100 (PBT) for 1 h. Sections were then incubated for 48 h at 4°C in antiserum generated against white-crowned sparrow GnIH (PAC 123a) diluted at 1:100,000 with 0.1% PBT. After incubation in anti-GnIH, brains were incubated for 1 h in biotinylated goat anti-rabbit (1:300; Vector Laboratories), followed by incubation in avidin-biotin-horseradish peroxidase complex (ABC Elite kit, Vector Laboratories). Brains were then incubated in a biotinylated tyramide solution (0.6%) for 30 min. This protocol allowed for the amplification of the highly diluted anti-GnIH antiserum required for double-labeling with two antibodies generated in the same species. Cells were then labeled by using Cy-2 conjugated to streptavidin (The Jackson Laboratory) as the fluorophore. After labeling for GnIH, sections were incubated for 48 h in a guinea pig anti-gonadotropin-releasing hormone (GnRH) antibody (antigenic determinants are amino acids 6-10; Advanced ChemTech) diluted at 1:10,000 with 0.1% PBT. GnRH cells were labeled with Cy-3 donkey anti-guinea pig (The Jackson Laboratory) as the secondary antibody/fluorophore. Sections were mounted onto gelatin-coated slides and coverslips were applied after dehydration in a graded series of alcohols.

DNA Sequencing of the Partial Syrian Hamster GnIH cDNA Fragment.

Total RNA of the hypothalamus was isolated by the sepasol extraction method. Three forward (5′-CAGCCTACAGGAATCTCA-3′, 5′-GATGCCCCATTTTCACAGC-3′, and 5′-GTTGACTTTAGCCACTTC-3′) and three reverse (5′-CCATGAATGCTTGTCTCC-3′, 5′-TCCCTTCTTCATCGTCTG-3′, and 5′-TGCTGGCAAGTCATGGCGGT-3′) primers were synthesized (Sigma Genosys) based on the known sequence for the Siberian hamster (*Phodopus sungorus*) GnIH precursor cDNA (K.I., T.U., K.U., L.J.K., and K.T., unpublished work). First-strand cDNA was synthesized by RT-PCR (ImProm-II reverse transcription system, Promega) in accordance with the manufacturer's instructions, using nine different primer combinations. After gel electrophoresis, the strongest band resulted from the combination of forward primer 5′-GATGCCCCATTTTCACAGC-3′ and reverse primer 5′-TGCTGGCAAGTCATGGCGT-3′. cDNA PCR product was purified with Amersham Pharmacia MicroSpin columns according to the manufacturer's instructions. The purified PCR products were subcloned into a pGEM-T Easy vector in accordance with the manufacturer's instructions (Promega). The DNA inserts of the positive clones were amplified by PCR with universal M13 primers. The nucleotide sequence was determined by cycle sequencing reactions, using fluorescent dideoxynucleotide terminators and loading onto an ABI 3730XL DNA analyzer (Applied Biosystems).

***In Situ* Hybridization of GnIH mRNA.**

The localization of GnIH mRNA expression was identified by *in situ* hybridization. Two female Syrian hamsters were deeply anesthetized with sodium pentobarbital before transcardial

perfusion with PBS, followed by fixative solution (4% paraformaldehyde in PBS). Brains were collected in the middle of the light/dark cycle on the day of diestrus to maximize GnIH detection. Ten-micrometer coronal sections of the medial basal hypothalamus were made by using a cryostat at –18°C and were placed onto 3-aminopropyltriethoxysilane-coated slides.

For *in situ* hybridization, the fixed sections were first rehydrated with PBS and treated with proteinase K (10 mg/ml) for 30 min, followed by postfixation with 4% paraformaldehyde in PBS for 10 min. After treating the sections in 0.2 M HCl for 20 min, the slides were kept in 40% deionized formamide in 4′ SSC (1′ SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0) for 30 min. Hybridization was carried out at 50°C for 15–17 h with 200 ng/ml digoxigenin-labeled RNA probe (antisense or sense) mixture dissolved in 50% Sigma hybridization solution 1 and 40% formamide. After washing six times with 50% formamide-2′ SSC at 50°C for 10 min each time, the sections were treated with 1.5% blocking reagent (Roche Diagnostics) in TBS and incubated with alkaline phosphatase-labeled sheep antidigoxigenin antibody (1:1,000 dilution in the blocking solution; Boehringer Mannheim) for 1 h. After this, the sections were washed three times with 0.05% Tween in TBS for 10 min each time. Immunoreactive products were detected by immersing the sections for 12 h in a substrate solution [0.035% nitro blue tetrazolium and 0.018% 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris•HCl/100 mM NaCl (pH 9.5)], and the expression of GnIH mRNA was observed by using a Zeiss M1 microscope. Control for specificity of *in situ* hybridization of GnIH mRNA was performed by using a digoxigenin-labeled sense RNA probe complementary to the antisense probe sequence.

Effects of GnIH on LH Concentrations.

Eighteen female hamsters were anesthetized i.p. with 60 mg/kg ketamine and 5 mg/kg xylazine and bilaterally ovariectomized. One week after this surgery, animals were anesthetized, placed into the stereotaxic apparatus (Kopf Instruments), and prepared for aseptic surgery. Animals were then implanted with a 9-mm, 22-gauge guide cannula (Plastics One) aimed at the lateral ventricle (stereotaxic coordinates relative to bregma are as follows: flat skull, anteroposterior, +1.1 mm; mediolateral, +1.5 mm; dorsoventral, –3.0 mm from the top of the skull). The cannula was fixed in place with three stainless-steel screws and cranioplastic cement.

Ovariectomized animals were injected either i.c.v. (5 μl of 0, 100, 300, or 500 ng of GnIH in saline; $n = 8$ per dose) or peripherally (0.2 ml, i.p. with 0 or 600 ng GnIH; $n = 10$ per dose). All injections commenced 3 h after light onset (light/dark, 14/10; lights on at 1500 hours). Injections were infused over a 5-min period, and a blood sample was collected either 5 (i.c.v.) or 30 (i.c.v. and i.p.) min after the end of the injection. At least a 1-week recovery was allowed between each sample collection. Animals fitted with cannulae were injected with each i.c.v. dose in a counterbalanced design. For i.p. treatments, all animals injected were injected with either saline or GnIH in a counterbalanced design.

LH Radioimmunoassay.

LH was assayed in blood plasma collected from female hamsters by the National Hormone and Pituitary Program. Reference preparation RP-3 was used as standard, NIDDK rLH I-9 (AFP-10250C) was used for iodination, and NIDDK S-10 anti-LH was used. Bound LH was precipitated by using goat anti-rabbit IgG. Standard and hamster serum produce parallel inhibition curves in this assay system. All samples were run in a single assay.

Estrogen Injections.

Twelve ovariectomized hamsters were injected (s.c.) with either estradiol (10 μg in 0.1 ml in sesame oil) or oil vehicle 3 h after light onset (L/D, 14/10; lights on at 1500 hours). This amount and route of estradiol administration resulted in elevated estrogen concentrations within 1 h, peaking after 5–6 h (unpublished results). Animals were killed either 3 (n = 3 per group) or 6 (n = 3 per group) h after the injection, and their brains were double-labeled for GnIH and FOS (see above).

Conventional Light Microscopy.

GnRH-ir cell counts and GnIH contacts on GnRH-ir cell bodies were investigated by using a Nikon Eclipse E800 microscope. Sections were examined by using the standard wavelengths for Cy-2 (488 nm) and Cy-3 (568 nm). Every fourth section from the medial septum to the caudal aspect of the anterior hypothalamus was evaluated. Specifically, the medial septum/diagonal band (MS/DBB), medial and lateral preoptic areas, and anterior hypothalamus, were investigated. GnRH cells were counted, and putative axosomatic contacts of GnIH-ir fibers on GnRH soma were screened at ×200. Contacts were assessed at ×400 and ×1,000. A contact was scored only if a GnIH-ir bouton-like structure was observed in close proximity to a GnRH cell body (with both the bouton and cell body being in the same plane of focus), and with examination of the fine focal plane revealing the continuity of the GnIH-ir fiber. All contacts were digitally captured to further confirm GnIH contacts in 8-bit grayscale by using a cooled charge-coupled device camera (SPOT, Diagnostic Instruments). Each image was captured as a single image without moving the position of the stage or plane of focus between captures. Images were superimposed digitally by using spot software (Diagnostic Instruments).

Confocal Microscopy.

Brain sections used for light microscopy were also used for the confocal scans to confirm that the close contacts were on the same 0.5-to 1.0-μm plane. To this end, GnRH-ir cells with GnIH-ir contacts identified at the light level were evaluated. Cells were observed under a Zeiss Axiovert 100TV fluorescence microscope with a Zeiss LSM 410 laser scanning confocal attachment. The sections were excited with an Argon-Krypton laser by using the standard excitation wavelengths for Cy-2 and Cy-3. Stacked images were collected as 0.5-μm multitract optical sections (with sequential excitation by each laser to avoid "cross-talk" between the two wavelengths). Using lsm 3.95 software (Zeiss), red and green images of the sections were superimposed. Each cell was examined through its entirety in 0.5-μm steps, and axosomatic appositions were assessed.

Results

Rodent Brain Exhibits Robust GnIH-ir Peptide Expression.

We first sought to determine the precise distribution of GnIH-ir cell bodies and fibers in hamsters, rats, and mice to explore GnIH connections. By characterizing GnIH-ir distribution, we aimed to gain further insight into the role that GnIH may play in mammalian reproduction and other functions. In all three rodent species, GnIH-ir cell bodies were concentrated in the dorsomedial nucleus of the hypothalamus (DMH) (Fig. 2.1 *A*, *B*, and *D* and Fig. 2.2 *E–H*), with

no other brain regions showing evidence of cell body staining. GnIH cells were mostly bi- and tripolar with a cell area of (622.52 ± 17.81 μm^2). In hamsters, cell numbers (Fig. 2.1 *C*) and fiber distribution did not differ between the sexes.

Deduced Amino Acid Sequence for the Syrian Hamster GnIH Precursor Polypeptide.

The deduced amino acid sequence of GnIH (Fig. 2.3) contains two LPXRF (X = L or Q) sequences that were both flanked by glycine residues as C-terminal amidation signal. Comparison of this partial preproprotein between those of mouse and rat RFamide-related peptides, white-crowned sparrow, and quail GnIHs were 81%, 77%, 43%, and 42%, homologous, respectively (Fig. 2.3).

GnIH mRNA Is Expressed in the DMH.

In situ hybridization using an antisense probe generated from the partial Syrian hamster GnIH clone revealed cell body labeling in the DMH, the neural locus where cell bodies stained immunohistochemically for GnIH are seen (Fig. 2.3). Extended processes and cell nuclei were devoid of reaction product. Importantly, the distribution of cells was identical to that seen with immunohistochemistry, with cell bodies spreading laterally from the third ventricle, just dorsal to the ventromedial hypothalamus, and lateral to the dorsal tip of the third ventricle. Staining was not seen in tissue processed using the GnIH sense probe (data not shown).

GnIH-ir Cells Project to GnRH Neurons.

Because the GnIH system projects to brain areas rich in GnRH cells, we used double-label immunohistochemistry to determine whether GnIH fibers directly contact GnRH cells. Contacts from GnIH-ir fibers were examined using both conventional and confocal microscopy. A large percentage of GnRH cells (>40%) receive projections from the GnIH system in female Syrian hamsters, suggesting the potential for direct inhibition (Fig. 2.4 *A*–*C*). In rats and mice, similar contacts were identified but not quantified (Fig. 2.5). Although we only quantified axo-somatic contacts, a number of axo-dendritic contacts were observed. Although most GnRH cells are concentrated rostrally in septal regions and the preoptic area with few cells in the AH, an equal percentage of cells was contacted within each subregion (Fig. 2.4 *A*). Whereas confocal and light microscopic analyses cannot be used to identify synaptic connections, the existence of pronounced close contacts suggests important functional implications.

GnIH Inhibits LH Secretion.

Given the similarity in the distribution of GnIH-ir cells and fibers in both sexes and among rodent species studied, we focused on exploring the functional role of GnIH using the hamster model. We investigated female Syrian hamsters because the precise regulation of hormones of the reproductive axis is critical for female reproductive function (ovulation, mating, pregnancy, and lactation). Furthermore, the timing of reproductive events is most precisely temporally regulated (estrus occurs at the same time every 4 days) in hamsters relative to rats and mice, making female hamsters an excellent model system to establish a role for GnIH. Ovariectomized Syrian hamsters were injected with GnIH either intracerebroventricularly (i.c.v.) (0, 100, 300, or 500 ng) or peripherally (0 or 600 ng). When injected i.c.v., GnIH rapidly reduced LH concentrations with suppression sustained 30 min after the most effective dose (Fig. 2.4 *D*

and *E*). Similar results were obtained with peripheral injections. Together, these findings provide compelling evidence for a role of GnIH as a mammalian gonadotropin inhibitory factor.

GnIH-ir Cells Express Sex Steroid Receptors and Respond to Gonadal Steroids with Increased Immediate Early Gene Expression.

To assess the role of GnIH in negative feedback of the reproductive system, we explored whether GnIH-ir cells express sex steroid receptors. In female hamsters, double-label immunofluorescence for GnIH and ERα indicates that a large subset (39.41 ± 2.06%) of GnIH-ir cells express ERα (Fig. 2.6 *E*). Similar results were obtained in male hamsters when brains were labeled for AR expression in GnIH-ir cells (data not shown).

We reasoned that if estrogen acts on GnIH-ir cells to stimulate their secretion, then these cells should be "activated" (express FOS) in ovariectomized females after estrogen administration. To determine whether this FOS activation occurs in GnIH-ir cells in the DMH, hamsters were ovariectomized and injected subcutaneously (s.c.) with either estrogen (10 μg in 0.1 ml in sesame oil) or oil vehicle. GnIH-ir cells (number of GnIH-ir cells investigated: 3 h oil, 103.0 ± 4.16; 3 h estrogen, 112.3 ± 5.01; 6 h oil, 107.5 ± 5.50; 6 h estrogen, 115.2 ± 4.26) from four brain sections were assessed for FOS expression by observers unaware of the conditions to which the animals had been exposed. FOS expression was markedly elevated in the DMH of estradiol-treated females relative to oil-injected control animals both 3 and 6 h after estradiol treatment. GnIH-ir cells from oil-injected controls exhibited low levels of FOS expression compared to the robust FOS expression seen after estrogen injections (Fig. 2.6 *A–D* and *F*). Together with the expression of ERα in GnIH-ir cells, these findings suggest that estrogen feeds back to the brain to act (at least in part) on GnIH cells.

Discussion

The results presented in this chapter provide evidence for a previously uncharacterized mammalian RFamide-related peptide in the signaling pathway regulating negative feedback effects of gonadal steroids on the reproductive axis in mammals. The results herein characterize previously unidentified projections from GnIH-ir neurons to the GnRH system, provide evidence that GnIH inhibits activity of the reproductive axis, and suggest that this inhibitory pathway is sensitive to sex steroids.

It is noteworthy that GnIH-ir cells are clustered in the DMH. The DMH projects extensively to neuroendocrine and preautonomic hypothalamic regions, and to a lesser extent to extrahypothalamic sites (Thompson et al. 1996). The DMH, along with five other preoptic nuclei, forms a complex interconnected network that plays a role in coordinating neuroendocrine, autonomic, and somatic responses to external stimuli, sensory feedback, and cognitive/motivational input (Thompson and Swanson 2003). In male and female Syrian hamsters, lesions of the DMH prevent the seasonal onset of reproductive quiescence thought to be mediated through enhanced gonadal steroid negative feedback (Lewis et al. 2002; Maywood et al. 1996). The importance of the DMH in regulating motivated behaviors and endocrine function, along with the present results indicating extensive projections of GnIH-ir cells to hypothalamic and extrahypothalamic brain regions, suggests that GnIH may be an important neurochemical mediator contributing to a broad spectrum of physiological and behavioral systems. The observation that GnIH-ir cells send pronounced projections to limbic brain regions (e.g., septum, striatum, amygdala, and hypothalamus) important for motivated behaviors

(Simerly 2002) provides further support for this speculation and suggests opportunity for study of GnIH beyond reproductive events.

The reproductive axis integrates information from a wide range of systems via a number of direct and indirect neurochemical inputs (Clarke and Pompolo 2005; Gore 2004; Smith and Grove 2002). These neurochemical modulators allow the GnRH system to monitor the internal and external environments and adjust reproductive function according to current conditions. For example, during nutritional deficits, GnRH secretion is inhibited and reproduction is curtailed (Wade and Jones 2004). Likewise, acute and chronic stress can have marked effects on GnRH secretion and reproductive function (Belsham and Lovejoy 2005; Tilbrook et al. 2002). The distribution of GnIH-ir in the present studies, indicating substantial innervation of midline hypothalamic regions, suggests the possibility that GnIH neurons may act through intermediary systems to influence the hypothalamic–pituitary–gonadal axis. For example, RFamide peptides have been implicated in control of hormone release and feeding via interactions with the opiatergic systems (Adams et al. 1991; Chartrel et al. 2003; Fukusumi et al. 2003; Hinuma et al. 2000; Ibata et al. 2000). The presence of GnIH-ir terminals in the arcuate and PVH suggest that GnIH is in a position to monitor nutritional state and modify the reproductive axis accordingly. Additionally, projections of GnIH-ir fibers to the PVH provide the potential for GnIH to regulate corticotropin-releasing hormone to hormonally influence GnRH function.

In avian species, GnIH neurons are found only in the PVN with extensive fibers projecting rostrally to the ventral paleostriatum, lateral and medial septum, and preoptic area and caudally to the median eminence, optic tectum, and brainstem (Bentley et al. 2003; Ukena et al. 2003), and alterations in GnIH have been implicated in seasonal changes in reproduction (Bentley et al. 2003; Ciccone et al. 2004a; Ciccone et al. 2004b; Osugi et al. 2004; Yin et al. 2005), with melatonin acting directly on GnIH-expressing cells (Ubuka et al. 2005). Neuroanatomical and functional studies suggest that GnIH, in turn, modulates gonadotropin secretion by acting directly on GnRH neurons (Bentley et al. 2003) as well as the anterior pituitary (Ciccone et al. 2004a; Osugi et al. 2004; Tsutsui et al. 2000). The possibility that GnIH cells express sex-steroid receptors has not been investigated in avian species. As seen in the present study, the general rodent pattern of cell and fiber distribution is similar to that of birds. Although innervation of the median eminence is sparse, these projections may be functional. In rats, GnIH inhibits LH and GnRH-stimulated release of LH, indicating a potential role for GnIH at the pituitary level. Together, these findings point to convergent roles for GnIH across species and underscore the importance of comparative investigations for this peptide.

RFamide-related peptides (RFRPs) homologous to GnIH as well as its receptor have been identified previously in rats and mice (Hinuma et al. 2000; Ukena et al. 2002; Ukena and Tsutsui 2001). As with the Syrian hamster fragment identified in the present study, the rat and mouse prepro-RFRP polypeptide encodes two RFRPs, called RFRP-1 and RFRP-3, with these precursor polypeptides sharing ≈80% homology among all three species (**Fig. 2.3**). The rat homolog is a potent stimulator of prolactin secretion, with reportedly no effects on other pituitary hormones, including LH (Hinuma et al. 2000). However, this study was conducted in gonad-intact male rats; as a result, LH suppression via sex steroid negative feedback was maximal at the time of testing. In the present study, using ovariectomized animals to release the hypothalamic–pituitary–gonadal axis from negative feedback, a role for this RFamide in regulating LH secretion has now been established.

In summary, RFamide peptides are emerging as important regulators of neuroendocrine function (Hinuma et al. 2000; Hoek et al. 2005; Yang et al. 1985). The data presented within this

first chapter indicate an important role for the RFRP, GnIH, in regulating the mammalian reproductive axis. The present findings point to a previously unidentified neurochemical pathway by which sex steroids act on the brain to regulate the reproductive axis. The extensive fiber projections of GnIH throughout the brain point to an opportunity for investigating the role of this peptide in an array of motivated behaviors.

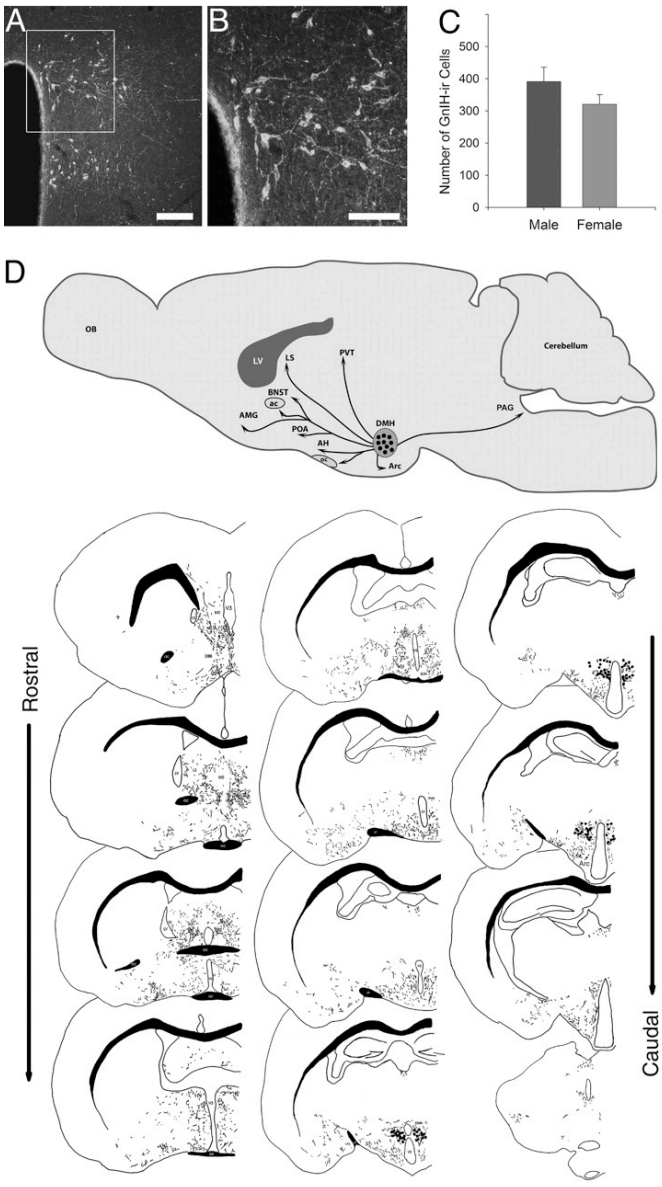


Figure 2.1

GnIH cells bodies are tightly clustered in the DMH and project throughout much of the brain of Syrian hamsters. (*A*) Medium-power photomicrograph depicting GnIH cell bodies clustered in the dorsal and ventral regions of the DMH. (Scale bar: 200 μm.) The box in the top image outlines the cells bodies shown at high power (*B*). (Scale bar: 100 μm.) The number of GnIH-immunoreactive cells was counted in both male and female hamsters (*C*). There were no differences between males and females in their cell counts or fiber distribution. A schematic diagram in the sagittal plane depicts the location of GnIH cell bodies and their projections (*D Upper*). Beneath the sagittal schematic is a tracing of the rostral-caudal extent of GnIH fiber projections and cell bodies (*D Lower*). Note that GnIH cell bodies are clustered in the dorsomedial region of the hypothalamus with diffuse projections throughout most of the brain, with a concentration of terminals in midline brain regions. ac, anterior commisure; AH, anterior hypothalamus; AMG, amygdala; Arc, arcuate nucleus; BNST, bed nucleus of the stria terminalis; LS, lateral septum; oc, optic chiasm; PAG, periaqueductal gray; POA, preoptic area; PVT, paraventricular nucleus of the thalamus.

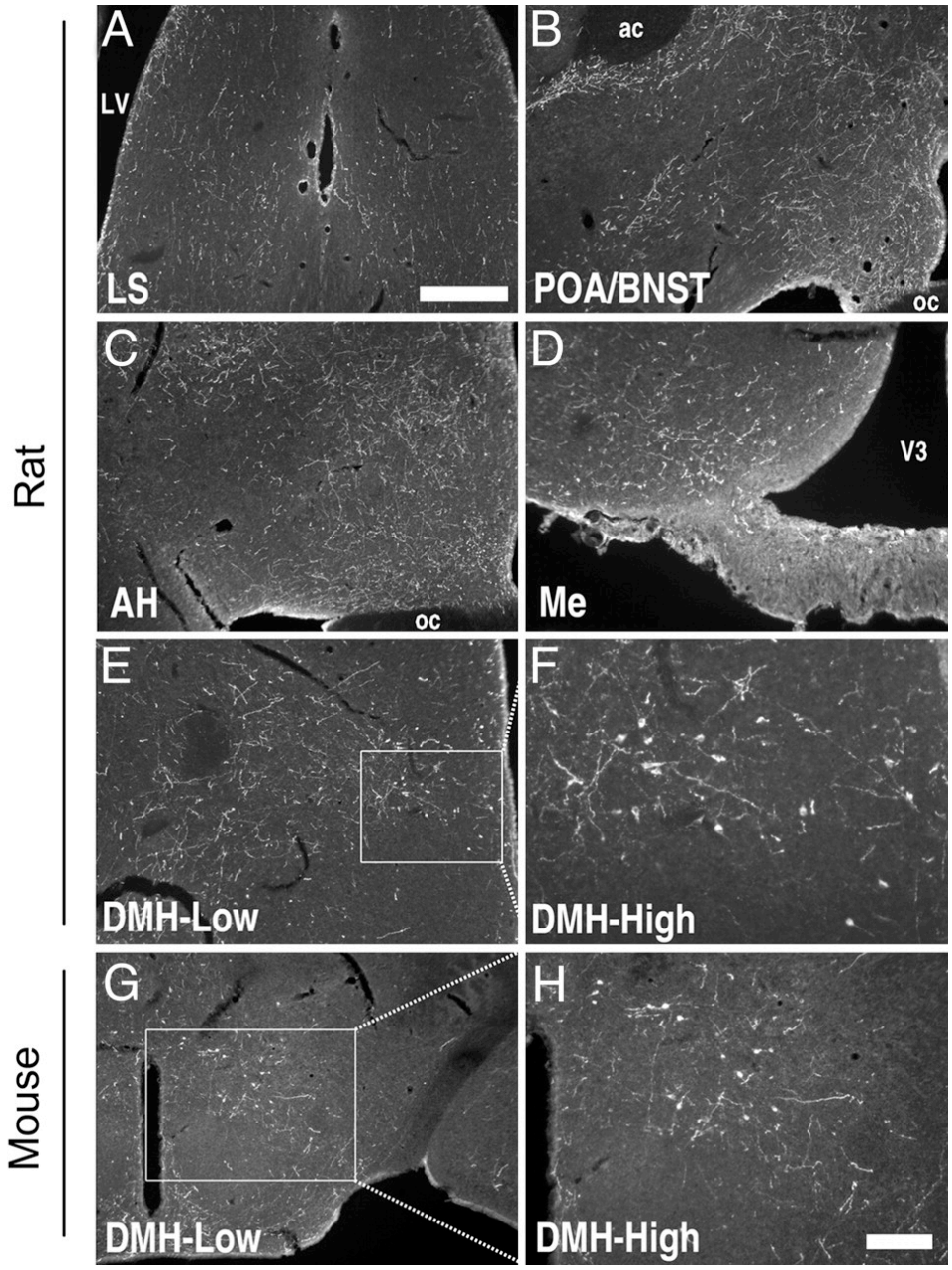


Figure 2.2

GnIH cell and fiber distribution in rats and mice. GnIH fibers are distributed throughout the rostrocaudal extent of the brain, with fiber terminals concentrated in midline brain regions in rat (*A–F*) and mice (*G* and *H*). In mice, the cell bodies tend to spread more lateral in the DMH in comparison with hamsters (*G* and *H*). The rat distribution of cell bodies is similar to that of hamster (*E* and *F*). ac, anterior commisure; AH, anterior hypothalamus; BNST, bed nucleus of the stria terminalis; LS, lateral septum; LV, lateral ventricle; POA, preoptic area. (Scale bars: 400 μm at low power, 100 μm at high power.)

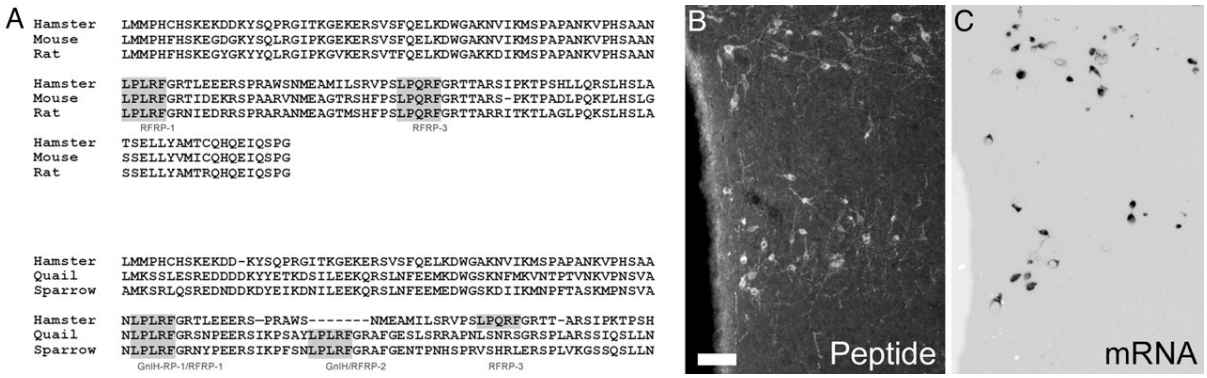


Figure 2.3

The amino acid sequence of the preproprotein that encodes the Syrian hamster GnIH homolog exhibits high homology with other mammalian LPXRFamide peptides and avian GnIH. (*A*) Alignment of preproproteins that encode RFamide-related peptides (RFRP-1, -2, and -3) and GnIH-related peptides (GnIH, -RP-1, and -RP-2) in mammals (hamster, rat, and mouse) and birds (quail and white-crowned sparrow). The predicted amino acid sequence for the Syrian hamster GnIH preproprotein homolog exhibits high homology to previously identified RFRPs in mouse and rat. The hamster amino acid sequence is highly homologous to the avian GnIH-RP-1. (*B* and *C*) Comparison of immunocytochemical staining (*B*) with RNA labeling (*C*) at similar levels of the DMH. Note that both labels exhibit a dorsal and ventral population in the same general pattern of expression. References for peptide sequences: mouse and rat, (Hinuma et al. 2000); white-crowned sparrow, (Osugi et al. 2004); quail,(Satake et al. 2001). (Scale bar: 100 μm.)

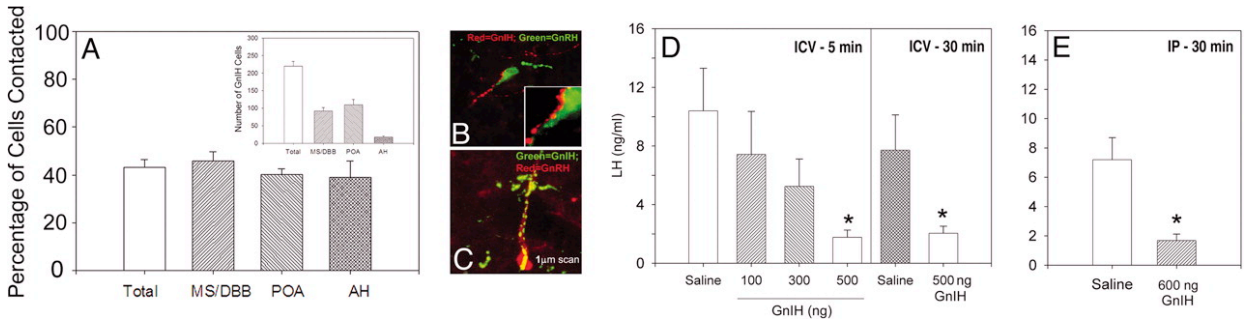


Figure 2.4

GnIH cells target a large proportion of GnRH somata. The GnRH system is distributed from the septum to the caudal aspect of the hypothalamus, and GnRH cells in all brain regions are similarly contacted (*Inset* depicts the distribution of GnRH-immunoreactive cells numbers across brain regions) (*A*). GnRH cells were investigated by using wide-field (*B*) and confocal (*C*) microscopy. Both *B* and *C* depict fibers for GnIH "tracking" the GnRH fiber and cell body; presumptive boutons are evident. GnIH administration either i.c.v. (*D*) or i.p. (*E*) leads to marked and rapid reductions in LH.

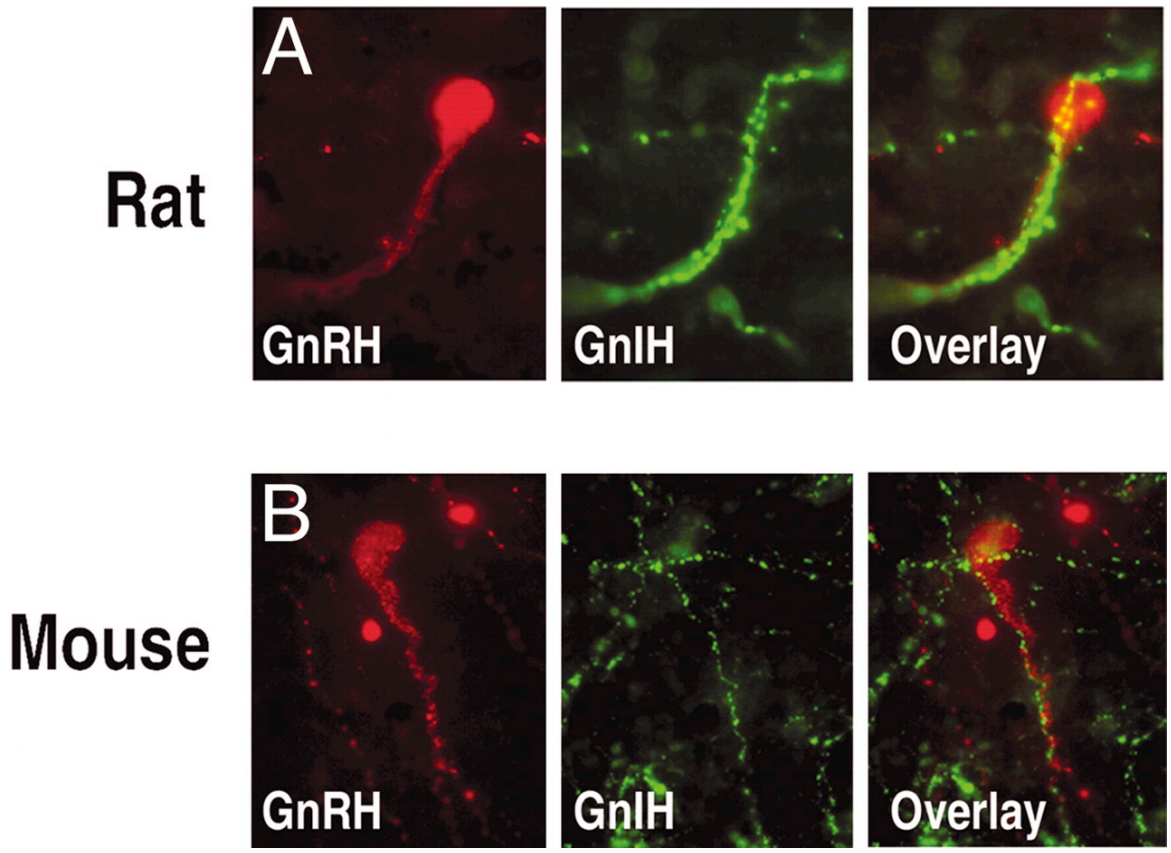


Figure 2.5

GnIH fibers contact GnRH cells in rats and mice. As in hamsters, GnIH fibers target GnRH cells in rats (*A*) and mice (*B*). Note how GnIH cells "track" the fiber following it to the cell body, with several presumptive boutons along the path. For visibility, images are shown as GnRH (red) alone and GnIH fibers (green) alone, followed by their respective overlays. Images were taken at ×1,000 at the light level.

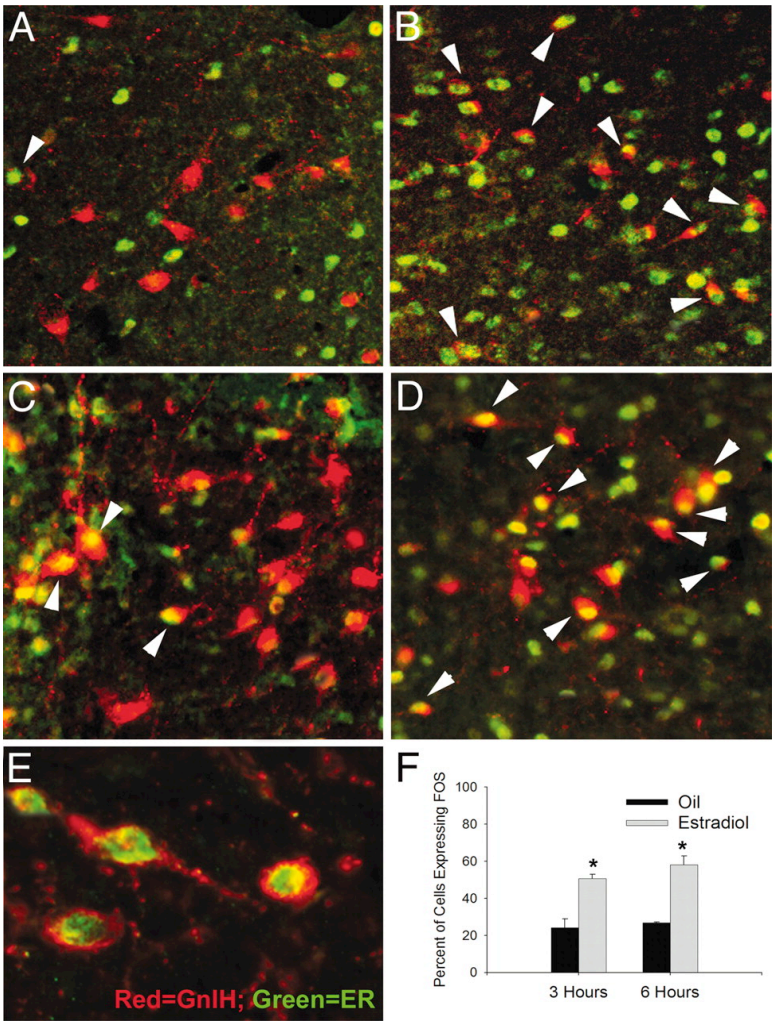


Figure 2.6.

GnIH cells are activated by sex steroid exposure. We pursued the role of GnIH in modulation of estrogen negative feedback because of the importance of this process in regulation of ovulation and the coordination of receptivity. Ovariectomized hamsters were injected with either estradiol (*B* and *D*) or oil vehicle (*A* and *C*) and killed either 3 (*A* and *B*) or 6 (*C* and *D*) h after injection. The percentages of double-labeled GnIH and FOS neurons were counted (*F*). In oil-treated controls, few GnIH cells expressed FOS (*A* and *C*), whereas robust expression of FOS was evident in GnIH cells after estradiol treatment (*B* and *D*). Because estrogen administration led to FOS expression in GnIH cells, it was necessary to see whether estrogen was acting on GnIH neurons or systems upstream. Double-label immunofluorescence was used to colabel GnIH cells and ERα. ERα is expressed in a subset of GnIH cells in female hamsters, suggesting direct actions of estradiol on GnIH cell activation (*E*).

CHAPTER 3

Photoperiod and Reproductive Condition Interact to Affect RFamide-Related Peptide (RFRP) Expression in Syrian Hamsters (*Mesocricetus auratus*)

Introduction

Environmental cycles, such as the annual progression of the seasons, have exerted potent selective pressures resulting in seasonally recurring changes in behavior and physiology across taxa. Survival and reproductive fitness are contingent on an individual's success at negotiating the challenges imposed by a fluctuating and often severe physical environment. Animals that inhabit temperate and boreal latitudes can gain a significant selective advantage by anticipating extreme seasonal shifts and inhibiting energetically expensive processes prior to winter months when food is scarce and weather inclement (Bronson 1989). Photoperiod, or day length, serves as the most salient proximate factor enabling the timing of seasonal adaptations (Goldman 2001). Reproduction and associated behaviors are precisely timed to coincide with abundant local food resources and other environmental conditions that are favorable for rearing offspring (Nelson et al. 1990). By inhibiting breeding during winter, animals with short gestation periods conserve significant energetic resources during times when a mistimed reproductive effort could be fatal for both mother and offspring. Despite the intense selective pressure to inhibit reproduction during winter, an alternative strategy of photoperiodic non-responsiveness occurs at a much lower frequency (Prendergast et al. 2001). Non-responsive individuals maintain an active reproductive system in the winter, a strategy with a presumed fitness advantage during mild winters, resulting in an evolutionarily stable co-existence of two seasonal reproductive phenotypes.

In mammals, day length information is communicated to the brain via nocturnal secretion of the pineal hormone melatonin (Goldman 2001; Malpaux et al. 2001). The duration of the melatonin signal is directly proportional to the length of night and allows animals to determine time of year precisely (Bartness et al. 1993; Carter and Goldman 1983). When held in day lengths of sufficiently short duration (or given melatonin of a significantly long duration), Syrian hamsters undergo testicular involution within eight weeks; when held in day lengths typical of summer conditions, full reproductive condition is maintained (Paul et al. 2008). Seasonal changes in reproductive state are governed by the interaction of two systems: a timing mechanism whose output is communicated by melatonin and the neuroendocrine substrate regulating reproductive function, the hypothalamic-pituitary-gonadal (HPG) axis.

The potency of the negative feedback actions of sex steroids is one of the principal mechanisms driving seasonal changes in reproductive function (Ellis and Turek 1979, 1980; Karsch 1987; Karsch et al. 1993; Meyer and Goodman 1986; Sisk and Turek 1982; Tamarkin et al. 1976). During the breeding season, testosterone exerts a relatively weak negative feedback influence on gonadotropin secretion. Following exposure to inhibitory day lengths, gonadal

hormones become markedly more effective at inhibiting gonadotropin secretion (Ellis and Turek 1979; Sisk and Turek 1982; Tamarkin et al. 1976; Turek 1977). Whereas it is well established that day length is encoded in the duration of the melatonin signal (Bartness et al. 1993), the neural locus(i) where this signal is decoded by the reproductive system to affect negative feedback processing remains undetermined. Given the absence of androgen receptors in GnRH neurons (Huang and Harlan 1993; Tilbrook and Clarke 2001), negative feedback presumably occurs upstream of the gonadotropin-releasing hormone (GnRH) system. The dorsomedial nucleus of the hypothalamus (DMH) has emerged as a key neural locus regulating the gonadotropic response to photoperiod in both male and female Syrian hamsters (*Mesocricetus auratus*) (Lewis et al. 2002; Maywood et al. 1996). High affinity melatonin receptors overlap with sex steroid receptors in the DMH, suggesting a possible site for melatonin-driven alterations in negative feedback (Maywood et al. 1996). Lesions of the DMH abolish reproductive regression in response to inhibitory photoperiods or long duration infusions of melatonin (Lewis et al. 2002; Maywood et al. 1996). These lesions destroyed the DMH and fibers of passage, making it impossible to determine whether the DMH is the locus for melatonin's actions, part of the circuitry mediating its effects, or whether fibers from one or more key nuclei decoding the melatonin signal pass through this brain region. However, the fact that the DMH exhibits a marked overlap in melatonin and androgen receptors strongly suggests participation in seasonal breeding. Despite suggestive evidence for the necessity of the DMH in mediating seasonal breeding in this species, the precise cellular identity of melatonin/androgen-sensitive neurons remains unknown.

The previous chapter of this dissertation documented a series of experiments that characterized a novel gonadotropin-inhibitory hormonal system, RFamide-related peptide [RFRP; as the mammalian homologue of gonadotropin-inhibitory hormone (GnIH) identified in birds (Tsutsui et al. 2000)] in the brains of rodents (Kriegsfeld et al. 2006). RFRP belongs to the family of neuropeptides containing the C-terminal Arg-Phe-NH_2 (RFamide)(Tsutsui et al. 2007; Ukena and Tsutsui 2005), whose prominent role in the regulation of various neuroendocrine functions is gaining recognition. Importantly, in the present context, RFRP cell bodies in hamsters are restricted to the DMH with projections directly to GnRH cell bodies, that this neuropeptide may link the reception of the melatonin signal in the DMH to the reproductive axis. Additionally, RFRP-ir cells express sex steroid receptors and have been implicated as a mediator of sex steroid negative feedback (Greives et al. 2008; Kriegsfeld 2006). In mice, direct administration of RFRP-3 rapidly and repeatedly inhibits firing rate in GnRH neurons (Ducret et al. 2009), pointing to direct actions on this neuronal system. In agreement with this possibility, intracerebroventricular (ICV) administration of RFRP attenuates FOS expression in GnRH neurons (Anderson et al. 2009). A direct hypophysiotropic action for RFRP has recently received support in rats (Murakami et al. 2008) and sheep, whereby RFRP suppresses GnRH stimulated LH release in cultured pituitary cells (Clarke et al. 2008). Together, these findings suggest that RFRP cells are in a position to receive photoperiodic information via melatonin, alter their response to negative feedback through changes in androgen receptors, and communicate this information to the reproductive axis.

Considering the potent inhibitory effect of RFRP on the reproductive axis in conjunction with the overlapping neural distribution of RFRP-producing neurons with a neural locus demonstrated to be critical for mediating the effects of photoperiod on the reproductive axis in

Syrian hamsters, I hypothesized that RFRP would be more abundantly produced and expressed under an inhibitory short day photoperiod relative to a stimulatory long day condition. Therefore, the goal of the present studies was to examine whether the pattern of RFRP peptide and mRNA expression is associated with changes in photoperiod and/or reproductive condition in the brains of Syrian hamsters. To achieve this aim I utilized two methods to differentiate the effects of photoperiod from sex steroids. First, I exploited the natural phenomenon of photoperiodic non-responsiveness, whereby individuals of a breeding population ignore day length cues and maintain a fully functional reproductive system in the winter (Prendergast et al. 2001). This variable response to short days offers a powerful tool to examine the effects of photoperiod exposure independent of changes in reproductive function in a natural context without manipulating sex steroids. To extend these findings and more closely examine the effects of photoperiod and hormonal status on RFRP expression, I measured photoperiodic effects on RFRP mRNA expression while manipulating steroid concentrations via castration and testosterone replacement.

Materials and Methods

Animals

Adult, male LVG Syrian hamsters (*Mesocricetus auratus*) (n=44 total for all studies) were used. All animals were purchased from Charles River (Wilmington, MA) at 4-5 weeks of age and allowed several weeks to acclimate to the laboratory conditions prior to the start of photoperiod manipulations. Animals were housed in translucent polypropylene cages (40 x 27 x 20 cm) and provided *ad libitum* access to food and water for the duration of the study. Animals were maintained in a colony room at 23 ± 1°C with a 24 hr light/dark cycle. All experimental protocols conformed to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley.

Experiment 1

Syrian hamsters (*Mesocricetus auratus*) were kept in long days (LD 16:8) until the start of the experiment, after which some (n=5) of the animals remained in long days while others (n=20) were moved to short-day (LD 8:16) conditions. All hamsters ranged in age from 60-80 days at the start of the study. Brains were collected from short-day hamsters after 3 wk (n=5) or 8 wk (n=15) of photoperiod exposure. Short-term photoperiod exposure (i.e., 3 wk) was used to separate the effects of photoperiod from hormonal status; these animals are acclimated to short day lengths but have not undergone alterations in the reproductive axis, and have testosterone profiles equivalent to long-day animals (Stirland et al. 1995; Stirland et al. 1996). Animals responsive to short days display fully regressed gonads and basal sex steroids by 8 wk in photoperiod. Animals in short days for 8 wk that do not respond to photoperiod have a fully functioning reproductive axis (Prendergast et al. 2001). Hamsters from the 8 wk short day condition were separated into 'responders' (SD-R) (n=9) and 'non-responders' (SD-NR) (n=6) based on gonadal size as described below.

Perfusions and Tissue Collection

At the conclusion of the experiment, hamsters were weighed to the nearest 0.1 g, anesthetized deeply with sodium pentobarbital (200mg/kg) and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.3). All hamsters were sacrificed between the hours of 12 and 3 pm PST. A previous report (Revel et al., 2008) noted that there was no circadian fluctuation in levels of RFRP expression in the brains of photoperiodic hamsters. Brains were postfixed for 3h at room temperature in 4% paraformaldehyde and cryoprotected in 20% sucrose in 0.1 M PBS and stored at 4°C until processed. Coronal sections (40 μm) were cut on a cryostat and processed as free-floating sections beginning rostrally at the medial septum/diagonal band of Broca and extending caudally to the brain stem. Necropsies were performed and reproductive organs (paired testes, epididymides, and seminal vesicles) were collected, cleaned of fat and connective tissue and weighed to confirm the effects of photoperiodic treatments on reproductive condition. After 8 wk in short days animals that had paired testes weighing more than 0.15 g were classified as non-responders; animals with paired testes weighing < 0.15 g were classified as responders. The threshold value of 0.15 g constitutes 2 standard deviations above the mean gonadal mass observed for a typical responder and therefore should only be observed less than 2.5% of the time by chance.

Single-Label Immunofluorescence

For visualization of RFRP, sections were washed in PBS, incubated in 0.5% H_20_2, and incubated in normal goat serum in 0.1% Triton X-100 (PBT) for 1 h. Sections were then incubated for 48 h at 4°C in antiserum generated against white-crowned sparrow GnIH (PAC 123a) diluted at 1:100,000 with 0.1% PBT as previously validated in Syrian hamsters with this antibody (Gibson et al. 2008; Kriegsfeld et al. 2006). After incubation in anti-GnIH, brains were incubated for 1 h in biotinylated goat anti-rabbit (1:300; Vector Laboratories), followed by incubation in avidin-biotin-horseradish peroxidase complex (ABC Elite kit, Vector Laboratories). Brains were then incubated in a biotinylated tyramide solution (0.6%) for 30 min. Cells were then labeled by using Cy-2 conjugated to streptavidin (The Jackson Laboratory) as the fluorophore. Control staining was accomplished by preadsorbing the primary RFRP antibody with rat or hamster RFRP peptides (each of which eliminated staining).

Measures

RFRP cell numbers, size, and optical density were measured. To gain a more detailed understanding of peptide expression, fiber density was evaluated for major RFRP terminal zones. Briefly, sections were investigated using a Zeiss Z1 microscope, using the standard wavelength for Cy-2 (488 nm). Every fourth section from the medial septum to the brainstem was assessed. RFRP-immunoreactive (ir) cell bodies were located by visually scanning the brains under 200x magnification and were found to be restricted to the DMH, as previously reported. All RFRP-ir

cells were counted through the rostrocaudal extent of the DMH. Immunoreactive cells were photographed in grayscale with a Zeiss Axiocam Cooled CCD camera at 400x magnification for cell size and density analyses. Cell bodies were outlined and the two dimensional area was calculated using NIH Image 1.61. Each pixel in the grayscale image capture, which is easily produced by inverting (i.e., negative image) a single label immunofluorescent image, has a measurable specific intensity, with values ranging from 0 (white) to 256 (black). The average value for all pixels in an outlined area is taken as the mean intensity of staining for a given region of the image. OD measures were normalized to minimize differences between replications of immunohistochemistry. First, a background measurement was taken by placing a square outline, four times, on non-overlapping, unstained areas of each section. The mean of these four measures provided the background OD for each section. The OD for each cell body was assessed by outlining the cell body, obtaining a density measure using NIH Image and subtracting the background OD from the OD of each cell. To account for potential over counting, an Abercrombie correction was applied to cell count data prior to analysis.

Experiment 2

In order to further investigate the role of photoperiod and hormonal status on RFRP mRNA expression, animals were castrated and either given blank, control SILASTIC (Dow Corning, Inc) capsules, or capsules filled with testosterone (15-mm length, 1.45-mm id, 1.93-mm od). This capsule length has been shown to restore circulating T levels to average basal levels of an intact LD Syrian hamster (Bittman et al. 1999; Faruzzi et al. 2005). Animals (n=20) were held in long days (LD 16:8) prior to surgery. More specifically, all hamsters (>60 days of age) were gonadectomized under isoflurane anesthetic, after which half (n=10) were implanted with testosterone and the other half (n=10) received an empty capsule. Upon recovery from surgery half of the animals from each hormone condition (n=5/group) remained in long days (LD 16:8) whereas the other half were transferred to short days (LD 8:16) for 8 wk prior to brain collection for *in situ* hybridization.

In situ hybridization

Fresh frozen brain tissue was used for this experiment. Coronal sections (20 μm) through the medial basal hypothalamus were cut on a cryostat and thaw mounted onto silane-coated slides (Electron Microscopy Sciences, Hatfield, PA). Briefly, fixed slices were hybridized with DIG-labeled sense or antisense RNA probes (200 ng / ml) at 50°C overnight followed by an additional 30 minutes RNase digestion step to further reduce the background caused by free DIG-labeled single strand RNA probe. The DIG-labeled probes were produced by a dual promoter (SP6 and T7) plasmid (Topo-II vector from Invitrogen) with hamster RFRP partial sequence inserted (GenBank accession number: DQ371799). After digestion and washing, the sections were treated with 1.5% blocking reagent (Roche Diagnostics, Indianapolis, IN) in PBS and incubated with alkaline phosphatase-labeled sheep anti-digoxigenin antibody (1:1000 dilution in the blocking solution; Roche Diagnostics, Indianapolis, IN) for 1 h. Immunoreactive products were detected by immersing the sections for 12 h in NBT/BCIP substrate solution

(Roche Diagnostics, Indianapolis, IN), and the expression of RFRP mRNA was observed with a Zeiss M1 microscope.

Statistics

All statistical analyses were conducted using SigmPlot statistical package. Data from experiment 1 were analyzed as a series of one-way ANOVAs. Experiment 2 was analyzed with a two-way ANOVA. All values are reported as means (+SEM) and all tests were considered significant at the $P < 0.05$ level. Group differences were assessed using *post hoc* Tukey tests.

Results

Experiment 1

Reproductive organ masses are influenced by photoperiod.

Short-day exposure for 8 wk significantly reduced reproductive organ (i.e., testes, seminal vesicles, and epididymides) masses in reproductively responsive animals relative to long-day controls, short-day 3 wk animals, and short-day 8 wk non-responders ($p<0.05$ in each case) (Fig 3.1). Seminal vesicle mass did not differ among non-responsive animals exposed to short days for 8 wk, long-day animals and short-day 3 wk hamsters ($p>0.05$ in each case). In contrast, epididymal and paired testes masses of short-day 8 wk non-responders were lower than long-day hamsters and short-day 3 wk animals ($p<0.05$ in each case) and greater than those of short-day 8 wk responders ($p<0.05$). There was no effect of photoperiod on body mass ($p> 0.05$).

RFamide related peptide immunoreactive (RFRP-ir) cell numbers and optical density are associated with reproductive condition, independent of photoperiod.

To determine the effects of photoperiod and reproductive condition on RFRP peptide expression, Syrian hamster brains were processed immunohistochemically to examine RFRP peptide (Fig 3.2). As previously reported, RFRP-ir cells were restricted to the dorsomedial nucleus of the hypothalamus (DMH), with extensive projections to midline brain regions. Reproductive state significantly affected the number and optical density ($p<0.05$, in both cases) of RFRP-ir neurons in the DMH. Animals that responded with reproductive inhibition to 8 wk exposure in short (LD 8:16) day photoperiod displayed significantly fewer ($p<0.05$) and less dense ($p<0.05$) RFRP-ir neurons than all other groups; all other groups displayed a similar number and size of RFRP-ir neurons ($p>0.05$) (Fig 3.3). Photoperiod alone did not significantly affect RFRP-ir cell number or size, as hamsters non-responsive to 8 wk of short day photoperiod, as well as ones only held in short days for 3 wk, resembled their long day (LD 16:8) counterparts in measures of cell number and size ($p>0.05$).

Fiber density is associated with changes in photoperiod and reproductive condition.

In an effort to characterize further the changes in RFRP-ir expression in different photoperiods, a systematic examination of the density of RFRP-ir fiber staining in many of the key targets of RFRP terminals (lateral septum (LS), preoptic area (POA), bed nucleus of the stria terminalis (BNST), anterior hypothalamus (AH), paraventricular nucleus of the thalamus (PVT), and the arcuate nucleus (Arc)) was carried out. Hamsters with regressed gonads held for 8 wk in short (LD 8:16) day photoperiods had significantly decreased ($p<0.05$) fiber optical density in the LS, POA, AH, and the PVT compared to either long day (LD 16:8) or short day non-responder groups. Hamsters held for 3 wk in short (LD 8:16) days had significantly ($p<0.05$) decreased fiber optical density in all of the same target nuclei (Figure 3.4).

Experiment 2

RFamide related peptide mRNA expression is affected by photoperiod treatment independently of hormone levels.

In order to determine whether differences in peptide expression in Experiment 1 were the result of changes in RFRP transcription I examined RFRP mRNA expression using *in situ* hybridization in animals whose testosterone concentrations were manipulated in each photoperiodic condition. Hamsters held in short (LD 8:16) days for 8 wk exhibited significantly fewer ($p<0.05$) RFRP mRNA expressing cells than groups held in long days, regardless of testosterone treatment. There was no significant effect of testosterone in either photoperiod ($p>0.05$), suggesting that RFRP expression is modulated by day length, and not a secondary result of changes in sex steroid concentrations (Figs 3.5 and 3.6).

Discussion

The present findings uncover an unanticipated pattern of RFRP expression associated with seasonal changes in reproductive function in Syrian hamsters. Males exposed to short, winter-like photoperiods with a regressed reproductive apparatus exhibit a marked reduction in RFRP-ir and mRNA expression relative to their long day counterparts. Importantly, these differences were not secondary consequences of diminished sex steroid concentrations, as testosterone replacement did not elevate RFRP mRNA expression to long-day values in short-day hamsters. Likewise, RFRP-ir cells were unaffected in short-day animals with a reproductive system that did not undergo regression, suggesting an association between changes in RFRP and reproductive competency.

Although the data herein are in agreement with recent findings (Revel et al. 2008), the results are counter to the original prediction that an increase in RFRP-ir and mRNA would be observed in animals with a quiescent reproductive system. Although it appears paradoxical that an inhibitory neuropeptide is decreased during inhibitory day lengths, these findings do not rule out a role for RFRP in seasonal changes in reproductive function. One possibility is that Syrian hamsters require enhanced RFRP activity to suppress GnRH during the initial period of

regression, but this level of inhibition is not necessary in hamsters with a fully regressed reproductive apparatus. Further empirical examination of the pattern and kinetics of RFRP expression throughout the development of reproductive quiescence is necessary to fully explore this possibility. The literature in birds is supportive of a transient effect of GnIH on the reproductive axis. For example, hypothalamic GnIH content is increased at the onset of gonadal regression in seasonally-breeding song sparrows, but this increase is not seen following gonadal regression (Bentley et al. 2003). Likewise, Calisi et al. observed an increase in hypothalamic GnIH content in response to stress in house sparrows at the start of the breeding season but not at the end (Calisi et al. 2008).

Immunohistochemical findings for RFRP expression in hamsters after three weeks of short day exposure are consistent with the possibility that the release of RFRP is increased during initial stages of inhibition, before the reproductive axis begins to regress (Stirland et al. 1996). More specifically, although cell counts in long day animals and hamsters held for three weeks in short days were identical, fiber density in RFRP cell targets was decreased in this latter group. The parsimonious explanation for this pattern of expression is that RFRP production is static during early stages of regression whereas peptide release is increased at hypothalamic targets. However, it is also possible that RFRP production and transport are decreased at this time and this peptide does not participate in short-day-induced gonadal regression. It should be noted that, because 3 weeks is too early to assess the photoperiodic responsive phenotype of Syrian hamsters, it is possible that a small number of animals that ultimately would be classified as non-responders may have been included in the analysis at the 3 week time point. At 8 weeks, decreased fiber expression, along with reduced RFRP-ir and mRNA expression in SD-R animals, suggests that transcription/translation is reduced at this time point relative to long-day animals, inconsistent with a role for RFRP in maintaining gonadal regression.

Theoretically, the trigger for changes in the RFRP system could be the coincidence of the long duration melatonin signal with androgen receptor binding in, or upstream of, RFRP neurons. Results examining RFRP mRNA from short-day animals given testosterone suggest that photoperiod acts independent of testosterone negative feedback to suppress RFRP production. Recent studies using an immortalized RFRP cell line generated from rat hypothalamus indicate that these cells express melatonin receptors and respond to melatonin administration with increased expression, suggesting a steroid-independent mechanism of photoperiodic control (Gingerich et al. 2009). Whether or not RFRP cells express melatonin receptors in hamsters requires further investigation. It is also possible that the RFRP system responds secondarily to melatonin's impact on other target loci. For example, a thyroid hormone-dependent cascade has recently been implicated in modulating the effects of photoperiod on the reproductive axis in a number of species. In sheep, the melatonin signal in decoded in the pars tuberalis through the local secretion of thyroid stimulating hormone (TSH). In turn, TSH activates type II deiodinase (DIO2) leading to increased tri-iodothyronine (Hanon et al. 2008). A similar mechanism of control has been identified in Siberian hamsters (Watanabe et al. 2004). The possibility that changes in DIO2 precede or contribute to changes in RFRP, or that these two pathways act in parallel to adjust the suite of reproductive traits that change seasonally, represents an interesting avenue for further inquiry.

The present findings uncover novel changes in RFRP-ir and mRNA expression associated with day length and reproductive status. The findings are consistent with the notion

that RFRP contributes to the initial suppression of reproductive function, but not to the maintenance of reproductive quiescence following its completion. However, further empirical studies are required to fully understand the role of this neuropeptide in mediating seasonal changes in reproductive function.

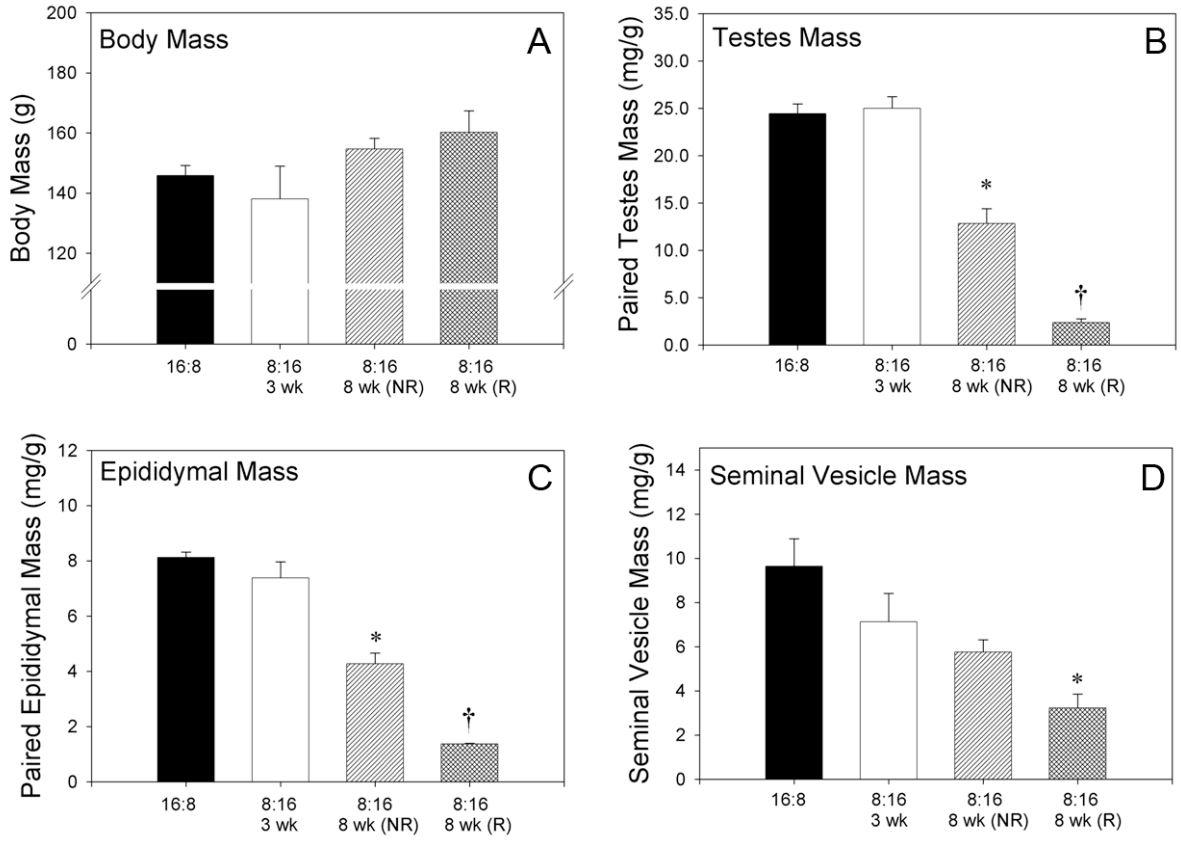


Figure 3.1

Body and reproductive organ masses from animals held in long (LD 16:8) or short (LD 8:16) day lengths. Animals held in short days for 8 wk were separated into 'responders' (R) or 'non-responders' (NR) based on gonadal size. Body size (A) was unaffected by photoperiodic treatment. Testes mass (B) and epididymal mass (C) were significantly diminished in SD-R. SD-NR had significantly decreased testes and epididymal mass than either long day or short day-3 week animals. Seminal vesicle mass (D) was significantly less in SD-R 8 week animals than all other groups.

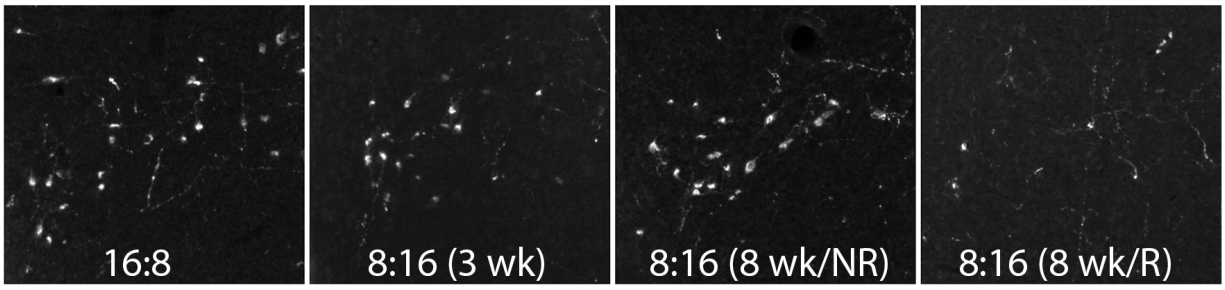
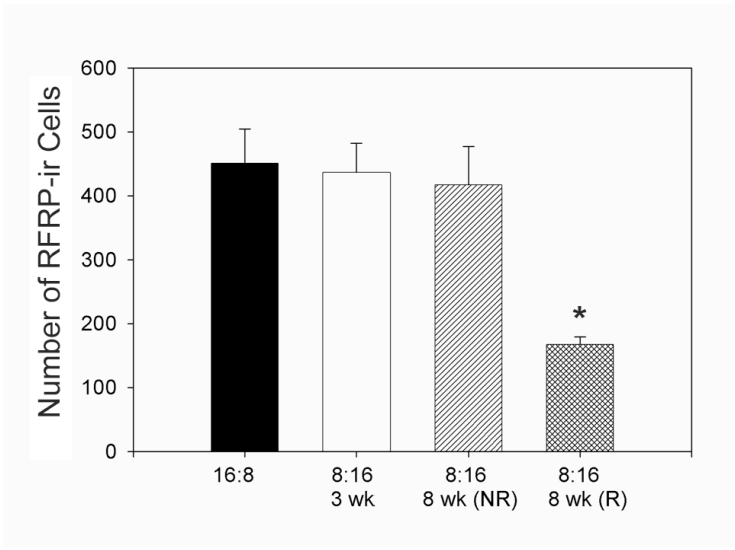


Figure 3.2

RFRP immunohistochemical labeling. Representative photomicrographs of RFRP-ir staining in the DMH of hamsters held in long day (LD 16:8) photoperiod (A) for 8 weeks, short day (LD 8:16) photoperiod (B) for 3 weeks, short day non-responders (NR) (C) for 8 weeks, and short day responders (D) for 8 weeks. High power (200x) photomicrographs are shown for each condition



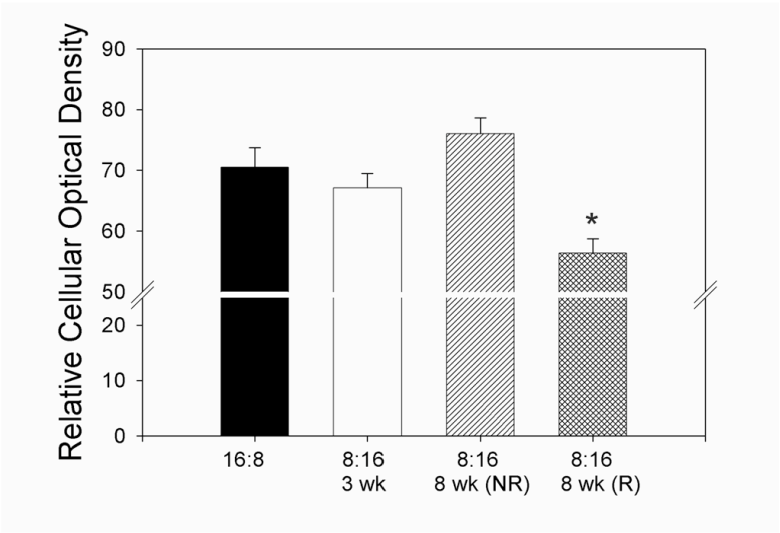


Figure 3.3

RFRP-ir cell numbers and optical density are associated with reproductive condition, independent of photoperiod. Mean (±SEM) number of RFRP-ir cells (top) and cellular optical density (bottom) of animals held in long days for 8 wk or short days for 3 wk or 8 wk (responders and non-responders). Short day responsive hamsters held for 8 wk in short days display significantly fewer number of RFRP-ir cells. Cellular optical density was unaffected by photoperiod. *= $p<0.05$

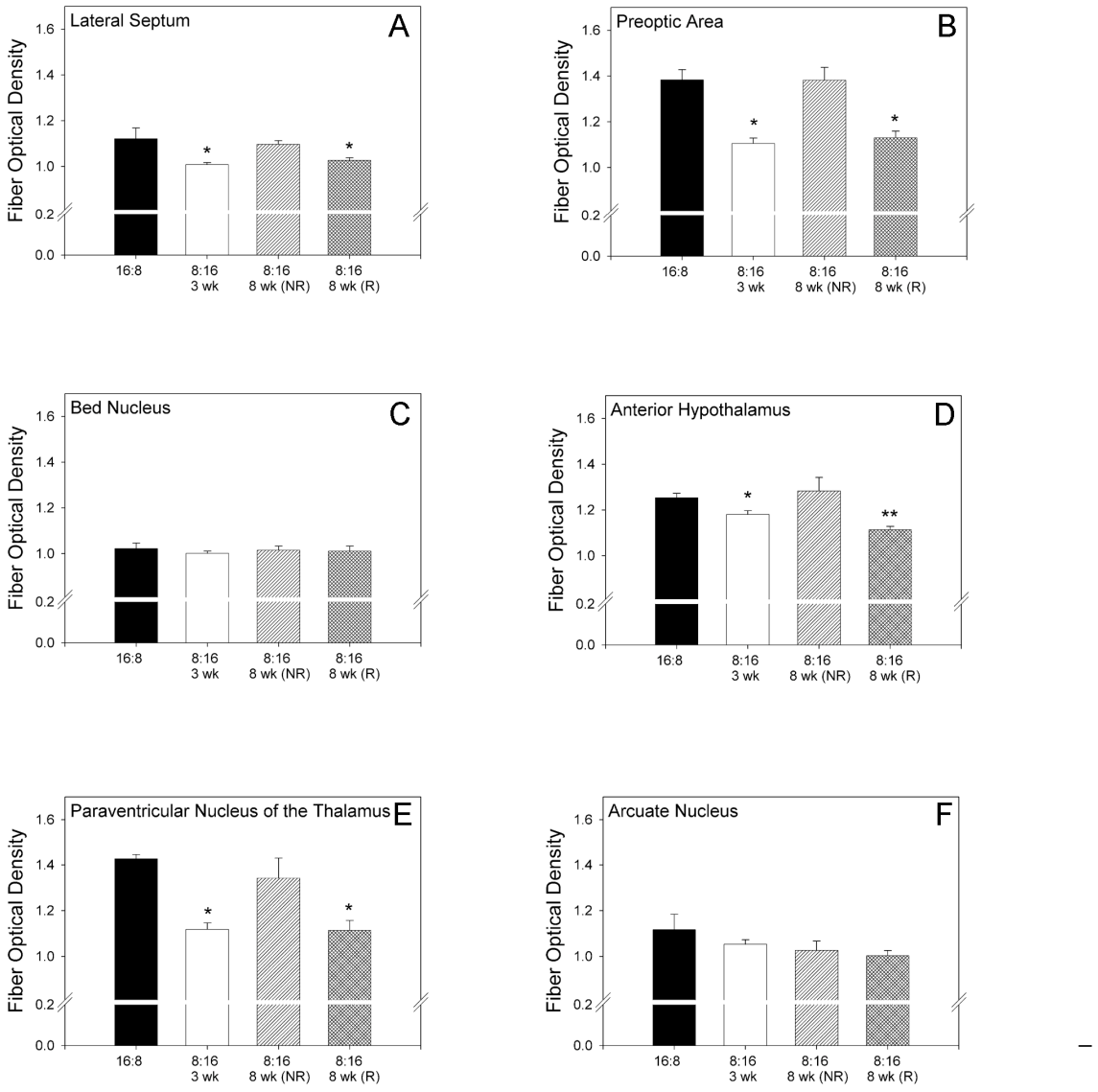


Figure 3.4

RFRP-ir optical fiber density is associated with changes in photoperiod and reproductive condition in some brain targets. Fiber optical density in the (A) lateral septum, (B) preoptic area, (D) anterior hypothalamus, and (E) paraventricular nucleus of the thalamus was significantly affected by photoperiod in short day responsive animals held for 8 wk in short days. In these same nuclei, fiber density was significantly decreased in animals held in short day for 3 wk. Fiber density was unaffected by photoperiod in the (C) bed nucleus of the stria terminalis and the (F) arcuate nucleus. *=significantly less than all other groups not sharing the same symbol (p<0.05). **=significantly less than all groups (p<0.05).

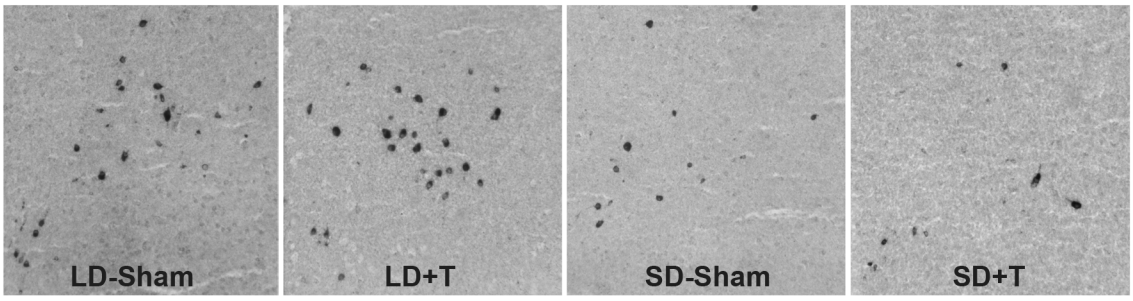


Figure 3.5

RFRP mRNA labeling. Representative photomicrographs depicting RFRP mRNA expression across long day (LD16:8) and short day (LD 8:16) photoperiods in gonadectomized animals receiving either testosterone replacement (+T) or empty capsules (sham).

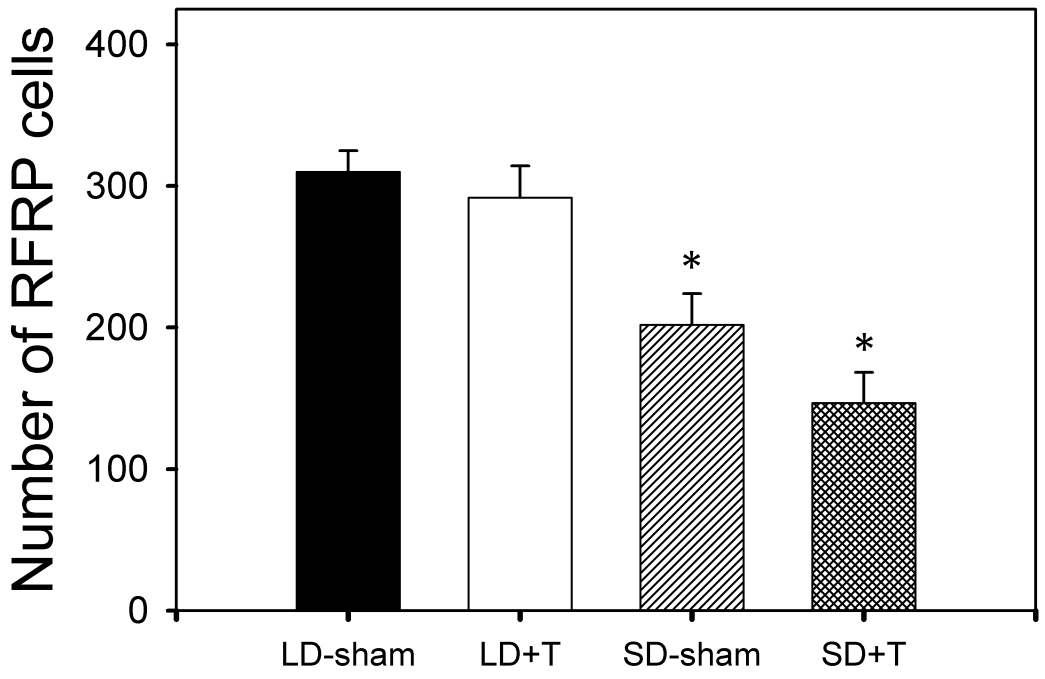


Figure 3.6

RFRP mRNA expression is affected by photoperiod, independent of testosterone levels. Mean (±SEM) number of RFRP mRNA expressing cells of gonadectomized animals held in long day (LD 16:8) and short day (LD 8:16) photoperiods and receiving either testosterone replacement (+T) or empty capsules (sham). Hamsters held in short (LD 8:16) days exhibited significantly fewer ($p<0.05$) RFRP mRNA expressing cells than groups held in long days, regardless of testosterone treatment. There was no significant effect of testosterone in either photoperiod ($p>0.05$). *= $p<0.05$

CHAPTER 4

Role of RFRP in Reproductive Photoresponse of Artificially Selected Lines of White-footed Mice (*Peromyscus leucopus*)

Introduction

Phenotypic traits that enhance an individual's reproductive fitness in natural animal populations have been selected throughout evolutionary time. One such trait is differential behavioral and physiological responsiveness to environment cues that signal predictable seasonal changes (Heideman 2004). Small mammals that inhabit temperate zones are faced with severe challenges during winter that necessitate significant behavioral and physiological adjustments aimed at inhibiting nonessential processes and allocating scarce energetic stores for maintenance of processes that are necessary to sustain life until favorable environmental conditions return (Bronson 1989; Bronson 1985). The maintenance of an active reproductive system is an energetically costly (Speakman 2008), nonessential process that is commonly curtailed during winter, as the burden of providing nourishment and parental care in a harsh climate would likely result in death of both parent and offspring (Bronson 1989). The pronounced seasonal variation in climatic conditions has been the major driving force behind the evolution of temporally defined breeding seasons. Despite the adaptive value of reproductive inhibition, most populations of seasonally breeding rodents contain a substantial level of variability in individual reproductive responses. A single breeding population often consists of reproductive phenotypes ranging from maximally inhibited to effectively nonresponsive to environmental cues (Prendergast et al. 2001). Significant understanding of the neuroendocrine mechanisms that allow predictive cues in the environment to impact the reproductive axis has already been achieved. Therefore, seasonally breeding rodents are a useful mechanism for exploring the genetic contribution to neuroendocrine traits that have adaptive value for animals in their natural environment (Heideman 2004).

The timing cue of greatest utility for anticipating predictable seasonal transitions is day length, or photoperiod, because of its relatively invariant relationship with the annual cycle of ambient conditions and food availability in a specific environment throughout evolution (Goldman 2001). All mammals process day length cues through a well-characterized photoneuroendocrine circuit that allows seasonal change in day length to cause seasonal change in reproduction, metabolism, fur density, and other important physiological traits. Briefly, light information enters the retina and is communicated to the circadian clock located in the suprachiasmatic nucleus of the hypothalamus (SCN). From the SCN, the photoperiodic signal is communicated via a multisynaptic pathway that terminates at the pineal gland where this neural signal is transduced into a hormonal signal as the nightly secretion of melatonin (MEL) (Prendergast 2002). MEL is the temporal code that acts as an internal representation of day

length for the brain and body. MEL receptors, found widely in various brain areas and tissues throughout the body, decode the MEL signal duration and respond with adjustments appropriate for conditions predicted by the particular day length (Bartness et al. 1993). Whereas the specific mechanism for interpreting the melatonin signal remains unspecified, the ultimate effect of seasonal changes in day length is to alter the activity of the GnRH neural network, the final common neural pathway regulating pituitary and gonadal function (Belsham and Lovejoy 2005; Wray 2002). The range of reproductive responses observed in both field and laboratory settings inevitably results from variability in the specific molecules, receptors, neurons, or circuits that process and transmit information about photoperiod from the eye to the reproductive axis (Heideman 2004). A genetic basis for some phenotypic variation in reproductive photoresponse has been suggested by research showing that a single generation of artificial selection can significantly alter the proportion of responsive individuals in laboratory populations of deer mice, field voles, and Djugarian hamsters (Desjardins et al. 1986; Lynch et al. 1989; Spears 1988). However, the specific genes that selection is acting upon and their precise phenotypic consequences within the photoneuroendocrine pathway remain to be determined.

White-footed mice (*Peromyscus leucopus*) are an ideal system for investigating the seasonal control of reproduction and the genetic consequences of selection for level of reproductive response, as nonresponsiveness has been particularly well studied in this species (Avigdor et al. 2005; Glass 1986; Heideman and Bronson 1991; Heideman 1999; Johnston and Zucker 1980). Wild populations of white-footed mice consist of individuals with a range of responses to photoperiod, with many displaying maximally inhibited reproductive function in winter and others reproducing irrespective of season. A previous selection experiment on a wild population of white-footed mice suggested that much of the variation in photoresponsiveness is genetic in origin (Heideman 1999). After only three generations, very little overlap is apparent in testes size between the lines selected for nonresponsiveness and for maximal response (Heideman 1999). Earlier studies in unselected white-footed mice, as well as deer mice suggest that differences in GnRH neuronal activity could explain differential responsiveness (Glass 1986; Korytko et al. 1995; Korytko et al. 1998). More recently, studies examining wild-derived white footed-mice selected for responsiveness, found that a line nonresponsive to short photoperiod had significantly greater total numbers of mature GnRH immunoreactive neurons than a line reproductively inhibited by short photoperiod (Avigdor et al. 2005). As a whole, these findings suggest that variation in GnRH neuronal activity may underlie some of the natural reproductive and life history variation observed in wild populations of *P. leucopus* (Avigdor et al. 2005).

We have recently identified a novel inhibitory modulator of the reproductive axis in mammals mediating the inhibitory response to photoperiod in seasonally breeding Syrian hamsters (*Mesocricetus auratus*) (Kriegsfeld et al. 2006). This novel neuropeptide, RFamide related peptide (RFRP), is gaining recognition as a general inhibitory modulator of the reproductive axis in all mammals, including both seasonal and non-seasonal species (Clarke et al. 2009; Tsutsui et al. 2010). Our previous findings in Syrian hamsters suggest that variability in the response of the RFRP system to short days may account for the nonresponsive phenotype in this species (Chapter 3). The goal of the present study was to take advantage of the white-footed mice model to determine whether variation in the RFRP system would account for some of the changes in reproductive function in the two previously characterized lines of mice.

Because it is known that the GnRH system is altered in these selected lines and that RFRP can act to inhibit reproductive function directly at the level of GnRH neurons as well as the pituitary, we hypothesized that we would observe changes in RFRP expression corresponding to the different levels of GnRH expression. This finding would suggest a mechanism upstream of the GnRH system responsible for genetic variation in reproductive response to photoperiod. To achieve this aim we examined levels of RFRP immunoreactivity in the brains of male white-footed mice from an artificially selected nonresponsive (SD-NR) and responsive (SD-R) line. The broad goal of this study is to further explore the neural and genetic basis of phenotypic variation of an important life-history trait in a wild population of seasonally breeding rodents to better understand how selection operates to produce organisms well adapted to their ecological niche.

Methods

Animals and Housing

Mice were obtained from a laboratory colony at the Population and Endocrinology Laboratory of the College of William and Mary. The wild founders of the population were captured at latitude 37° 16'N, near Williamsburg, VA (Heideman 1999). A detailed explanation of the methods and criteria for producing the artificially selected lines of either responsive (short day R) or nonresponsive (short day NR) mice is available in a previous report (Avigdor et al. 2005). In brief, males and females were classified as SD-NR or SD-R based on gonadal and reproductive organ size measures. Males and females classified as NR were paired together to generate a photo-nonresponsive line (SD-NR), and the same strategy was used to produce a photo-responsive line (SD-R).

The present experiment was carried out on two groups of young adult *P. leucopus* from the F7 or F8 generation of the two selected lines, producing four total groups. A total of 32 male mice were used for this study. SD-R (n=8) and SD-NR raised in SD photoperiod (LD 8:16) (n=8) were produced by transferring mothers and their pups from LD photoperiod to SD photoperiod within 3 days of birth of the litter. Male offspring were weaned at 21-23 days, singly housed until 70 ± 3 days of age when their testis index was assessed, and singly housed again until perfusion. RI (n=8) and NR in LD (LD 16:8) (n=8)

Perfusions and Tissue Sectioning

All perfusions were conducted in mice aged 70-100 days. Mice were weighed, euthanized with an overdose of isoflurane (Abbot Laboratories, North Chicago, IL), and allowed to enter respiratory arrest before perfusion. Mice were perfused through the left ventricle at ~4 ml/min using a perfusion pump and bled via the right atrium. Perfusion of 5 ml of 0.1 M PBS at a pH of 7.4 was followed by a perfusion of 50 ml of fresh, cold (5°C) 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) and saturated picric acid (Sigma) in PBS. Brains were removed and postfixed overnight at 4°C in 0.1 M PBS with 30% sucrose for cryoprotection. After perfusion the mass of the seminal vesicles was assessed in all animals. All brains were

sliced within 4 days of perfusion. Frozen coronal sections (30 μm) were cut on a freezing sliding microtome and separated into four wells, each containing every fourth section. Wells were filled with brain antifreeze [37.5% sucrose, 37.5% ethylene glycol, and 10 g PVP-40 in 500 ml 0.02 M Tris-buffered saline (TBS)]. Brains were stored at -20°C until ICC.

Single-Label Immunocytochemistry (ICC)

For visualization of RFRP, sections were washed in PBS, incubated in 0.5% H_20_2, and incubated in normal goat serum in 0.1% Triton X-100 (PBT) for l h. Sections were then incubated for 48 h at 4°C in antiserum generated against white-crowned sparrow GnIH (PAC 123a) diluted at 1:100,000 with 0.1% PBT as previously validated in Syrian hamsters with this antibody (Gibson et al. 2008; Kriegsfeld et al. 2006). After incubation in anti-GnIH, brains were incubated for 1 h in biotinylated goat anti-rabbit (1:300; Vector Laboratories), followed by incubation in avidin-biotin-horseradish peroxidase complex (ABC Elite kit, Vector Laboratories). Brains were then incubated in a biotinylated tyramide solution (0.6%) for 30 min. Cells were then visualized by using 0.03% 3,3-diaminobenzidine (DAB) as a chromogen, following three washes in PBT. Following visualization, tissue was mounted onto subbed slides and allowed to dry overnight. The next day slides were rinsed in a series of alcohols and xylenes prior to application of coverslips.

Measures

Slides were examined under bright field illumination on a Zeiss Z1 microscope by an independent observer naïve to the experimental conditions. RFRP-immunoreactive (ir) cells were located by visually scanning the brains under 200× magnification. Cell populations were restricted to the dorsomedial hypothalamus (DMH), in agreement with other rodent species. All cells were confirmed at a minimum of 400×. Cells were photographed with a Zeiss Axiocam Cooled CCD camera at 400× magnification for cell size and density analyses. All cells in every 4th section were counted through the rostrocaudal extent DMH. Only those cells with a visible nucleus were counted. Soma size and optical density (OD) measurements were performed on images captured at 400×. Soma size and optical density provide a semi-quantitative measure of protein/peptide content visualized immunocytochemically (Nishio et al. 1994). Whereas this measure is unlikely to uncover subtle differences in peptide content across groups, more significant changes should be observed. Cell bodies were outlined and the two-dimensional area was calculated using NIH Image 1.61. Each pixel in the grayscale image capture has a measurable specific intensity, with values ranging from 0 (white) to 256 (black). The average value for all pixels in an outlined area is taken as the mean intensity of staining for a given region of the image. OD measures were normalized to minimize differences between replications of immunohistochemistry. First, a background measurement was taken by placing a square outline, four times, on non-overlapping, unstained areas of each section. The mean of these four measures provided the background OD for each section. The OD for each cell body was assessed by outlining the cell body, obtaining a density measure using NIH Image and subtracting the

background OD from the OD of each cell. To account for potential overcounting, an Abercrombie correction was applied to cell count data prior to analysis.

Statistics

All statistical analyses were conducted using SigmPlot statistical package. Cell number, size, and OD measures were analyzed as a series of one-way ANOVAs. All values are reported as means (+SEM) and all tests were considered significant at the $P < 0.05$ level. Group differences were assessed using *post hoc* Tukey tests.

Results

RFamide related peptide immunoreactive (RFRP-ir) cell numbers are affected by genetic line and photoperiod.

To determine the effects of photoperiod and genetic line (SD-R vs. SD-NR) on RFRP peptide expression, white-footed mice brains were processed immunohistochemically to examine RFRP peptide (Fig 4.1). In common with studies in several different rodent species (Kriegsfeld et al. 2006), RFRP-ir cell bodies were also restricted to the DMH of white-footed mice. The specific genetic line that a mouse belonged to significantly affected the number of RFRP-ir neurons in the DMH. Mice from the responsive line (SD-R) displayed significantly fewer RFRP-ir neurons than mice selected for nonresponsiveness ($p<0.05$) (SD-NR) (Fig. 4.2). In addition, photoperiod significantly affected RFRP-ir cell number regardless of genetic line identity ($p<0.05$). Both SD-R and SD-NR mice held in short days (LD 8:16) displayed significantly fewer RFRP-ir cell numbers than their long day (LD 16:8) counterparts ($p<0.05$) (Fig. 4.2). The magnitude of decrease in RFRP-ir cell numbers in short day photoperiod was equivalent across the two lines of mice.

RFamide related peptide immunoreactive (RFRP-ir) cell size and OD are non-systematically affected by genetic line and photoperiod.

There were no main effects of photoperiod or genetic line upon RFRP-ir cell size or optical density measures ($p>0.05$ in all cases). However, planned pair-wise comparisons revealed several significant differences. Within the SD-R group, cell size was significantly decreased in short day relative to long day photoperiods ($p<0.05$) (Fig. 4.3). Within short days, the SD-NR group had significantly larger cell size than the SD-R group ($p<0.05$) (Fig 4.3). Also within short days, the OD of RFRP cells was significantly greater in SD-NR than SD-R mice ($p<0.05$) (Fig 4.4).

Discussion

The present results indicate that artificial selection for reproductive responsiveness in white-footed mice results in significant changes in a key, inhibitory neuropeptidergic system. Specifically, we found that mice bred to exhibit a robust reproductive response to photoperiod (SD-R) exhibit a decrease in RFRP-ir neuron numbers relative to a line with a negligible reproductive response (SD-NR). Cell size and optical density analysis suggest that transcription/translation are impacted by reproductive genotype, as SD-NR mice had larger and more dense RFRP-ir neurons than SD-R mice in short days. Additionally, these results provide further support for a role of RFRP in mediating the effects of photoperiod on the reproductive axis. Interestingly, regardless of the genetic line to which a mouse belonged, exposure to "winter-like" photoperiods resulted in a significant reduction in the number RFRP-ir neurons detected in the DMH. This observation is consistent with the notion that photoperiod, mediated by melatonin, is likely the primary driver of the expression of this peptide, an observation that is consistent with the results of previous studies on RFRP in other seasonally breeding rodent species (Revel et al. 2008). Whether or not MEL acts directly on RFRP cells, or on systems upstream of RFRP requires that RFRP cells be examined for MEL receptors in this species.

As discussed previously, within-species, genetic variation in photoperiod responsiveness in this species is associated with variation in the number and function of GnRH neurons (Avigdor et al. 2005). Specifically, the non-responsive line that maintains reproductive competency all year has significantly more GnRH expressing cells in both long and short day lengths than the responsive line. Because neurons that secrete GnRH are the master regulators of pituitary gonadotropins and gonadal function, it is likely that variation in the number or morphological characteristics of these neurons is responsible for much of the disparity in reproductive condition between selected lines. Although GnRH is the master molecule regulating reproductive function in all vertebrates, GnRH neuron function is susceptible to regulation by an array of neurochemical and neuromodulatory factors that convey reproductively relevant information about internal and external conditions to be integrated by the final common GnRH pathway (Clarke and Pompolo 2005; Gore 2004). Therefore, a complete understanding of how selection acts to modify the GnRH system should explore the effect on upstream systems known modulate GnRH neural activity and function.

RFRP peptide levels are decreased in Syrian hamsters that are exposed to 8 weeks of short photoperiod, the time required to achieve full reproductive quiescence (Chapter 3). Likewise, RFRP gene transcription is also diminished after 8 weeks of short photoperiod exposure. By manipulating testosterone concentrations we determined that changes in RFRP were due to photoperiod and not changes in sex steroid levels. Our current results are consistent with findings in Syrian hamsters, with short photoperiod exposure leading to decreased RFRP peptide expression in both lines of mice. As indicated previously, data from Syrian hamsters suggests that RFRP initiates reproductive regression but is likely not required for its maintenance. The present results suggest that the level of GnRH present in nonresponsive mice cannot be suppressed by RFRP inhibition.

In summary, the present findings show that selection on an important life-history trait from a wild founder population of white-footed mice affects the number of RFRP expressing neurons in the brain. In nature, organisms are in a constant struggle to survive and reproduce

under changing environmental conditions. Successful adaptation to changing climates requires neuroendocrine and physiological systems that can process predictive cues in order to alter physiological state in advance of major seasonal transitions in climate. Genetic variability leading to phenotypic variation provides the raw material for natural selection to act upon. The present and previous studies on these artificially selected lines of white-footed mice provide evidence for heritable and selectable variation in a specific neuronal trait that manifests as variable reproductive phenotypes that would likely impact fitness in nature.

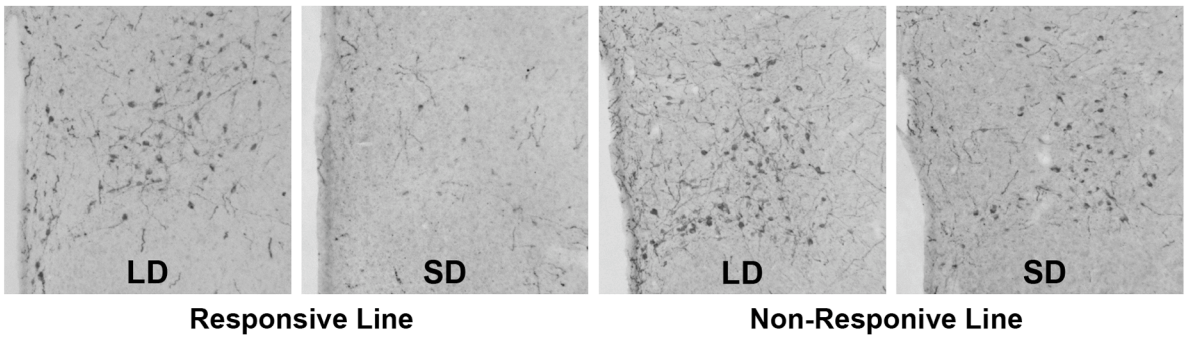


Figure 4.1

RFRP immunohistochemical labeling. Representative photomicrographs of RFRP-ir staining in the DMH of two genetic lines of mice, Responsive or Non-Responsive, held in long day (LD) or short day (SD) photoperiod for 8 weeks. Medium power (100x) photomicrographs are shown for each condition

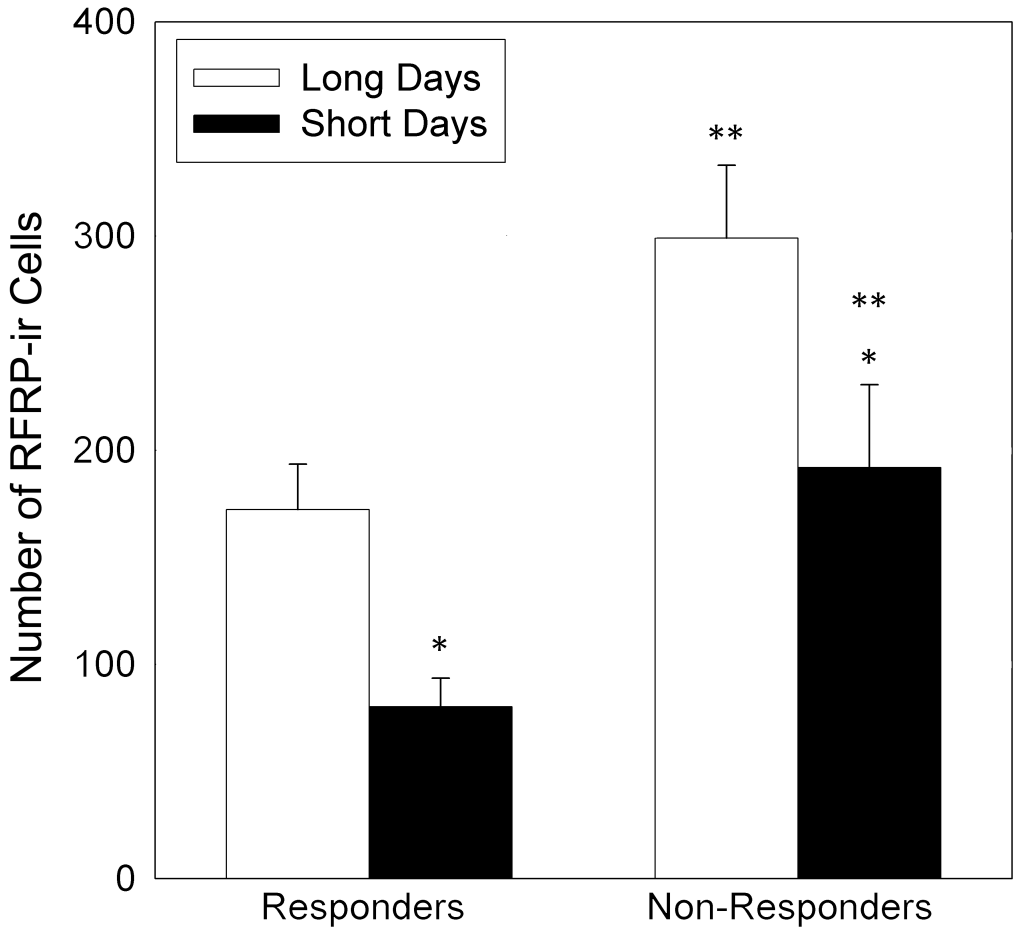


Figure 4.2

RFRP-ir cell numbers are affected by genetic line and photoperiod. Mean (±SEM) number of RFRP-ir cells of Responsive or Non-responsive lines of mice held in long or short days for 8 wk. Short day mice of both genetic lines display significantly fewer numbers of RFRP-ir cells than their long day counterparts. Responsive line exhibited significantly fewer numbers of RFRP-ir cells regardless of photoperiodic condition. *= $p<0.05$

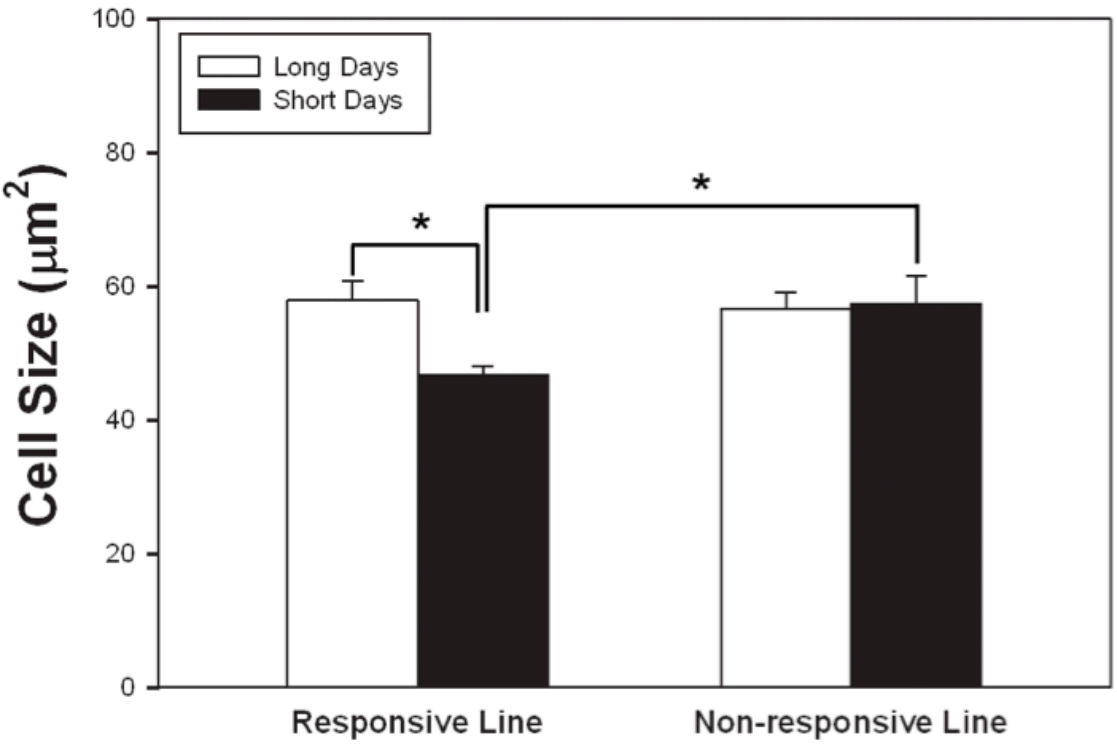


Figure 4.3

RFRP-ir cell size is non-systematically affected by genetic line and photoperiod. Mean (±SEM) size of RFRP-ir cells of Responsive or Non-responsive lines of mice held in long or short days for 8 wk. Within the Responsive line of mice, short days significantly reduced measure cell size of RFRP-ir neurons in the DMH compared to their long day counterparts. Within the short day condition, the Non-responsive line exhibited significantly larger RFRP-ir cells than the Responsive line. *= $p<0.05$

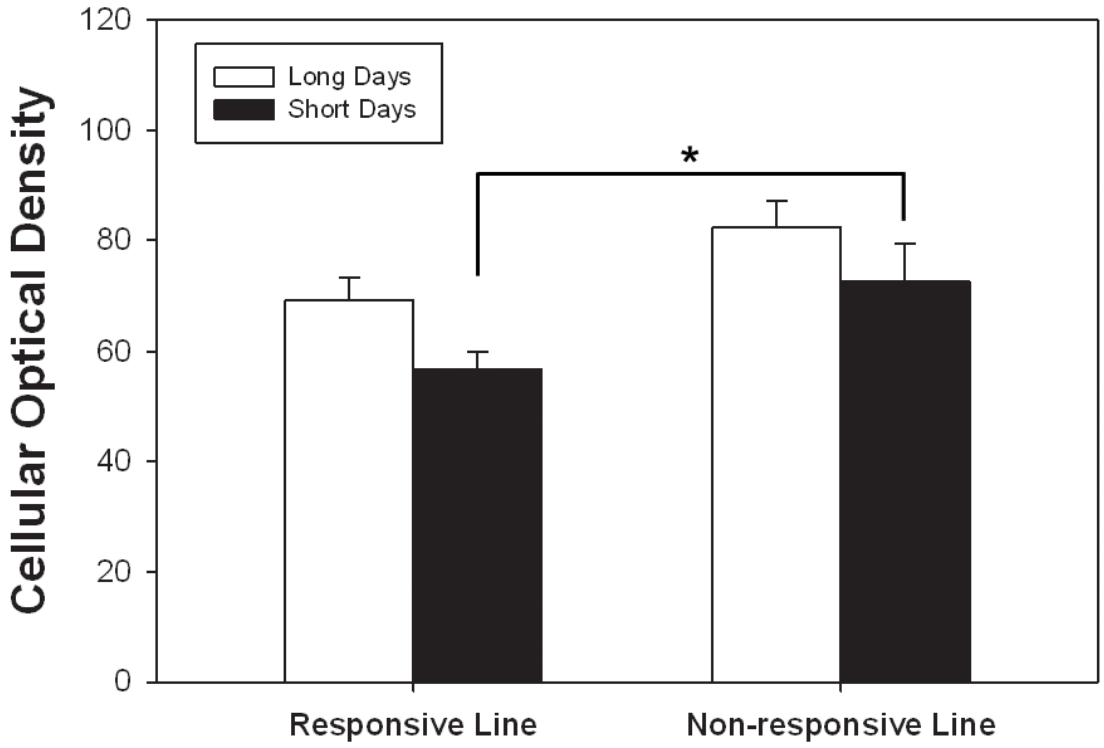


Figure 4.4

RFRP-ir cellular optical density (OD) is non-systematically affected by genetic line and photoperiod. Mean (±SEM) OD of RFRP-ir cells of Responsive or Non-responsive lines of mice held in long or short days for 8 wk. Within the short day condition, the Non-responsive line exhibited significantly more dense RFRP-ir cells than the Responsive line. *= $p<0.05$

CHAPTER 5

Environmental Control of Kisspeptin: Implications for Seasonal Reproduction

Introduction

To maximize reproductive success and avoid breeding during inappropriate conditions, animals must integrate neural signals that convey both external and internal status and appropriately alter the activity of the hypothalamo-pituitary-gonadal (HPG) axis. The final common pathway in which these stimuli are integrated to influence reproductive function is the GnRH neuronal system (Bronson 1989; Herbison 2006). The GnRH system has traditionally been considered the pinnacle of hierarchical control regulating downstream pituitary and gonadal function. Upstream mechanisms responsible for interpreting internal or external status and relaying this information to the GnRH system, however, remain largely unspecified.

Examining changes in neuropeptide levels in response to environmental stimuli in specific neuronal populations that mediate reproductive function can provide important insight into potential systems that act at the interface between the environment and the GnRH system. The previous three chapters described the results of experiments I, along with collaborators, have conducted on the RFRP/GnIH system, one specific neuropeptidergic population that is inhibitory to the reproductive axis. Another recently identified class of peptide hormones, kisspeptins, are the product of the antimetastatic *KiSS-1* gene, which encodes a large 145-amino acid chain that is subsequently enzymatically cleaved into shorter, biologically active peptides (*i.e*. kisspeptin-54, -14, -13, -10) (Kotani et al. 2001). These peptides are the natural ligands of the previously orphaned G protein-coupled receptor GPR54 (Kotani et al. 2001; Ohtaki et al. 2001) and exert a profound influence on the HPG axis (Funes et al. 2003; Shahab et al. 2005). Specifically, administration of exogenous kisspeptin leads to marked, dose-dependent increases in the gonadotropins LH and FSH across all mammalian species studied to date (Gottsch et al. 2004; Kaiser and Kuohung 2005; Navarro et al. 2004a; Navarro et al. 2005a; Navarro et al. 2005b; Shahab et al. 2005; Thompson et al. 2004), including humans (Dhillo et al. 2005). This response appears to be mediated via the actions of kisspeptin on the GnRH system, rather than a direct action on the pituitary; kisspeptin depolarizes GnRH neurons (Han et al. 2005), and gonadotropin release can be blocked by pretreatment with a GnRH antagonist (Gottsch et al. 2004; Irwig et al. 2004; Matsui et al. 2004; Shahab et al. 2005). Furthermore, treatment of pituitary tissues or cultured cells with kisspeptin *in vitro* fails to elicit gonadotropin release (Irwig et al. 2004; Thompson et al. 2004); but see (Navarro et al. 2005b). Within the brain, kisspeptin cell bodies are concentrated in the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei of the hypothalamus, with scattered cells in the periventricular and anterodorsal preoptic nuclei (Gottsch et al. 2004; Smith et al. 2006b; Smith et al. 2005). These regions likely play an important role in kisspeptin regulation of HPG activity and thus reproductive functions.

An ideal model system to investigate the mechanisms by which endogenous and exogenous stimuli impact the GnRH neuronal system is seasonally breeding rodents. Temperate zone rodents breed seasonally, restricting reproduction to the time of year when environmental conditions are optimal (*i.e*. spring/summer). Virtually all seasonally breeding rodents use

photoperiodic signals, which provide a noise-free cue to precisely time reproduction. Changes in photoperiod alter HPG axis activity and reproductive function, allowing reproduction to be coordinated with favorable ambient conditions (Bronson 1989; Dawson et al. 2001; Goldman 2001; Lincoln and Richardson 1998). Under controlled laboratory conditions, animals maintained on long summer-like photoperiods (>12 h light/d) remain reproductively active with fully functional gonads, whereas animals' maintained in short winter-like photoperiods (<12 h light/d) exhibit down-regulation of HPG axis activity and pronounced regression of the gonads and internal reproductive ducts (Goldman 2001).

For the present studies, I hypothesized that kisspeptin acts as a relay point for integrating and interpreting reproductively relevant stimuli, including photoperiod. In these experiments I used Siberian hamsters (*Phodopus sungorus*) to capitalize on several aspects of their reproductive physiology. As with other seasonally breeding rodents, Siberian hamsters display marked changes in reproductive physiology in response to changes in photoperiod (Goldman 2001). Furthermore, this species, like the Syrian hamsters and white-footed mice described in preceding chapters, displays the polymorphism in which a subset of individuals, called reproductive nonresponders, fails to respond to photoperiodic information and remain reproductively active despite exposure to short days (Kliman and Lynch 1992; Prendergast et al. 2001); the remaining animals, in contrast, display the typical gonadal regression in response to short days. This differential response to short days provides a powerful tool to assess how the same environmental stimuli can be differentially interpreted by the central nervous system and relayed to the reproductive axis. The goal of the present study was to determine the role of kisspeptin in mediating the pronounced changes in reproductive state observed in seasonal breeders exposed to differing photoperiodic stimuli. I previously reported that kisspeptin staining in the AVPV is significantly reduced in short-day, compared with long-day hamsters. Based on these initial observations, my specific hypothesis was that expression of the neuropeptide kisspeptin in the hypothalamus would change in response to photoperiod and that these changes would track the reproductive state of the animal. In addition, I hypothesized that exogenous kisspeptin would stimulate the HPG axis regardless of photoperiod treatment. Collectively, these data will elucidate a possible key role for kisspeptin in mediating reproductive responses to relevant environmental stimuli.

Materials and Methods

Animals and housing

Adult (>60 d of age) male Siberian hamsters (*Phodopus sungorus*) (n = 56) were obtained from the breeding colony maintained at Indiana University. All animals were group housed at weaning with same-sex siblings in a long-day photoperiod (light-dark 16:8). Before the start of the study, animals were housed individually in polypropylene cages (27.8 x 7.5 x 13.0 cm) for 1 wk and then placed in either a long- (16:8) or short-day (8:16) photoperiod. Temperature was kept constant at 20 ± 2 C and relative humidity was maintained at 50 ± 5%. Food (rat chow; Purina, St. Louis, MO) and tap water were available *ad libitum* throughout the experiments.

Experiment 1: effects of photoperiod and reproductive state on kisspeptin neurons

Hamsters were held for either 2 or 8 wk in long- (2 wk n = 5; 8 wk n = 5) or short-day (2 wk n = 5; 8 wk n = 10) photoperiods. These two time points were chosen because animals responsive to short days display fully regressed gonads and basal sex steroid levels by 8 wk in photoperiod. An additional time point was included at 2 wk of short day exposure, because gonadal regression has not yet occurred and circulating testosterone remains elevated (Demas, G. E., A. Lutz, and D. A. Zysling, unpublished data). This allowed for the capture of any dynamic changes in kisspeptin labeling that may occur before full reproductive regression.

Perfusions and tissue preparation

At the conclusion of the experiment, hamsters were weighed to the nearest 0.1 g and then deeply anesthetized with 0.3 ml of a ketamine (20 mg/ml)/xylazine (4 mg/ml) cocktail in 0.9% saline and perfused transcardially with 50 ml of 0.9% saline, followed by 100–150 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.3). Brains were postfixed for 3 h at room temperature in 4% paraformaldehyde and cryoprotected in 20% sucrose in 0.1 M PBS and stored at 4 C until processed. Coronal sections (40 μm) were cut on a cryostat and processed as free-floating sections beginning rostrally at the medial septum/diagonal band of Broca and extending caudally to the brain stem.

Necropsies were performed and paired testes were collected, cleaned of fat and connective tissue and weighed. Animals that, after 8 wk in short days, had paired testes weighing more than 0.15 g (n = 6, mean = 0.65 ± 0.12 g) were classified as short-day nonresponders; animals with paired testes weighing < 0.15 g (n = 4, mean = 0.07 ± 0.03 g) were classified as short-day responders.

Antibody characterization and immunohistochemistry

Kisspeptin-immunoreactive (ir) cells were labeled using a rabbit antihuman kisspeptin serum (T-4771; Peninsula Laboratories Inc., Bachem, San Carlos, CA) raised against the following amino acids Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2, corresponding to amino acids 4–13, diluted at 1:7500. In preliminary trial runs, nonspecific staining strikingly similar to the distribution of gonadotropin inhibitory hormone (GnIH) peptide and mRNA was noted in the dorsomedial hypothalamus (DMH) whereas labeling in the AVPV and ARC resembled that of kisspeptin mRNA across species (Franceschini et al. 2006; Gottsch et al. 2004; Kriegsfeld et al. 2006; Smith et al. 2006b; Smith et al. 2005). This nonspecificity likely resulted from the fact that kisspeptin and GnIH share common amino acids at their C terminus (see (Kriegsfeld 2006)for review). Double-label immunohistochemistry using anti-Syrian hamster GnIH (PAC1365) and kisspeptin antisera resulted in colabeling of all cells in the DMH, whereas cells in the AVPV and ARC remained single labeled for kisspeptin only (Fig. 5.1). To eliminate potential GnIH staining, we preadsorbed the kisspeptin antiserum with GnIH peptide (generous gift of Dr. George Bentley, University of California, Berkeley, Berkeley, CA) for 24 h at 4 C before application. This procedure eliminated the DMH population of cells, whereas maintaining the AVPV and ARC populations in all cases (Fig. 5.1). Preadsorption with both GnIH and kisspeptin eliminated all staining.

We further confirmed the specificity of Bachem T-4771 (preadsorbed with GnIH) by double-labeling tissue with T-4771 and a rabbit antihuman kisspeptin antibody raised against amino acids 43–52 (a generous gift of Dr. Alain Caraty and Dr. Isabelle Brailiou, UniversityTours/Haras Nationaux, Nouzilly, France). This second antibody has been previously validated to show high specificity for kisspeptin (Franceschini et al. 2006). Antisera were detected using two biotinylated goat antirabbit secondary antibodies (CY2-T4771 and CY3-Caraty antibody; Vector Laboratories Inc., Burlingame, CA). The dilution of T-4771 was 10-fold greater (1:7500) than that which is optimal for direct immunohistochemistry (*i.e.* 1:750). This dilution prevented the second, secondary antibody from nonspecifically binding to the first primary. Amplification of the T4771 signal was accomplished by using a modified biotinylated tyramide procedure previously described (Kriegsfeld et al. 2004). The antibody provided by Dr. Caraty and colleagues was directly labeled using a CY3 goat antirabbit secondary (Vector Laboratories). All double-label experiments using this procedure resulted in 100% colabeling of cells in the AVPV and ARC (Fig. 5.2). To confirm that this procedure was effective at preventing nonspecific labeling with the second rabbit antibody, the second kisspeptin antibody (*i.e.* provided by Dr. Caraty and colleagues) was eliminated and all other procedures implemented. In these controls trials, CY3 did not label T4771-ir neurons. Sections were mounted onto gelatin-coated slides, dehydrated in a graded series of ethanol solutions (70, 95, and 100%), and cleared in xylenes (Fisher Scientific, Hanover Park, IL) before the application of coverslips. Brains were processed immunohistochemically in three separate immunohistochemical runs (n = 10, 10, and 5 brains per assay). Variability between immunohistochemical replications was controlled by having an equal number of animals for each group in each run of immunohistochemistry. For each run, incubation times for every procedure were strictly controlled.

Microscopy, cell counts, and OD

Slides were examined under bright field illumination on a Zeiss Z1 microscope by independent observers naïve to the experimental conditions. Kisspeptin-ir cells were located by visually scanning the brains under x200 magnification. Cell populations were restricted to the AVPV region of the preoptic area and ARC. All cells were confirmed at a minimum of x400. Counted cells were photographed with a Axiocam Cooled CCD camera (Zeiss, New York, NY) at x400 magnification for cell size and density analyses. All cells in every fourth section were counted through the rostrocaudal extent of the AVPV and ARC. For all animals this resulted in counting three sections through the AVPV and eight sections through the ARC. Both cells with a clearly discernable nucleus and cells showing clear soma and processes without a clear, unstained nucleus were counted. Because the inclusion of cells without a clearly defined nucleus may result in counting overestimates, an Abercrombie correction was applied before data analysis.

Soma size and OD measurements were performed on images captured at x400. All cells examined had ODs at least 2 SD above the mean background OD measures for an individual brain. Cell bodies were outlined and the two-dimensional area was calculated using Image J v1.32. Each pixel in the gray-scale image capture has a measurable specific intensity, with values ranging from 0 (white) to 256 (black). The average value for all pixels in an outlined area is taken as the mean intensity of staining for a given region of the image. OD measures were normalized

to minimize differences between replications of immunohistochemistry. First, a background measurement was taken by placing a square outline, four times, on nonoverlapping, unstained areas of each section. The mean of these four measures provided the background OD for each section. The OD for each cell body was assessed by outlining the cell body, obtaining a density measure using Image J, and subtracting the background OD from the OD of each cell.

Experiment 2: endocrine response to exogenous kisspeptin

Hamsters were held in long- (n = 11) or short-day (n = 19) photoperiods for 8 wk before kisspeptin injections. Hamsters were injected with kisspeptin-10 [KiSS-1 (112–121)/metastin (45–54) (human); Phoenix Pharmaceuticals, Inc., Belmont, CA], a commercially available product with known ability to stimulate the HPG axis (Gottsch et al. 2004), or a 0.1 M PBS vehicle injection based on a previously published protocol (Messager et al. 2005). Briefly, an initial blood sample was drawn from all hamsters via the retroorbital sinus to measure baseline hormone levels. Next, long- and short-day hamsters received ip injections of either 100 µl PBS (long day: n = 5; short day: n = 10) or 100 µl of a PBS solution containing 10 µM kisspeptin-10 (Phoenix Pharmaceuticals) (long day: n = 6; short day: n = 9) every 30 min for a total of four injections. Thirty minutes after the last injection, all hamsters were again bled. The injection protocol, described in more detail elsewhere (Messager et al. 2005), was chosen because it previously demonstrated the ability to elicit a significant increase in serum LH levels in mice, a similarly sized rodent to Siberian hamsters (Messager et al. 2005). Blood was centrifuged at 2500 rpm for 30 min, and serum was collected and stored at –80 C until assayed for hormones.

After the last blood sample was collected, necropsies were preformed and paired testes were removed and weighed. Animals were categorized *post hoc* as either short-day responders or nonresponders based on paired testes mass as described in experiment 1. One short-day animal that received kisspeptin injections displayed the nonresponsive phenotype (gonadal mass > 0.15 g), whereas five animals that received PBS injections displayed this phenotype (gonadal mass > 0.15 g), leaving five short-day responders injected with vehicle and eight short-day responders injected with kisspeptin.

Hormone measurements

Serum LH concentrations were measured in duplicate via a single RIA with reagents obtained from the National Institutes of Health based on a previous protocol (Chappell et al. 1997). 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 2.9% for the low pool and 8.5% for the high pool. Serum testosterone was measured from samples with adequate serum after LH analysis (long day kisspeptin n = 6; long day vehicle n = 5; short day kisspeptin n = 4; short day vehicle n = 6) via a commercial enzyme immunoassay kit (Correlate-EIA kit no. 900–065; Assay Designs, Ann Arbor, MI). Serum samples were diluted 1:20 and run in duplicate for each sample. The sensitivity of the assay was 3.82 pg/ml, the intraassay coefficient of variation was 9.2%, and the interassay of variation was 2.14%. The antisera used in both assays were highly specific for the hormones measured, with low cross-reactivity with other hormones. Both the LH and testosterone assays have been previously validated for use in Siberian hamsters (Demas et al. 2004; Wolfe et al. 1995).

Statistical analyses

Data in experiment 1 were grouped according to the photoperiod, duration in photoperiod, and reproductive state, yielding five groups: long days/2 wk, long days/8 wk, short days/2 wk, short days/8 wk (responders), and short days/8 wk (nonresponders). The effects of photoperiod and reproductive state on body and gonadal masses as well as kisspeptin-ir neuron number, size, and OD were each analyzed in separate one-way ANOVAs. Pair-wise comparisons were probed with Tukey's *post hoc* tests when the overall ANOVA was significant.

In experiment 2, only one short-day, nonresponsive morph received kisspeptin injections (five received vehicle); thus, statistical comparisons between responsive and nonresponsive morphs were not possible. As such, all nonresponsive animals (n = 6) were removed from subsequent analysis. The effects of photoperiod on body and gonadal mass and baseline levels of the hormones LH and natural log (ln) testosterone were assessed using a one-way ANOVA; testosterone levels were natural log transformed to meet the parametric assumption of equal variance. The effects of peripheral kisspeptin injections on LH and testosterone were each analyzed using separate repeated-measures ANOVAs, with pre- and posthormone levels as the within-subjects factor and photoperiod and injection as the between-subject factors. In all cases, differences were considered statistically significant if $P < 0.05$. All analyses were performed using SPSS 14 for Windows (SPSS, Inc., Chicago, IL).

Results

Experiment 1: effects of photoperiod and reproductive state on kisspeptin neurons

Consistent with other rodent species, kisspeptin-ir neurons were concentrated in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) (Fig. 5.3). Photoperiod and reproductive state significantly affected the number ($F_{4,20} = 3.989$, $P = 0.015$) and size ($F_{4,20} = 3.819$, $P = 0.018$) but not OD ($P > 0.1$) of kisspeptin-ir neurons in the AVPV (Fig. 5.4). Animals that regressed their gonads in response to 8 wk of short-day photoperiod displayed significantly fewer ($P < 0.05$) and smaller ($P < 0.05$) kisspeptin-ir neurons than all other groups; all other groups displayed a similar number and size of kisspeptin-ir neurons ($P > 0.05$) (Fig. 5.4).

The number of kisspeptin-ir neurons in the ARC was significantly affected by photoperiod and reproductive state ($F_{4,20} = 9.144$, $P < 0.001$) (Fig. 5.5), with animals reproductively responsive to 8 wk of short-day photoperiod (*i.e.* regressed gonads) displaying significantly more kisspeptin-ir neurons than all other groups ($P < 0.05$). Sixty percent of hamsters with regressed gonads had the most robust increase in ARC kisspeptin-ir cells labeling, whereas a modest increase was seen in the remaining 40% of hamsters. In sharp contrast to reproductively competent hamsters that had between 0 and 3 labeled cells in ARC, 100% of animals with regressed reproductive axes had cell counts between 20 and 65. Groups with functional gonads did not differ in the number of kisspeptin-ir neurons observed in the ARC ($P > 0.05$) with a mean of 2.3 ± 0.38 cells across conditions. Because of the extremely small number of ARC cells labeled for kisspeptin in reproductively competent animals, OD and cell size measures were not taken in this brain region because these results may be misleading due to measurement of one to two cells in most animals.

Photoperiod significantly affected gonadal ($F_{4,20} = 13.76$, $P < 0.001$) (Fig. 5.5B) and body mass ($F_{4,20} = 9.798$, $P < 0.001$), with responsive animals held on short days for 8 wk having significantly smaller testes ($P < 0.001$ in all cases) and lower mass ($P < 0.02$ in all cases); no difference was observed between the other groups ($P > 0.05$ in all cases).

Experiment 2: endocrine response to exogenous kisspeptin

Animals held in long days for 8 wk had significantly heavier paired testes ($F_{1,20} = 411.59$, $P < 0.001$), higher body mass (long day: 42.1 ± 1.8; short day: 32.7 ± 1.06) ($F_{1,20} = 18.98$, $P < 0.001$) and higher levels of testosterone (T) ($F_{1,16} = 4.67$, $P = 0.046$), compared with animals held on short days (Fig. 5.6). Baseline LH levels were not affected by photoperiod ($P > 0.05$).

Animals that received injections of kisspeptin displayed significantly elevated LH levels, compared with animals receiving PBS ($F_{1,23} = 19.04$, $P < 0.001$) (Fig. 5.6), regardless of photoperiod. Photoperiod treatment had no main effect on LH level ($P > 0.05$), and there was no interaction between photoperiod and kisspeptin treatment ($P > 0.05$).

Kisspeptin, compared with vehicle, significantly elevated levels of testosterone in long- but not short-day animals (photoperiod * injection; $F_{1,14} = 5.44$, $P = 0.035$) (Fig. 5.6). There was a main effect of photoperiod on testosterone ($F_{1,14} = 16.18$, $P = 0.001$), with long-day animals displaying higher levels of testosterone than short-day animals. There was no effect of injection treatment on testosterone ($P = 0.06$) because short-day animals displayed no elevation of testosterone.

Discussion

The results of the present study demonstrate an important role for the peptide kisspeptin in the interpretation of environmental information and subsequent regulation of the neuroendocrine reproductive axis. Furthermore, the differences observed in the staining patterns between animals exhibiting a polymorphism in reproductive responsiveness to short-day lengths demonstrates a role for kisspeptin in driving the disparate response of the reproductive system to the same reproductively relevant stimulus, namely photoperiod. Specifically, in experiment 1, kisspeptin-ir neurons in the ARC and AVPV tracked reproductive state, with significantly more staining in the AVPV of animals reproductively active, compared with those with regressed gonads. Interestingly, the ARC kisspeptin cell population exhibited the opposite pattern of staining with regressed animals showing robust cell staining that was virtually absent in animals with active reproductive systems. In experiment 2, peripheral injections of exogenous kisspeptin resulted in robust increases in gonadotropin release in both long- and short-day hamsters, demonstrating that the GnRH system remains sensitive to kisspeptin regardless of reproductive condition. These results suggest that differential release of kisspeptin from the hypothalamus in response to differing day lengths mediates reproductive physiology in seasonally breeding animals.

As mentioned previously, kisspeptin staining in the hypothalamus (AVPV and ARC) was altered in response to photoperiodic treatment and reproductive state. This observed pattern may be regulated directly via photoperiodic signals (*i.e.* the duration of melatonin secretion) or may be

the result of changes in circulating sex steroids due to photoperiod-induced changes in gonadal morphology (*e.g.* regressed gonads in short day responsive animals). Patterns of melatonin secretion are known to influence reproductive state in many seasonal rodents, including Siberian hamsters (Goldman 2001). The nonresponsive morph of Siberian hamsters produces the same melatonin signal as a long-day hamster, causing these hamsters to code for long days, even while in short-day photoperiods (Prendergast et al. 2001). The observation that short-day nonresponsive hamsters displayed the same pattern of brain kisspeptin expression as long-day hamsters supports a possible mechanistic role for melatonin in the regulation of kisspeptin expression. Similar staining patterns as those observed in the present studies have been reported previously for *KiSS-1* mRNA expression; gonadectomized mice have low *KiSS-1* expression in the AVPV but high expression in the ARC, whereas testosterone replacement results in the opposite expression pattern (Smith et al. 2005). Additionally, *KiSS-1* neurons express both androgen and estrogen receptors (Smith et al. 2005) suggesting that kisspeptin may be responding to photoperiodic changes in sex steroids. Future studies examining melatonin receptor expression in kisspeptin cells along with manipulations of melatonin, photoperiod, and gonadal steroids will address the relative contribution of these potential regulatory factors to hypothalamic kisspeptin expression.

The two hypothalamic nuclei staining positively for kisspeptin, the AVPV and ARC, contain neurons projecting to the medial preoptic area, a brain region containing GnRH cell bodies (Hahn and Coen 2006). Furthermore, more than half of GnRH neurons express mRNA for the kisspeptin receptor, *GPR54* (Han et al. 2005; Messager et al. 2005). The specific contribution of AVPV *vs*. ARC kisspeptin neurons in regulating GnRH cell function remains to be determined. It has been suggested that the opposing peptide expression patterns observed between the AVPV and ARC (Smith et al. 2006) and in the present study may participate in positive and negative feedback, respectively (Smith et al. 2006). It is noteworthy, however, that a peptide able to potently stimulate the HPG axis is expressed in high concentrations in the ARC in nonreproductive animals, a finding incompatible with a stimulatory action of ARC kisspeptin on the HPG axis. It remains possible that, whereas the kisspeptin neurons in the AVPV may act as a potent stimulator of the HPG axis, the kisspeptin neurons within the ARC may instead serve other, yet-unidentified neuromodulatory functions unrelated to reproduction. Alternatively, increased kisspeptin-ir labeling in the ARC may be the result of inhibited peptide release in this brain region, allowing greater immunodetection. Recent evidence in goats and rats has suggested a role in modulating the GnRH pulse generator frequency by the ARC population of kisspeptin neurons (Li et al. 2009; Ohkura et al. 2009). Additionally, new work in rats has suggested that ARC kisspeptin neurons stimulate prolactin secretion through direct inhibitory contacts upon dopaminergic neurons that are the principal inhibitory regulators of prolactin secretion from the pituitary (Szawka et al. 2010). These findings are particularly intriguing, considering the well characterized seasonal regulation of prolactin secretion in many seasonal breeders; however, prolactin is markedly suppressed during winter which would suggest that kisspeptin in the ARC should be decreased if it is providing stimulatory input to these neurons. Despite these new developments, the fact remains that further research aimed at determining the precise neuroendocrine functions of kisspeptin within these two brain regions will help to select among these hypotheses.

As with kisspeptin staining in the ARC, mRNA expression for *Vgf* mRNA in the ARC is greater in short- compared with long-day animals (Ross et al. 2005). The function of this gene is still unknown, but it has been implicated in energy balance (Hahm et al. 1999). Kisspeptin neurons in the ARC respond to signals of energy availability and balance (Castellano et al. 2005; Navarro et al. 2004b; Smith et al. 2006a), and Siberian hamsters are typically used as models for studies of energy balance because they exhibit marked seasonal changes in food intake and metabolism (Wade and Bartness 1984). Given the pronounced role of the ARC in feeding regulation, along with seasonal changes in energy balance in Siberian hamsters, it is possible that kisspeptin neurons in the ARC are altered in response to energy status in addition to modulation through negative feedback in response to sex steroids (Smith et al. 2005) and photoperiod. Interestingly, a previous report (Pompolo et al. 2006) has demonstrated kisspeptin staining in sheep in brain regions comparable with those seen in Siberian hamsters (*e.g.* the ARC and periventricular nuclei). Sheep, like hamsters, are a seasonally breeding species. In contrast to hamsters, however, sheep are short-day breeders, restricting reproduction to the short days of winter and inhibiting reproduction during long day lengths. A recent study investigated the the effect of photoperiod on kisspeptin and RFRP cell body and fiber staining as well as terminal contacts upon GnRH neurons in ewes (Smith et al. 2008). It was found that kisspeptin and *Kiss1* mRNA levels were elevated in the ARC during short days and that RFRP levels were decreased by the same photoperiod exposure that is typical of the breeding season. Additionally, kisspeptin-ir fiber contacts were increased with a concomitant RFRP-ir fiber contact reduction upon GnRH cell bodies. These results strongly support a role of kisspeptin in the seasonal regulation of reproduction in sheep, pointing to a possible conserved role in all seasonally breeding mammals. It is interesting that the staining pattern of ARC kisspeptin in ewes in response to photoperiodic manipulations was the same as those seen in the long-day breeding Siberian hamsters of the present study despite the fact that the reproductive states of the two species are in completely opposite conditions. This finding suggests that the involvement of kisspeptin in the control of seasonal reproduction may be common among diverse mammalian species, but the precise function that it exerts may have been modified.

All individuals, regardless of photoperiod or reproductive state, displayed significant elevation in LH in response to peripheral injections of kisspeptin, demonstrating that animals are able to respond to the peptide regardless of photoperiodic signal. In addition, animals with functional gonads exhibited a robust increase in testosterone in response to exogenous kisspeptin, presumably stimulated by the observed elevated LH response to kisspeptin. In the current study, only one dose of kisspeptin (*i.e.* 10 μM/injection across four injections) was used in a manner known to elicit a significant LH surge in mice (Messager et al. 2005). Although both long-day and short-day-responsive animals displayed a comparably robust response to kisspeptin administration in the current study, the dose of kisspeptin used may have been sufficiently high to mask potential subtle differences in hypothalamic sensitivity to the peptide. Future investigations using a range of kisspeptin doses will allow a more direct examination of this possibility. Despite these potential subtle alterations in responsiveness to kisspeptin, the present results demonstrate that short-day animals are capable of activating the hypothamo-pituitary system in response to a kisspeptin signal.

Both long-day and short-day animals displayed elevated LH levels in response to kisspeptin. However, short-day-responsive animals did not alter serum testosterone

concentrations. The fact that kisspeptin administration does not increase testosterone in short-day responders is likely due to the regressed, nonfunctional state of the testes in these animals. Whether this is driven by a reduction in LH receptors or a lack of functional Leydig cells remains to be determined. Because kisspeptin has previously been shown to act at the level of the hypothalamus, and not the pituitary, to directly stimulate pituitary release of LH (Matsui et al. 2004; Thompson et al. 2004) but see (Navarro et al. 2005b), the peripheral injections used in the present study likely exerted their effects centrally. In seasonally breeding rodents, short-day lengths result in a marked down-regulation of the HPG axis and subsequent gonadal regression, whereas exposure to long days induces an up-regulation of the HPG axis followed by gonadal recrudescence. These changes result from actions upstream of the pituitary, as GnRH injections stimulate pituitary LH and FSH to a comparable degree in short-day, regressed and long-day, reproductively competent animals (Kriegsfeld et al. 1999; Pickard and Silverman 1979; Turek et al. 1977; Wingfield et al. 1979). Whereas the pituitary response to GnRH is not altered by photoperiod or reproductive state, GnRH release is markedly reduced in animals with regressed reproductive gonads (Caillol et al. 1998; Kriegsfeld et al. 1999; Pickard and Silverman 1979). In the present study, alterations in kisspeptin staining, combined with our results demonstrating comparable LH responses to exogenous kisspeptin in long- and short-day animals, indicate that kisspeptin is likely driving seasonal changes in GnRH. Furthermore, these data uncover a novel upstream mechanism of GnRH regulation whereby environmental factors can be interpreted, integrated, and relayed to the GnRH system.

The results of the present study demonstrate that the investigation of photoperiodic polymorphisms can provide a powerful tool for understanding how kisspeptin affects the HPG axis independent of photoperiod. Kisspeptin-ir expression in short-day nonresponders did not differ from long-day hamsters in any of the measurements. Interestingly, although the subset of kisspeptin injected animals used in experiment 2 included only one short-day nonresponsive animal (making statistical comparisons impossible), LH and testosterone levels in response to kisspeptin injections in this animal displayed the same pattern of values observed in long-day animals (baseline LH = 0.68 ng/ml, postinjection LH = 11.60 ng/ml; baseline T = 5.00 ng/ml, postinjection testosterone = 33.92 ng/ml). Collectively, these results indicate that kisspeptin may provide a mechanism for differential interpretation and response to the same reproductively relevant stimuli and lends insight into the neural mechanisms mediating individual differences in reproductive regulation. For example, the nonresponsive phenotype may fail to inhibit hypothalamic kisspeptin synthesis in the AVPV, leaving high levels of the peptide available for continued stimulation of the GnRH neuronal system. Although our data provide both morphological (kisspeptin staining in the AVPV) and functional (HPG response to kisspeptin) support for this hypothesis, future studies are necessary to directly examine this possibility.

The combined results of this investigation provide the first evidence for an important regulatory role of kisspeptin in mediating reproductive consequences resulting from exposure to reproductively relevant stimuli. In addition, these findings indicate an important role for kisspeptin in mediating seasonal changes in reproductive function and individual differences in responsiveness to seasonal information. Seasonally breeding species serve as an important tool with which to explore the role of kisspeptin in mediating reproductive consequences in response to a wide range of environmental factors including the social environment, disease states, and energy availability.

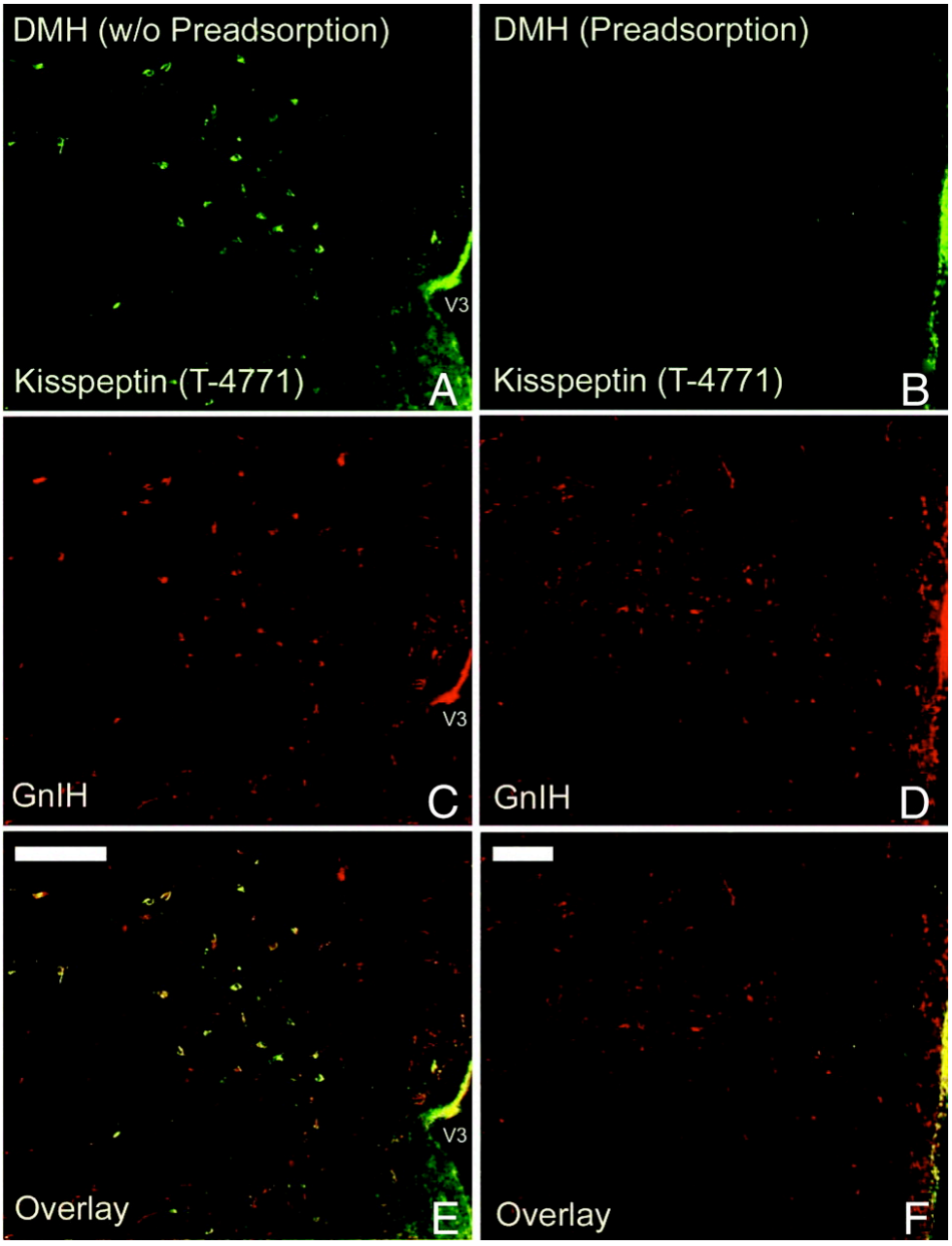


Figure 5.1

Elimination of nonspecific GnIH staining with peptide preadsorption. Medium-power photomicrographs showing putative GnIH neurons in the DMH staining positively using the Bachem kisspeptin antiserum (A). GnIH staining confirms that the same neurons labeled using the kisspeptin antiserum are colabeled for GnIH in single- (C) and double-label (E) images. Putative GnIH labeling using the kisspeptin antiserum is abolished after GnIH peptide preadsorption (B). GnIH staining in the same tissue confirms that the kisspeptin antibody does not label GnIH cells after GnIH peptide preadsorption (D and F). *Scale bars*, 100 µM.

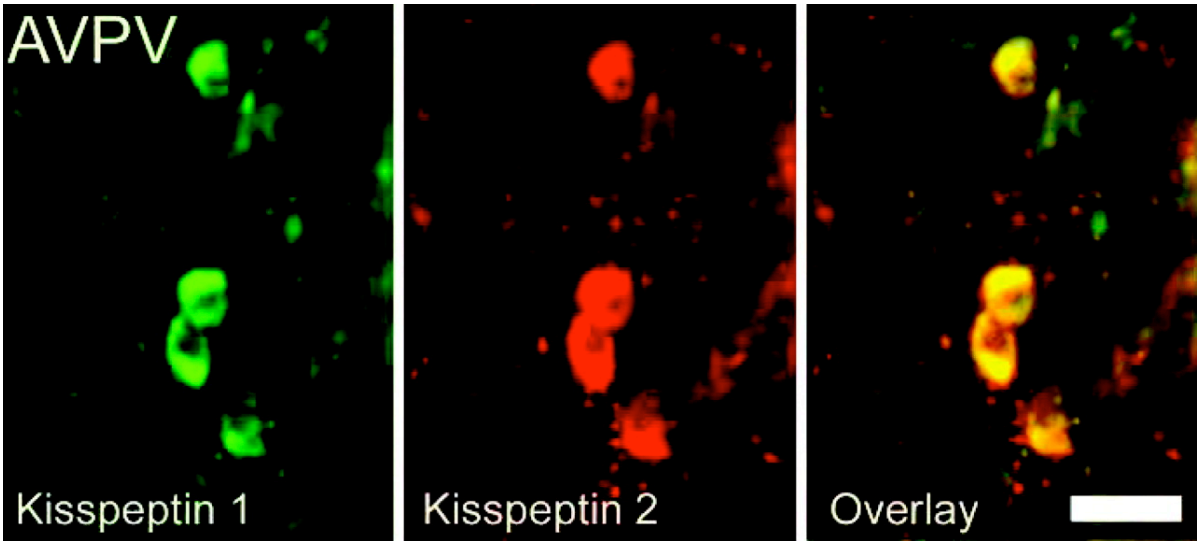


Figure 5.2

Kisspeptin staining in AVPV with two kisspeptin antibodies. Representative photomicrographs of neurons staining for kisspeptin using the Bachem kisspeptin antibody (kisspeptin 1) preadsorbed with GnIH peptide, the kisspeptin antibody kindly provided by Dr. Alain Caraty and Dr. Isabelle Brailiou (kisspeptin 2). *Scale bar*, 25 μM. Both antibodies label 100% of the same cells in the AVPV (shown here) and the Arc (data not shown).

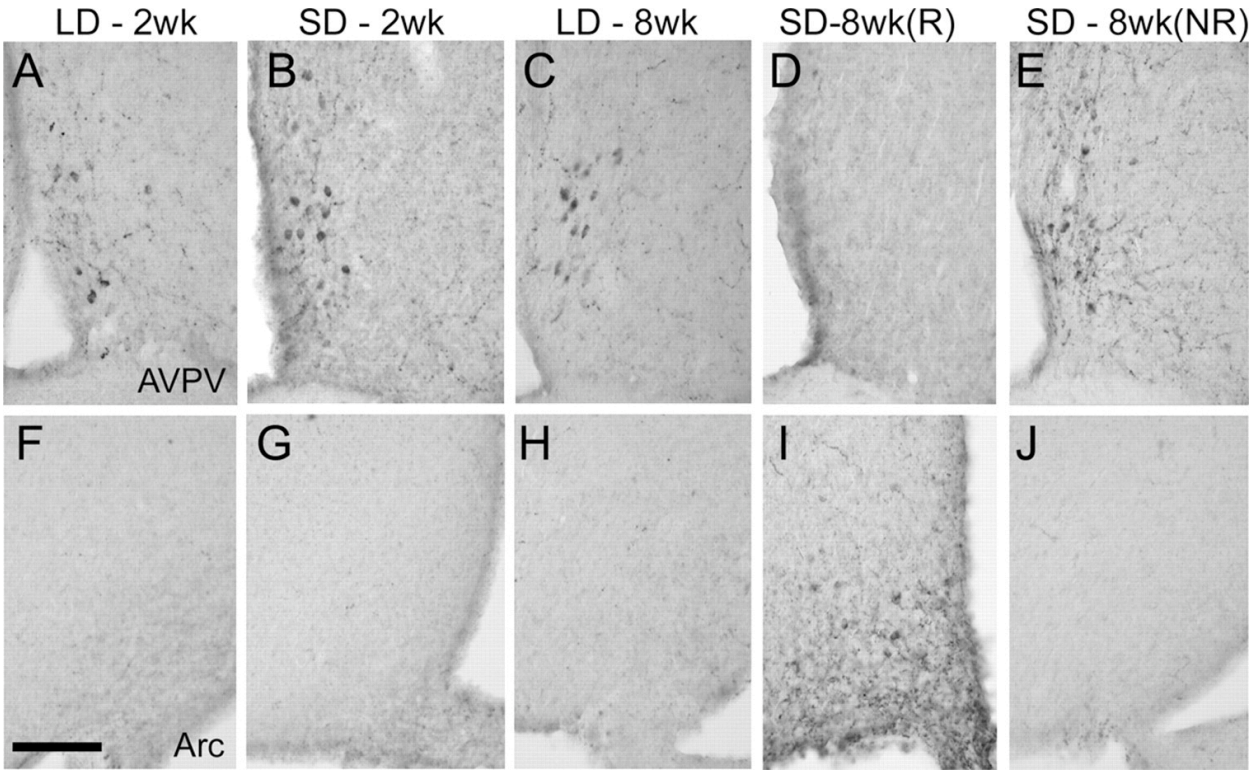


Figure 5.3

Response of kisspeptin-ir neurons to photoperiodic treatment. Photomicrographs of kisspeptin-ir neurons in the AVPV (A–E) and the ARC (F–J) in the brains of animals held in long-day photoperiod either 2 (LD-2wk) or 8 wk (LD-8wk), or short-day photoperiod for 2 wk (SD-2wk), and short-day-responsive [SD-8wk (R)] and nonresponsive [SD-8 wk (NR)] animals held on short-day photoperiod for 8 wk. *Scale bar*, 100 μM. Animals responsive to short-day photoperiod [SD-8wk (R)] display a marked reduction in kisspeptin-ir neurons in the AVPV and a significant increase in the ARC, compared with all other groups.

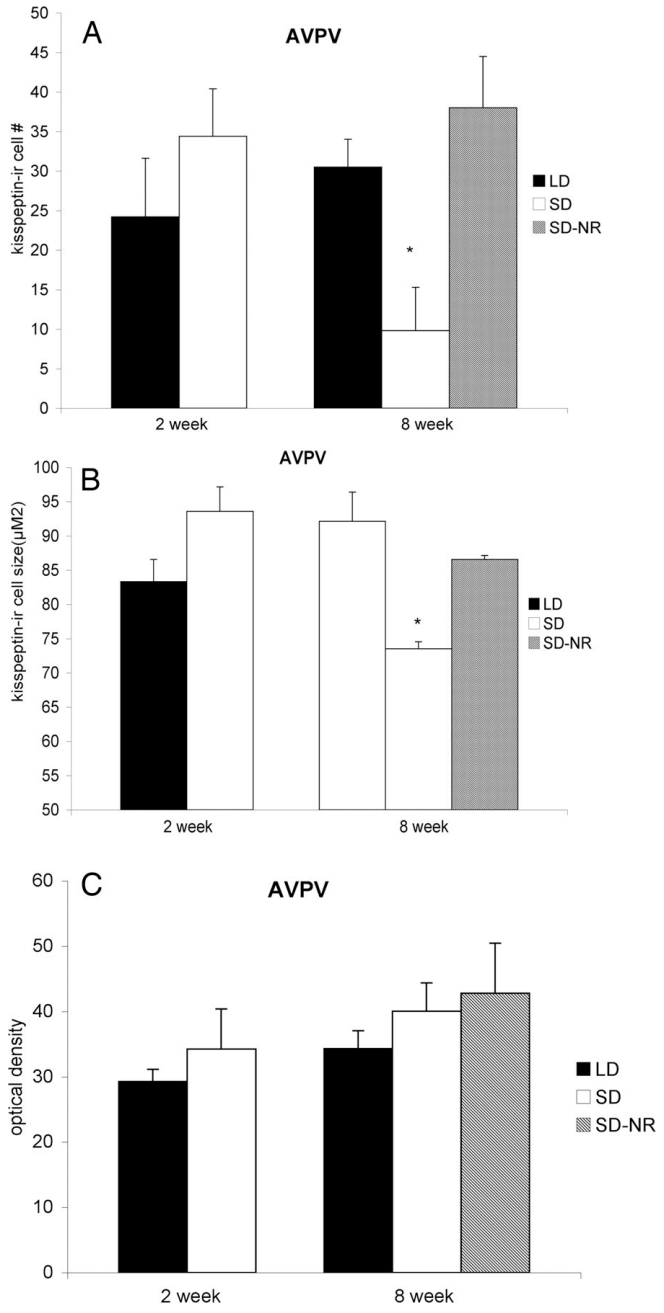


Figure 5.4

Effects of photoperiod on AVPV kisspeptin-ir neurons. Short-day-responsive animals (SD-R) held 8 wk on short days display significantly fewer numbers of cells (A) and smaller kisspeptin-ir neurons (B) in the AVPV; no change in OD was observed (C). An *asterisk* denotes $P < 0.05$. LD, Long day; SD, short day; SD-NR, short-day nonresponsive.

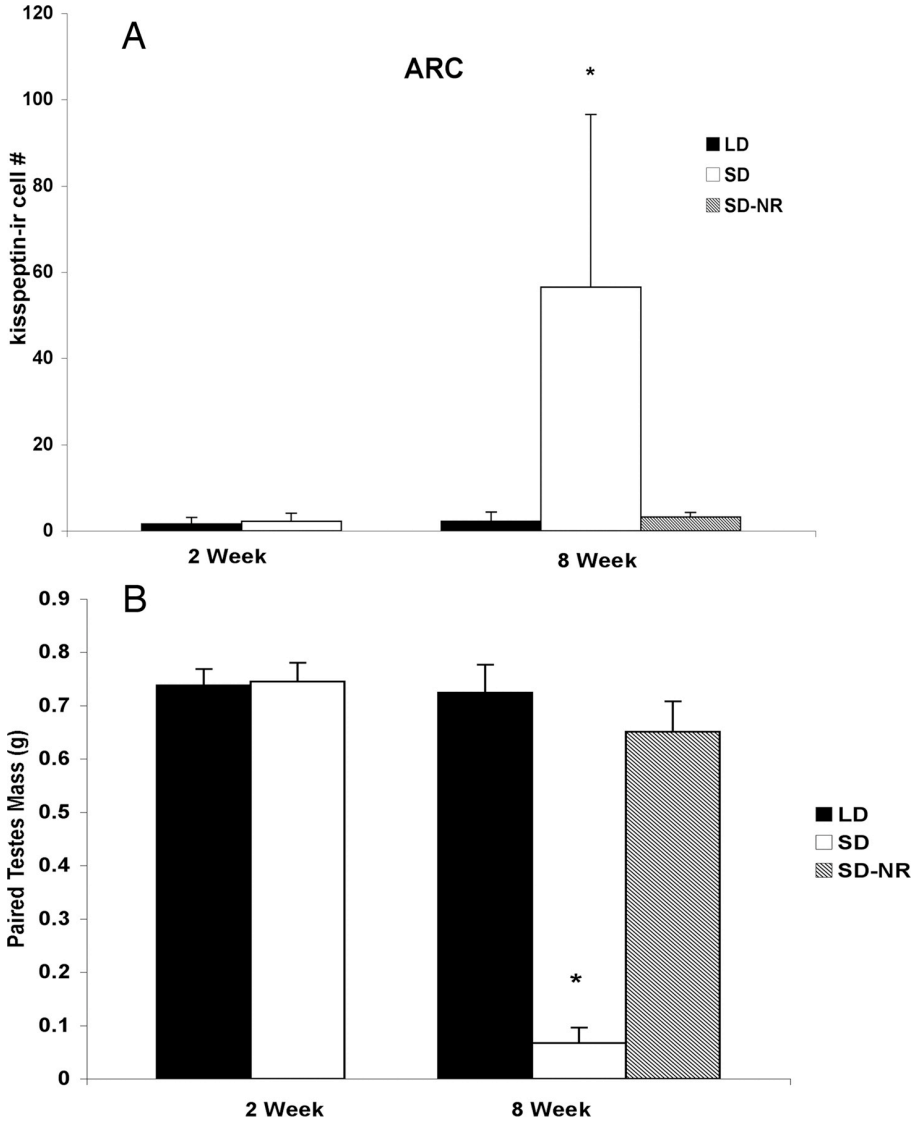


Figure 5.5

Effects of photoperiod on ARC kisspeptin-ir neurons. After 8 wk on short days, responsive animals had significantly more kisspeptin-ir neurons in the ARC, compared with animals held on long days (LD) or short days (SD) for 2 wk or nonresponsive (SD-NR) animals held on short days for 8 wk (A). Short-day-responsive animals had significantly smaller paired testes than all other groups. An *asterisk* denotes $P < 0.05$.

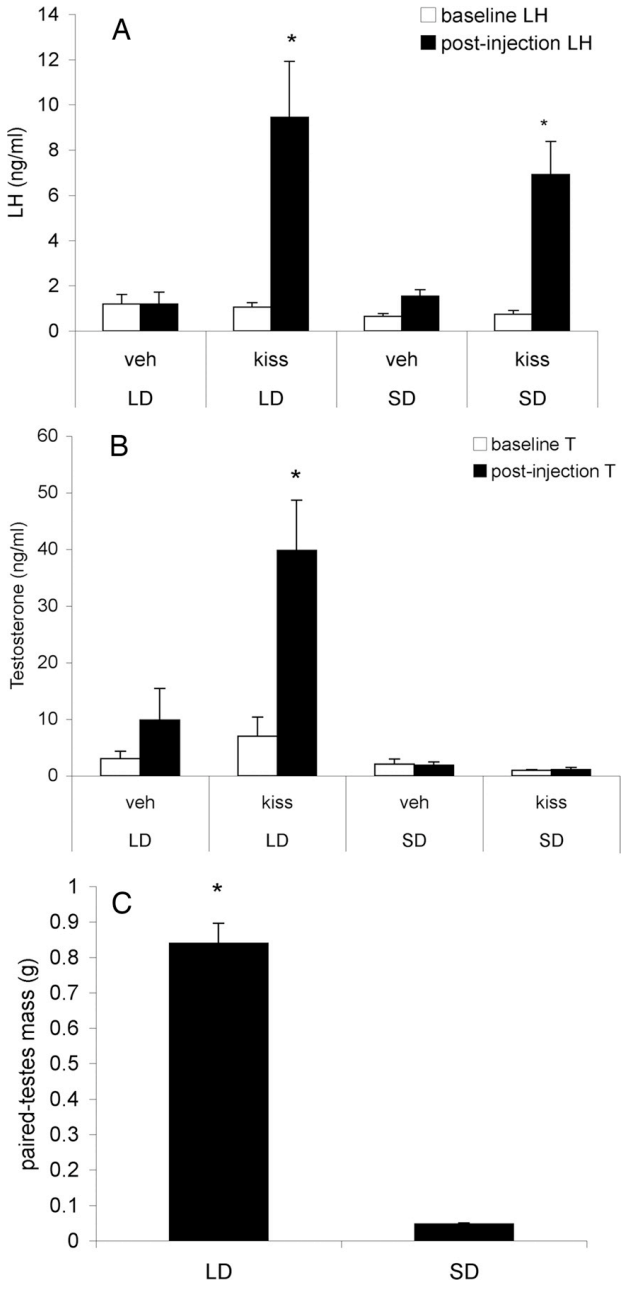


Figure 5.6

Effect of kisspeptin on gonadotropin release. Peripheral injections (ip) of kisspeptin (kiss) significantly elevated pituitary LH, compared with either baseline levels or vehicle (PBS injected) animals (A). Hamsters held in both long- (LD) and short-day (SD) photoperiods for 8 wk displayed a similar increase in LH levels in response to kisspeptin. In addition, animals held in long days displayed significantly elevated testosterone (T) concentrations in response to kisspeptin (B). Short-day animals had significantly smaller gonads than animals housed in long-day photoperiod (C). An *asterisk* denotes $P < 0.05$. veh, Vehicle.

CHAPTER 6

Suppression of Kisspeptin Expression and Gonadotropic Axis Sensitivity Following Exposure to Inhibitory Day Lengths in Female Siberian Hamsters

Introduction

Organisms inhabiting temperate climates confront numerous challenges during the winter months, when harsh weather conditions necessitate significant shifts in behavior and physiology. Many mammals have evolved strategies for partitioning energy reserves to essential physiological processes and inhibiting nonessential processes during these unfavorable climatic conditions (Bronson 1989; Bronson 1985; Voltura and Wunder 1998; Wade and Schneider 1992). One such adaptive strategy employed by many small rodent species occupying variable environments is the complete cessation of reproductive activity by inhibition of the reproductive axis (Nelson et al. 1990). This profound alteration in reproductive activity ensures offspring are not born during times when resources are scarce and weather inclement, improving the probability of both parent and offspring survival (Bronson 1989; Bronson 1985).

The principal environmental signal mediating seasonal reproduction is photoperiod (day length). Specifically, short, decreasing day lengths are interpreted as winter whereas long, increasing day lengths are perceived as summer. Changing day lengths accompanying the late summer to autumn transition are transduced via the pineal gland into a melatonin signal that then acts on the neural substrates mediating reproductive function (Carter and Goldman 1983; Goldman 2001). Specifically, long durations of melatonin are interpreted as short, winter-like days whereas short melatonin durations are interpreted as long, summer-like days. The final common neural pathway in which environmental information is integrated to modulate the reproductive axis is the gonadotropin-releasing hormone (GnRH) neuronal system (Bronson 1989). GnRH regulates pituitary secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn act on the gonads to regulate steroidogenesis and gametogenesis, respectively. Whereas the encoding of day length into a melatonin signal has been well established as the mechanism driving seasonal changes in reproductive function, the neural loci that decode the melatonin signal and communicate this information to the reproductive axis remain largely unspecified.

The neuropeptide kisspeptin has recently been identified as a potent stimulator of GnRH release. Kisspeptin is the endogenous ligand for the (formerly orphaned) G-protein-coupled receptor 54 (GPR54) (Gottsch et al. 2006) and is a potent positive regulator of the HPG axis. Exogenous administration of kisspeptin leads to marked, dose-dependent increases in circulating levels of LH in all species studied to date, including humans (Dungan et al. 2006; Seminara 2005). The effects of kisspeptin are mediated by its actions on GnRH neurons through GPR54. For example, the immediate early gene, c-Fos, is expressed in GnRH neurons following kisspeptin administration (Irwig et al. 2004; Matsui et al. 2004). Additionally, the GnRH receptor antagonist, acyline, blocks gonadotropin release elicited by kisspeptin (Gottsch et al. 2006; Irwig et al. 2004; Shahab et al. 2005). A large proportion of GnRH cells co-express

GPR54 mRNA, further supporting a direct mode of action by kisspeptin on GnRH neurons (Han et al. 2005; Irwig et al. 2004; Messager et al. 2005).

Seasonally breeding rodents provide an ideal model system for investigating the impact of external environmental cues on the reproductive axis. Siberian hamsters (*Phodopus sungorus*) are indigenous to the steppes and semi-arid deserts of Siberia, Mongolia and central Asia, an environment characterized by a marked, predictable decline in ambient temperature and food availability during the fall and winter months (Weiner 1987). The energetic costs of reproduction under such conditions may function as an important ultimate factor that selects against individuals that attempt to breed during fall and winter and that, instead, favors individuals that time parturition to coincide with times of moderate temperatures and greater access to high-quality food (Nelson et al. 1990; Prendergast et al. 2001).

As was detailed in the previous chapter, in earlier experiments I demonstrated that changes in kisspeptin expression are associated with photoperiod-induced suppression of reproductive function in male Siberian hamsters (Greives et al. 2007). Specifically, kisspeptin-ir cell bodies were localized to the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (Arc) of the hypothalamus. High expression was observed in the AVPV of reproductively competent hamsters whereas the inverse state was seen in animals with gonadal involution. Notably, the opposite pattern of expression was seen in the Arc (Greives et al. 2007). Despite being reproductively quiescent, short-day males were equally sensitive to a kisspeptin challenge, suggesting that mechanisms downstream of hypothalamic kisspeptin do not differ photoperiodically, at least in male hamsters.

Males and females differ in many significant ways with respect to morphology, physiology, and behavior. Many of these important differences are a directly related to the different roles that the sexes must assume in the sexual process as a result of differences in gamete numbers and size (Andersson, 1994). The energetic costs of reproduction are not borne equally by the sexes; rather, female mammals expend more energy than males in the form of gestation, lactation and maternal care (Beery et al. 2007). Given this differential investment in reproductive effort, males and females have likely evolved disparate mechanisms to regulate seasonal reproduction. Most research to date has focused on the mechanisms responsible for seasonal changes in male rodents, and one goal of these experiments was to examine whether similar mechanisms underlie changes observed in females. Specifically, the goals of the present study were (1) to identify the distribution of kisspeptin-ir neurons in the brains of reproductively active (i.e., long-day) and inactive (i.e., short-day) *female* Siberian hamsters, (2) to examine reproductive axis sensitivity in long- and short-day females exposed to exogenous kisspeptin peptide and (3) to determine the neural substrates on which kisspeptin acts to influence reproductive axis activity.

Materials and methods

Animals and housing

Adult (> 60 days of age), intact female Siberian hamsters (*P. sungorus*) ($n = 82$) were obtained from the breeding colony maintained at Indiana University. All animals were group housed with same sex siblings in a long-day photoperiod (light:dark [LD] 16:8) prior to the start of the study. Animals were housed individually in polypropylene cages (27.8 × 17.5 × 13.0 cm) and placed in either long- (LD 16:8) or short-day (LD 8:16) photoperiods. Temperature was kept

constant at 20 ± 2 °C and relative humidity was maintained at 50 ± 5%. Food (Purina Rat Chow) and tap water were available *ad libitum* throughout the experiments. All animal protocols were approved by the Bloomington Institutional Animal Care and Use Committee.

At the conclusion of each experiment, animals were weighed to the nearest 0.1 g, euthanized and necropsies were performed. Paired ovaries and uterine horns were collected, cleaned of fat and connective tissue and weighed together as "reproductive organ mass".

Experiment 1: effects of photoperiod on kisspeptin expression

To determine seasonal changes in the pattern of kisspeptin peptide expression, hamsters were held for 12 weeks in long (LD; $n = 10$) or short (SD; $n = 9$) photoperiods. After photoperiod treatment, hamsters were deeply anesthetized with 0.3 ml of a ketamine (20 mg/ml)/xylazine (4 mg/ml) cocktail in 0.9% saline and perfused transcardially with 50 ml of 0.9% saline, followed by 100–150 ml of 4% paraformaldehyde in 0.1 M PBS, pH 7.3. Brains were postfixed for 3 h at room temperature in 4% paraformaldehyde and were cryoprotected in 20% sucrose in 0.1 M PBS and stored at 4 °C until processed. Coronal sections (40 μm) were cut on a cryostat and processed as free-floating sections beginning rostrally at the medial septum/diagonal band of Broca and extending caudally to the brainstem. Kisspeptin immunoreactive cells were labeled using a rabbit anti-kisspeptin antiserum (Penninsula Laboratories Inc., Bachem, San Carlos, CA) diluted at 1:7500 and preadsorbed with GnIH peptide to eliminate cross-reactivity with this related RFamide peptide, as previously described (Greives et al. 2007). We have previously validated this staining procedure and confirmed specificity for kisspeptin peptide (Greives et al. 2007). Amplification of the signal was accomplished by using a modified biotinylated tyramide procedure previously described (Greives et al. 2007). Sections were mounted onto gelatin-coated slides, dehydrated in a graded series of ethanol solutions (70, 95 and 100%) and cleared in xylenes (Fisher Scientific) before the application of coverslips.

Microscopy, cell counts and optical density

Slides were examined under bright field illumination on a Zeiss Z1 microscope by an independent observer naïve to the experimental conditions. Kisspeptin-immunoreactive (ir) cells were located by visually scanning the brains under 200× magnification. Cell populations were restricted to the AVPV region of the preoptic area and the arcuate nucleus (Arc). All cells were confirmed at a minimum of 400×. Cells were photographed with a Zeiss Axiocam Cooled CCD camera at 400× magnification for cell size and density analyses. All cells in every 4th section were counted through the rostrocaudal extent of the AVPV and Arc. Only those cells with a visible nucleus were counted. Soma size and optical density (OD) measurements were performed on images captured at 400×. Soma size and optical density provide a semi-quantitative measure of protein/peptide content visualized immunocytochemically (Nishio et al. 1994). Whereas this measure is unlikely to uncover subtle differences in peptide content across groups, more significant changes should be observed. Cell bodies were outlined and the two-dimensional area was calculated using NIH Image 1.61. Each pixel in the grayscale image capture has a measurable specific intensity, with values ranging from 0 (white) to 256 (black). The average value for all pixels in an outlined area is taken as the mean intensity of staining for a given region of the image. OD measures were normalized to minimize differences between replications of immunohistochemistry. First, a background measurement was taken by placing a square outline, four times, on non-overlapping, unstained areas of each section. The mean of these four

measures provided the background OD for each section. The OD for each cell body was assessed by outlining the cell body, obtaining a density measure using NIH Image and subtracting the background OD from the OD of each cell. To account for potential overcounting, an Abercrombie correction was applied to cell count data prior to analysis.

Experiment 2: endocrine response to exogenous kisspeptin

To determine whether seasonal changes in reproduction are related to a reduction in HPG axis sensitivity to kisspeptin, I examined whether kisspeptin-induced LH secretion was altered by photoperiodic treatment. Hamsters were held in long- ($n = 12$) or short-day ($n = 19$) photoperiods for 8 weeks prior to kisspeptin injections. Animals were injected with either kisspeptin-10 [KiSS-1 (112–121)/ metastin (45–54) (human); Phoenix Pharmaceuticals, Inc., Belmont, CA], a peptide known to stimulate GPR54 (Gottsch et al. 2004) or a 0.1-M PBS vehicle injection. Injections were given between 8.5 and 11 h prior to lights out in LD animals and 4.5–7.0 h before lights out in SD animals. These time points were chosen to ensure low estradiol concentrations in LD animals (Dodge et al. 2002). The injection protocol has been previously described elsewhere (Messager et al. 2005). Briefly, an initial blood sample was drawn from all hamsters via the retro-orbital sinus to measure baseline hormone levels. Next, long- and short-day hamsters received i.p. injections of either 100 µl PBS (long-day: $n = 6$) (short-day: $n = 9$) or 100 µl of a PBS solution containing a 10-µM concentration of kisspeptin-10 (Phoenix Pharmaceuticals, Inc.) (long-day: $n = 6$) (short-day: $n = 10$), every 30 min for a total of four injections. Thirty minutes after the last injection, all hamsters were again bled. Blood was centrifuged at 2500 RPM for 30 min and serum was collected and stored at − 80 °C until assayed for hormones.

Experiment 3: endocrine response to exogenous kisspeptin after the administration of a GnRH antagonist

To examine whether the actions of kisspeptin are mediated at the level of the GnRH system, I examined the LH response to kisspeptin following treatment with the GnRH receptor antagonist, acyline, kindly provided by Dr. Richard Blye at the NIH/NICHD. Procedures were modified from a previously described protocol (Gottsch et al. 2004). Animals received injections of either the GnRH antagonist, acyline or vehicle, combined with injections of kisspeptin. Briefly, an initial blood sample was drawn from all hamsters via the retro-orbital sinus to measure baseline hormone levels. Next, long-day animals were given an injection i.p. of either 50µg acyline diluted in 100 µl mannitol (5 %) water ($n = 6$) or 100 µl of vehicle mannitol water ($n = 6$). Exactly 1 h after injection, animals received single 100 µl i.p. injections of 10 µM kisspeptin. Additionally, four animals received vehicle injections at both time points. Pilot data from our labs indicated that one injection of kisspeptin caused a comparable LH rise to that seen in animals injected following the four injection protocol described above (Greives et al., unpublished data). Thirty minutes following the final injection, a second blood sample was collected. Blood was centrifuged at 2500 RPM for 30 min. and serum was collected and stored at − 80 °C until assayed for hormones.

Hormone measurements

Serum LH concentrations were measured in duplicate via a single radioimmunoassay (RIA) with reagents obtained from the National Institutes of Health based on a previous protocol (Chappell et al. 1997). The antiserum was rLH-S-11 and the standard was rLH-RP3. The

sensitivity was 0.01 ng/tube and the intra-assay coefficient of variation was 2.9% for the low pool and 8.5% for the high pool. The antiserum used was highly specific to LH and has been previously validated for use in Siberian hamsters (Wolfe et al. 1995).

Statistical analyses

Data in Experiment 1 were analyzed using a two-tailed Student's *t*-test. Specifically, the effects of photoperiod on body mass, gonad mass, kisspeptin-ir neuron number, size and optical densities were each analyzed in separate analyses. For Experiment 2, the effects of peripheral kisspeptin injections on LH were analyzed using a 2 × 2 × 2 mixed model ANOVA, with pre- and post-hormone levels as the within-subjects factor and photoperiod and injection type as the between-subjects factors. LH data for experiment 3 were analyzed with a 2 × 2 × 2 (LD vs. SD × acyline vs. vehicle × kisspeptin vs. vehicle) ANOVA. All post hoc comparisons were analyzed using Tukey tests. In all cases, differences were considered statistically significant if $p < 0.05$.

Results

Experiment 1: effect of photoperiod on kisspeptin neurons

Kisspeptin-ir cell bodies were concentrated in the AVPV and Arc nuclei. In both nuclei, kisspeptin expression was significantly altered by photoperiodic condition ($p < 0.05$ in each case (Fig. 6.1 and Fig. 6.2). LD hamsters exhibited a significantly greater number of kisspeptin-ir neurons in the AVPV compared to SD animals ($p < 0.05$; Fig. 6.1 and Fig. 6.2). The inverse was true for kisspeptin-ir expression in the Arc; females held in SD conditions had a greater number of kisspeptin-ir cells compared to LD animals ($p > 0.05$; Fig. 6.1 and Fig. 6.2). Neither cell size nor optical density differed between groups in the AVPV ($p > 0.05$) (Figs. 6.1b and c). As expected, photoperiod significantly affected body and reproductive organ masses, with hamsters held in short-day conditions having lower body mass (mean ± SEM: LD = 39.1 ± 1.74 g, SD = 25.8 ± 1.07 g, $p < 0.05$) and smaller reproductive organ masses (mean ± SEM: LD = 0.15 ± 0.01 g and SD = 0.048 ± 0.004 g, $p < 0.05$) compared to LD females.

Experiment 2: endocrine response to exogenous kisspeptin

The effects of kisspeptin on LH secretion were dependent upon the photoperiod to which the animals were exposed. LD hamsters responded to injections of kisspeptin with a significant increase in LH concentrations compared to animals receiving vehicle ($p < 0.05$). In contrast, peripheral injections of kisspeptin did not elicit a rise in serum LH concentrations in SD hamsters ($p > 0.05$ relative to baseline and vehicle controls; Fig. 6.3). As in experiment 1, photoperiod significantly affected reproductive organ masses, with LD hamsters having significantly heavier (0.11 ± 0.017 g) ovary ± uterine horn masses than SD animals (0.047 ± 0.0039 g) ($p < 0.05$).

Experiment 3: endocrine response to exogenous kisspeptin after treatment with GnRH antagonist, acyline

A single kisspeptin injection significantly increased LH concentrations in LD hamsters ($p < 0.05$; Fig. 6.4). Pretreatment with acyline, however, prevented the rise in LH concentrations following administration of kisspeptin ($p < 0.05$ relative to vehicle-treated animals). These findings are consistent with previous studies indicating that kisspeptin is acting at the level of the GnRH system rather than the pituitary.

Discussion

The results of the present study demonstrate a critical role for kisspeptin in the interpretation and integration of reproductively relevant environmental signals and transmission of this information to the GnRH neuronal network. Striking differences in kisspeptin-ir expression were observed following manipulation of photoperiod in two cell populations, the AVPV and Arc. Female Siberian hamsters maintained in "summer" photoperiods exhibited robust kisspeptin expression in the AVPV, with virtually no labeling in the Arc. In contrast, females held in "winter" day lengths had minimal kisspeptin-ir expression in the AVPV, but significant expression in the Arc. These findings suggest divergent roles for these two populations of kisspeptin neurons. Unlike male Siberian hamsters, that inhibit reproduction by reducing kisspeptin expression in the AVPV while maintaining GnRH sensitivity to this peptide (Greives et al. 2007), females housed in short days failed to show an endocrine response to exogenous kisspeptin. These findings suggest important sex differences in HPG responsivity with females exhibiting a dual mechanism of control, both reducing AVPV kisspeptin expression and reproductive axis sensitivity to this peptide. Finally, consistent with other mammalian species studied to date (rat, mouse and macaque) (Gottsch et al. 2004; Irwig et al. 2004; Plant et al. 2006), kisspeptin produces its effects in Siberian hamsters by acting principally on the GnRH system rather than the pituitary.

The force of natural selection produces a state in which organisms must continuously adapt to their environment, and proper timing of reproduction is key to this process. Diverse selective pressures have resulted in a tightly regulated reproductive axis that is highly sensitive to a variety of internal and external environmental cues, which provide crucial information about the present state of the environment (Clarke and Pompolo 2005; Gore 2004; Kriegsfeld 2006). The fundamental role that GnRH plays in regulating reproduction and fertility has been unequivocally established, however, the upstream mechanisms that integrate the state of the organism and environmental conditions are less well understood.

The AVPV has been identified as a critical brain region mediating the positive feedback effects of estrogen, crucial to the initiation of the preovulatory LH surge (Wintermantel et al. 2006). *Kiss-1* mRNA expression is at a maximum in the AVPV at the time of the LH surge and these neurons show peak activity, as measured by FOS expression, on the afternoon of proestrus (Smith et al. 2006c). Results of the present study suggest an additional role for kisspeptin neurons in the AVPV, namely as an upstream center for the integration of reproductively relevant external stimuli with the steroidal signals that are necessary to trigger ovulation. Whereas inputs to kisspeptin cells have not been investigated extensively, melatonin does not act on the AVPV directly to induce reproductive quiescence. In Siberian hamsters, the SCN is a critical locus for the decoding of the short-day melatonin signal (Bartness et al. 1991; Teubner and Freeman 2007). Because the SCN projects extensively to the AVPV (Kriegsfeld et al. 2004; Leak and Moore 2001), it is likely that the SCN is driving seasonal changes in kisspeptin cells.

Unlike our previous findings in *male* Siberian hamsters (Greives et al. 2007), the present findings indicate that the effects of kisspeptin are regulated via two levels of control in females. Not only is kisspeptin expression altered in the AVPV and the Arc in response to changes in day length, but the sensitivity of the GnRH system to this peptide is also changed. In contrast to short-day male hamsters (Greives et al. 2007), the current study found that females with a

regressed reproductive axis did not respond to peripheral kisspeptin injections with an elevation in LH concentrations (Fig. 6.3). In males, LH release is elicited by exogenous kisspeptin treatment regardless of reproductive condition (Greives et al. 2007). The selective pressures impacting the timing of male and female fertility are likely to differ in significant ways and this may be reflected in the underlying mechanisms controlling seasonal reproduction (Beery et al. 2007). A winter pregnancy would likely result in death of both mother and offspring, due to the overwhelming increase in energetic demands, whereas males would not experience the pressures of gestation, lactation and parental care. Given the minimal likelihood of offspring survival during winter, the increased energetic demand required for maintenance of the reproductive axis outweighs the potential reproductive benefits in males. Females appear to have evolved a "fail-safe" mechanism to ensure that pregnancy will not occur during inappropriate times of year while males may have evolved an increased sensitivity to other environmental variables to breed opportunistically as ambient conditions allow.

Whereas a role for the AVPV in direct regulation of ovulation has been well established (Wintermantel et al. 2006), the role of the Arc in this process remains less well specified. The Arc monitors current energy state and relays this information to the reproductive axis (Smith and Grove 2002). In times of reduced energy availability, reproduction is inhibited (Bartness 1996; Schneider et al. 2000). In addition to mediating the reproductive axis through energetic signaling, estrogen-receptor-positive Arc projections also act on GnRH terminals in the median eminence to modulate their output (Lehman et al. 1997). Whether kisspeptin neurons specifically mediate these Arc effects on the reproductive axis represent an interesting topic for further inquiry. That Arc kisspeptin neurons exhibit high expression during times of reproductive quiescence argues against a stimulatory role for this population of cells. Alternatively, these cells may inhibit release of kisspeptin during winter, leading to their greater detection with immunohistochemistry. Distinguishing among these competing hypotheses will require converging approaches investigating release/production rates of kisspeptin in AVPV and Arc populations. An additional possibility is that the Arc kisspeptin cells perform a function unrelated to reproductive control yet to be determined.

Previous studies have revealed that kisspeptin expression is highly modulated by the level of circulating gonadal sex steroids. In mice, castration results in a significant increase in *Kiss-1* mRNA expression in the Arc, an effect reversed by testosterone replacement (Smith et al. 2005). These results, as well as those from the present study, suggest the possibility that kisspeptin responds to levels of sex steroids rather than drives reproductive system involution. However, recent findings in Syrian hamsters (*Mesocricetus auratus*) indicate that photoperiod drives changes in kisspeptin directly rather than through changes in gonadal steroids (Revel et al. 2006). More specifically, *Kiss-1* mRNA in short-day hamsters given testosterone replacement does not differ from short-day controls (Revel et al. 2006). Whether or not photoperiod-induced changes in kisspeptin expression in female hamsters are also independent of gonadal steroids requires further study. Whereas the estrous cycle of Syrian hamsters is remarkably precise (Dodge et al. 2002), cycles in female Siberian hamsters cannot be tracked reliably and the relationship between vaginal cell cytology and ovarian follicular development is questionable (Anand et al. 2004; Dodge et al. 2002; McMillan and Wynne-Edwards 1999). As a result, this precluded my ability to examine the role of estrogen on kisspeptin expression in Siberian females under natural conditions. Siberian and Syrian hamsters also differ in the brain regions that appear to be critical in the interpretation of photoperiodic information, with the dorsomedial hypothalamus being essential in Syrian hamsters (Maywood et al. 1996) and the SCN being crucial in Siberians

(Bartness et al. 1993; Bittman 1984; Bittman et al. 1991; Bronson 1985). Future studies investigating the similarities and differences in melatonin signal transduction and kisspeptin control among photoperiodic species will be necessary to fully appreciate the evolution of seasonality.

In summary, kisspeptin acts as an important integration point mediating the interpretation and relay of environmental stimuli relevant for reproduction. Inhibitory photoperiods produce divergent effects on AVPV and Arc populations of cells, suggesting that these cells may have different roles in reproductive axis regulation. Female hamsters utilize a dual mechanism of control over the GnRH neuronal network, altering kisspeptin peptide levels and sensitivity of the GnRH system to kisspeptin signaling. This mode of reproductive axis suppression ensures that females will not breed during inappropriate times of year. This finding contrasts with that seen in male Siberian hamsters, where kisspeptin expression is altered without changes in sensitivity to the peptide (Greives et al. 2007). Together, these findings further place kisspeptin in a position of marked importance in the regulation of reproduction and thus reproductive success. Furthermore, these results demonstrate the utility of a photoperiod model for investigations of both the ecological and physiological factors regulating mammalian reproduction.

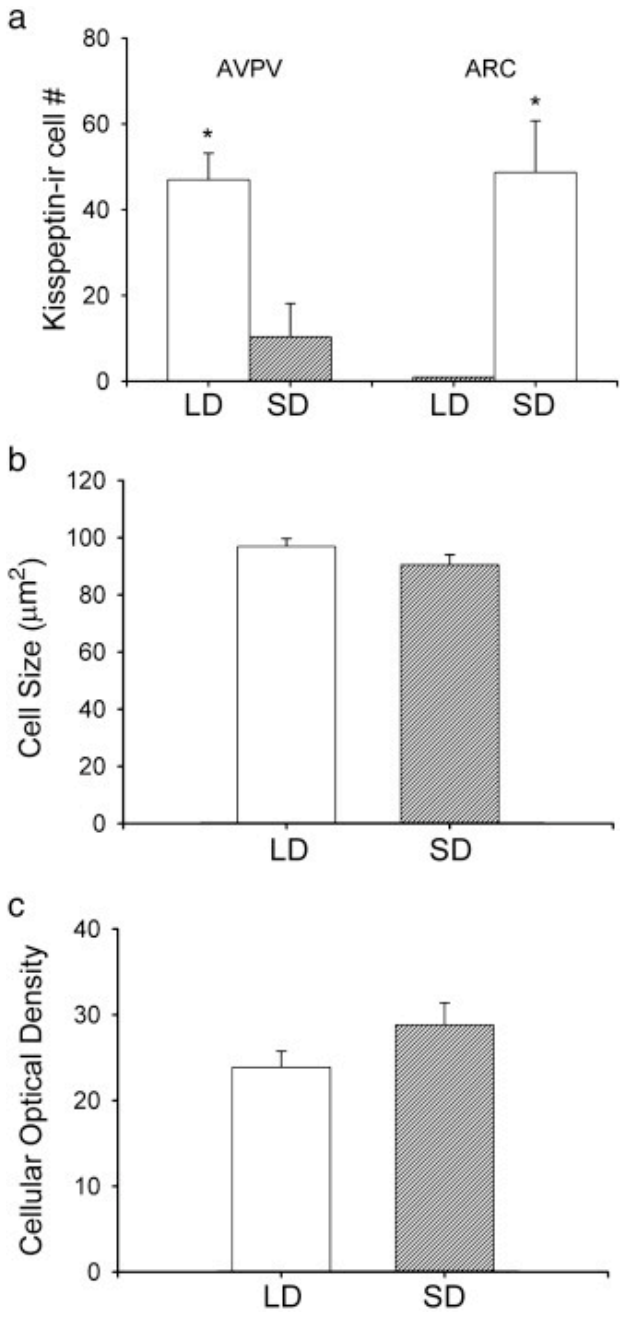


Figure 6.1

Photoperiod affects kisspeptin-ir cell numbers in the AVPV and Arc. Animals held for 8 wk in short-day (SD) photoperiods display significantly fewer cells in the AVPV and significantly more cells in the Arc compared to animals held in long-day (LD) photoperiods (a). Cell size (b) and optical density (c) were not affected in the AVPV by photoperiod. Because kisspeptin-ir cells were absent in the Arc of LD animals, cell size and optical density were not evaluated. *$p < 0.05$.

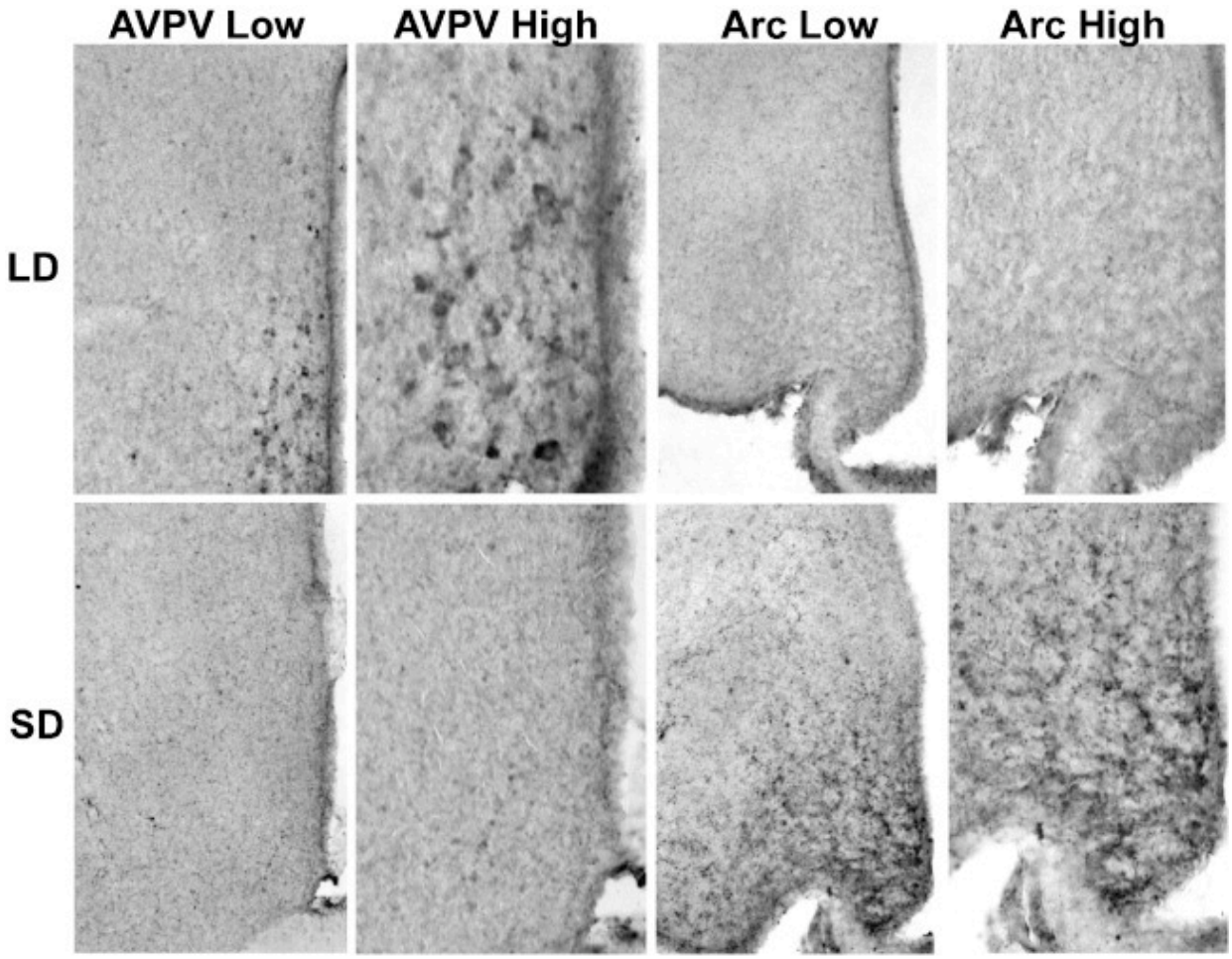


Figure 6.2

Representative photomicrographs of kisspeptin-ir cell labeling in the AVPV and Arc of hamsters held in either long (LD; top)- or short (SD; bottom)-day lengths. Low (100×) and high power (200×) photomicrographs are shown for each condition and brain region investigated.

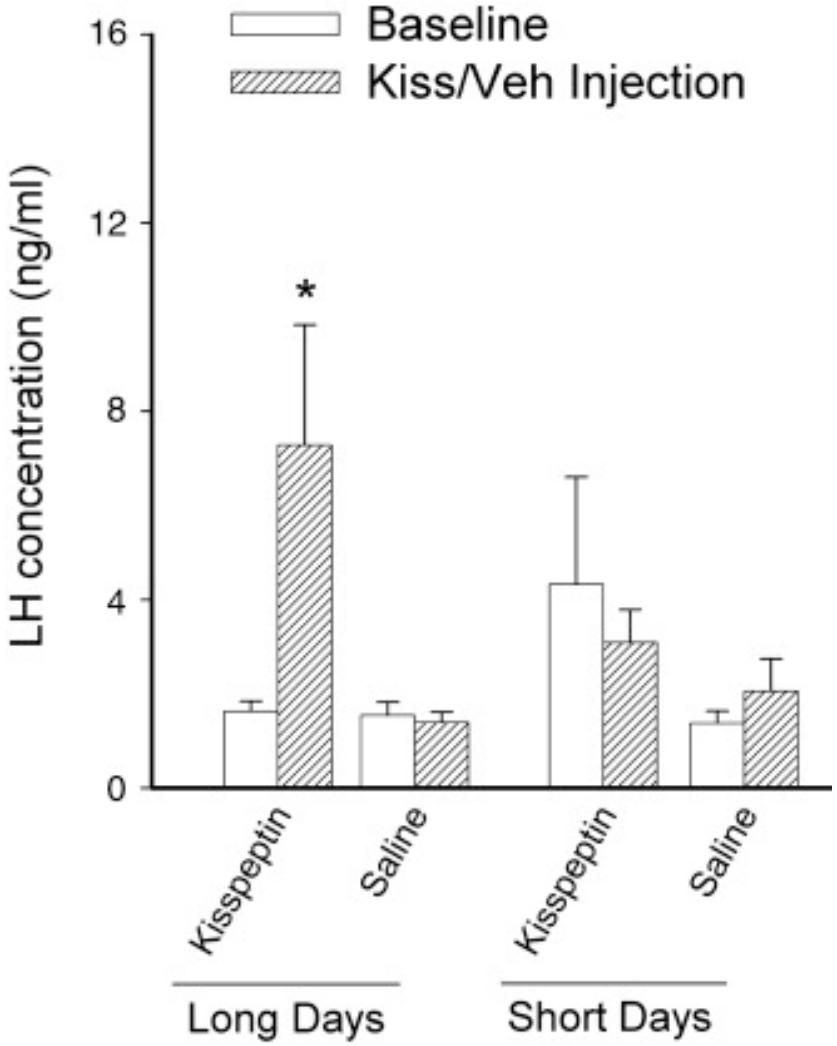


Figure 6.3

Effect of kisspeptin on gonadotropin release. Peripheral injection (i.p.) of kisspeptin significantly elevated levels of pituitary luteinizing hormone (LH) in animals held in long days relative to baseline or saline injections. Females held in short days for 8 weeks did not show a significant elevation in LH concentrations after kisspeptin injection relative to baseline or saline injections. *$p < 0.05$.

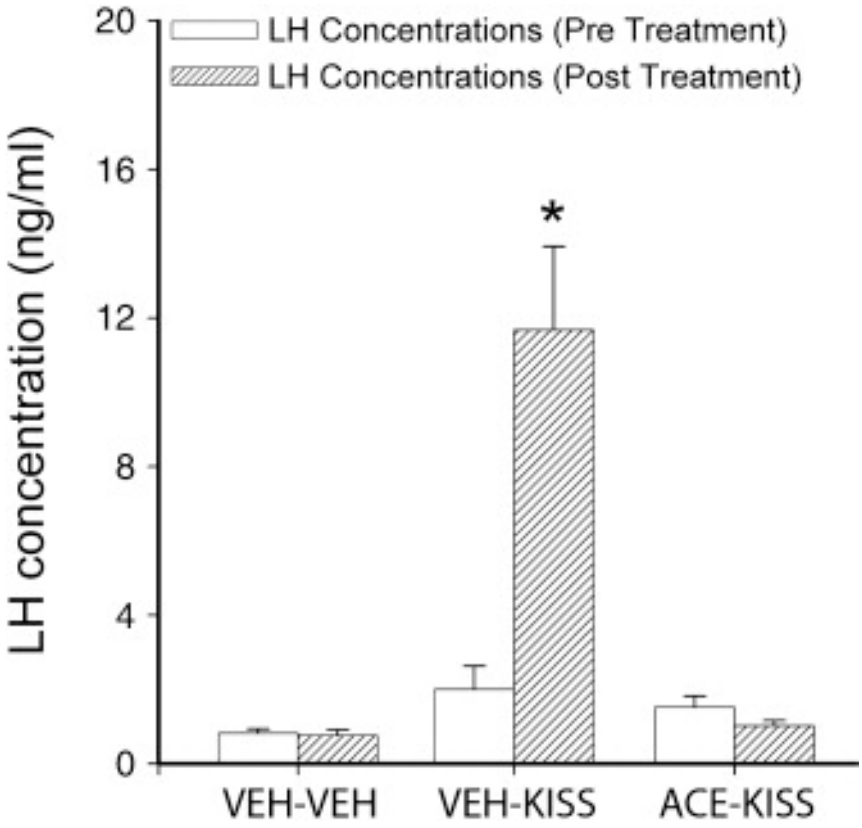


Figure 6.4

Mean (± SEM) concentrations of LH before and following kisspeptin (KISS) injections preceded by vehicle (VEH) or acyline (ACE). Animals receiving kisspeptin injections show a significant elevation in LH concentrations that is blocked by pretreatment with acyline. *$p < 0.05$.

Chapter 7

General Conclusions

The goal of the present series of experiments was to achieve a better understanding of the mechanisms by which organisms use day length information to modify their reproductive state to conserve energy for successfully coping with the changing seasons. These findings represent a significant contribution towards filling this knowledge gap. The findings indicate that the novel hypothalamic neuropeptidergic populations of kisspeptin and GnIH serve an important role in fine-tuning the activity of the GnRH neural network in response to photoperiodic cues by providing a balance of excitatory and inhibitory input that shifts with changes in day length.

Several of the specific contributions from these studies are important and deserve reiteration. Chapter 2 described the initial studies that identified an inhibitory reproductive role for the novel neuropeptide RFRP in mammals. RFRP is the homolog of gonadotropin inhibitory hormone, a recently characterized neuropeptide already established as an important inhibitory regulator of reproduction in birds. RFRP is positioned in the hypothalamus to interact directly with GnRH neurons and potentially mediate the negative feedback effects of sex steroids. This, in particular, is an important contribution as negative feedback regulation of the reproductive axis has been recognized for decades but the precise mechanism of control remains unspecified.

Chapter 3 presented two experiments that aimed to elucidate a role for RFRP in seasonal reproduction in Syrian hamsters. The specific motivation for this study was the finding that RFRP neurons are localized to the DMH, a neural locus previously identified as crucial for mediating the effects of photoperiod on the reproductive axis (Lewis et al. 2002; Maywood et al. 1996). The results were counter to my initial hypothesis that I would observe greater RFRP peptide levels and mRNA production in short, inhibitory day lengths. Nevertheless, the results do not preclude an inhibitory role for RFRP in initiating the cascade of events culminating in reproductive quiescence, as fiber density after short-day exposure is consistent with the possibility that RFRP release is initially increased following exposure to inhibitory day lengths.

Chapter 4 examined the possible involvement of the RFRP system in the genetic basis of photoperiodic nonresponsiveness using selected lines of white-footed mice. The two lines of mice that were artificially selected to either respond maximally to short days with reproductive inhibition or not respond at all had significantly different numbers of immunoreactively labeled RFRP neurons. This finding suggests that variation in the numbers of RFRP cells may result in an inability to maximally inhibit the GnRH system in response to photoperiod that would manifest as different reproductive responses to photoperiod. Therefore, the potential exists for variation in the RFRP system to account for some of the phenotypic variation observed in natural populations of seasonally breeding rodents.

Chapters 5 and 6 investigated the potential role for kisspeptin neurons as an integration site interpreting and transmitting reproductively relevant information to the GnRH neural network. For these studies I utilized Siberian hamsters as a seasonal model, and provided

evidence for an important association between kisspeptin levels and changes in reproductive function in response to photoperiod. In both males and females striking changes in expression occurred in both hypothalamic nuclei containing kisspeptin cell bodies, however the direction of change at these sites were in opposition. This finding is important as it suggests that these two populations of kisspeptin neurons may not serve the same function.

Another notable finding from the kisspeptin studies was that male and females in short day conditions differed in their response to an exogenous kisspeptin challenge. The divergent selective pressures faced by male and female mammals with respect to the reproductive process as a whole, from fertilization through parental care, could account for this differential response. As female mammals undertake a much greater energetic burden than males, having to undergo gestation and lactation, this sex may have evolved 'backup' mechanisms to ensure that reproduction does not occur when the environment is unsuitable. This finding is particularly significant in the context of a renewed interest in sex differences in brain function by neuroscientists (Cahill 2006), as it suggests a framework for interpreting sex differences in the context of differential reproductive investment.

As a whole, my research has shown that kisspeptin and GnIH/RFRP serve as a critical interface between the environment and the reproductive axis of the organism. These two neuropeptides provide a balance of stimulatory and inhibitory neuromodulatory input upon the HPG axis and are well positioned to receive direct and indirect information about environmental state that is necessary for maintaining optimal reproductive function in the face of fluctuating environmental conditions. As a final note, I believe my results strongly advocate for the utility of using seasonally breeding rodent species for the fruitful role they serve in research aimed at elucidating the neural and endocrine mechanisms that regulate reproductive function. Many practically important and valuable advances have been realized from biomedical research or other fields that focus exclusively on proximate mechanistic questions. However, I would argue that research that does not rely on the highly inbred traditional laboratory rodents, such as the research I have chosen for my research program provides a necessary counterpoint to the standard approach to research. Too often, biomedical research is loses sight of the larger evolutionary and environmental context in which the physiological and behavioral traits of interest arose. All organisms that present exist are the result of generations of selection pressures imposed by unique and diverse past environments. At the very least, one should be aware that organisms cannot be fully understood without some familiarity with the environmental conditions under which they evolved and currently inhabit. This fact is becoming more relevant and urgent with the growing recognition that many modern diseases that afflict humans are likely attributed, at least in part, to the significant mismatch between the current environment that we, in developed nations, inhabit relative to the environment that the human species evolved (Gluckman 2009). As humans have emancipated themselves from the natural environment and exert ever more control over their physical environments to meet the demands of society and culture, that deep-rooted connection between organism and environment is being tested in ways that are leading to negative consequences as revealed in the rise of major modern health epidemics such as obesity, certain cancers, or mental health problems. A greater understanding of the role that the environment has played in shaping the behavioral patterns and physiological systems should provide valuable insight into ways of ameliorating the detrimental physical effects of our diminishing connection to the major environmental rhythms of day and season.

This realization was an important motivating force behind the research projects I undertook and have described in my dissertation.

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