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Social and Environmental Control of the Reproductive Axis

By

Kimberly J. Jennings

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Psychology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Lance J. Kriegsfeld, Chair
Professor George E. Bentley
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Abstract

Social and Environmental Control of the Reproductive Axis
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In order to survive and reproduce, animals must be exquisitely sensitive to their environment. Factors such as season and social setting can have powerful influence on reproductive outcomes by determining availability of mates, food, safety, or housing. Therefore, many species have evolved mechanisms to detect changes in environmental context and modify reproductive physiology accordingly. This dissertation examines the neural substrates mediating social and environmental control of reproductive physiology and behavior. The studies presented here focus on the hypothalamic neuropeptides kisspeptin and RFamide-related peptide (RFRP). These neuropeptides regulate the reproduction by modulating release of gonadotropin releasing hormone (GnRH) and downstream luteinizing hormone (LH), which in turn regulates gonadal function and sex steroid production. Chapter 2 explores the impact of aggressive encounters on kisspeptin and RFRP neuronal systems. Chapter 3 investigates how chemosensory information about a potential mate is conveyed to the reproductive axis, and how processing of this information differs across breeding and non-breeding conditions. Chapter 4 examines in detail how expression of kisspeptin and RFRP changes as males and females transition from breeding to non-breeding back to breeding condition in response to changes in photoperiod (proportion of light:dark in a 24 h day). These studies, utilizing a variety of rodent species, indicate that RFRP but not kisspeptin is sensitive to social information, whereas both neuropeptide systems integrate information about photoperiod, although in different ways. These findings inform our understanding of the complex circuitry mediating social and environmental control of physiology and behavior.

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Chapter 1: General Introduction

To maximize reproductive success, organisms must be sensitive to information about their environment and align mating to the most opportune contexts. Reproducing is one of the most energetically expensive behaviors an animal can engage in during its lifespan. Animals of both sexes must expend energy on mating behaviors while also forgoing opportunities to gather additional resources. Males must maintain reproductive capability and, in many species, patrol and defend acquired resources, and potentially provide paternal care. Females invest metabolic resources in growing the developing offspring and, in mammals, providing lactation and other maternal care. Thus, reproducing under unfavorable conditions threatens the survival of both the offspring and potentially of the parents. To synchronize breeding to the most favorable conditions, animals have evolved numerous mechanisms to detect and integrate information about their external environment for the control of reproductive behavior and physiology. This chapter will begin by briefly reviewing central regulation of reproductive physiology (focusing on mammals) before describing selected types of external information known to influence reproductive physiology and concluding with an overview of the questions addressed in the following chapters.

1.1 Central control of reproduction

1.1.1 The hypothalamic-pituitary-gonadal axis

Reproductive physiology is regulated by the central nervous system through the hypothalamic-pituitary-gonadal (HPG) axis. Canonically, neurons of the hypothalamus release gonadotropin releasing hormone (GnRH) into the hypophyseal blood portal system. GnRH is quickly transported to the anterior pituitary, where it acts on gonadotropic cells to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into general blood circulation. These gonadotropins act on the gonads to promote gametogenesis and the production of sex steroid hormones, such as androgens (e.g., testosterone) and estrogens. Sex steroids exert negative feedback on upstream elements of the HPG axis to keep circulating concentrations within the physiological range. Because sex steroids also influence expression of sexual behaviors in most mammalian species, the HPG axis regulates both reproductive physiology and behavior. Over the last 17 years, two additional neuropeptides have been established to operate upstream of GnRH neurons and exert potent influence on HPG axis activity: kisspeptin and RFamide-related peptide (RFRP).

1.1.2 Kisspeptin

Kisspeptin, encoded by the *kiss1* gene, was initially recognized to have reproductive relevance as a puberty regulator (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003). Kisspeptin potently stimulates release of GnRH through its g-protein coupled receptor, GPR54 (Castellano et al., 2005; Gottsch et al., 2004; Han et al., 2005; Irwig et al., 2004; Messager et al., 2005). Approximately 70-95% of GnRH cells express GPR54 in sheep and rodents (Han et al., 2005; Herbison et al., 2010; Messager et al., 2005; Poling et al., 2012). In rodents, kisspeptin is produced within two hypothalamic nuclei: the anteroventral periventricular nucleus (AVPV), and

the arcuate nucleus (Franceschini et al., 2006; Gottsch et al., 2004; Kauffman et al., 2007; Kim et al., 2009; Smith et al., 2005a; Smith et al., 2005b). These populations both positively regulate HPG axis activity, but are implicated in distinctly separate functions.

Kisspeptin cells of the AVPV drive the pre-ovulatory surge in LH, which is necessary for ovulation to occur. These kisspeptin cells are positively regulated by circulating sex steroids (Kauffman et al., 2007; Smith et al., 2005a; Smith et al., 2005b). As circulating estrogen concentrations increase across the estrous cycle, as does AVPV kisspeptin expression. At the appropriate circadian time on the day of estrus, these cells release kisspeptin to stimulate a surge of GnRH (Kriegsfeld, 2013a; Smarr et al., 2012; Williams et al., 2011). In keeping with this female-oriented function, AVPV kisspeptin expression is also sexually dimorphic with many more kisspeptin-expressing cells in the AVPV of females (Homma et al., 2009; Kauffman et al., 2007). The function of AVPV kisspeptin cells in males is less clear.

Kisspeptin expressing cells of the arcuate nucleus have been implicated as an important component in the GnRH pulse generator. GnRH is not released tonically, but instead is released in pulses occurring at regular intervals throughout the day (Fink, 1988). The pulse interval and amplitude are determined by a complex interaction amongst arcuate nucleus kisspeptin-expressing cells producing synchronous burst firing, causing release of kisspeptin onto GnRH cells (Goodman et al., 2013; Han et al., 2015; Verma et al., 2014; Wakabayashi et al., 2010). These neurons have been nicknamed "KNDy" neurons due to their high co-expression of kisspeptin, neurokinin B, and dynorphin (Goodman et al., 2007; Lehman et al., 2010a; Navarro et al., 2009). Arcuate kisspeptin expression is negatively regulated by circulating sex steroids (Kauffman et al., 2007; Smith et al., 2005a; Smith et al., 2005b). Thus, kisspeptin expressing cells of this region aid in the homeostatic regulation of sex steroid concentrations by negative feedback (Lehman et al., 2010a). Whereas AVPV kisspeptin cells primarily target GnRH cell bodies, arcuate kisspeptin cells project heavily to GnRH terminals in the nearby median eminence (Uenoyama et al., 2011; Yip et al., 2015). There, kisspeptin acts as a neuromodulator to facilitate release of GnRH (d'Anglemont de Tassigny et al., 2008; Glanowska and Moenter, 2015; Smith et al., 2011).

1.1.3 RFRP

RFRP is the mammalian ortholog to the avian peptide gonadotropin inhibitory hormone (GnIH). Discovered by Tsutsui and colleagues in 2000, GnIH was the first peptide found to directly inhibit LH release (Hinuma et al., 2000; Tsutsui et al., 2000). In mammals, the gene *rfrp* (also known as *npvf*) encodes a preproRFRP peptide that is cleaved into two secreted peptides, RFRP-1 and RFRP-3. These peptides act on two g-protein coupled receptors, GPR174 (also known as NPFFR1) and GPR47 (NPFFR2). GPR47 has greater affinity for RFRP-1 whereas GPR147 has greater affinity for RFRP-3, although both peptides may bind to either receptor (Ubuka et al., 2013b). Pharmacological investigations of RFRP functioning have primarily used RFRP-3 with less attention to RFRP-1. However, there is currently no available antibody specific for RFRP-3 and only one antibody specific for RFRP-1 (Jorgensen et al., 2014). All others, including the antibodies used in the research reported in the following chapters, cross-react with RFRP-1, RFRP-3, and the RFRP precursor peptide. Therefore, the term "RFRP" is

used hereafter in contexts in which the study does not or cannot distinguish between the RFRP-1 and RFRP-3 peptides, or when discussing expression of *rfrp* mRNA.

RFRP was initially reported to inhibit release of LH, functioning similarly to its avian counterpart (Anderson et al., 2009; Johnson et al., 2007; Kriegsfeld et al., 2006). However, further examination has uncovered additional sex, species, and environment specific effects of RFRP on LH release. In females, RFRP cells appear to tonically inhibit LH release and lift this inhibition around the initiation of the pre-ovulatory LH surge (Ancel et al., 2017; Gibson et al., 2008; Henningsen et al., 2016a; Kriegsfeld et al., 2006; Russo et al., 2015). However, central infusions of RFRP peptides have been reported to increase or decrease LH concentrations in seasonally-breeding hamsters depending on the animal's reproductive state (breeding or non-breeding) (Ancel et al., 2012; Henningsen et al., 2016a; Ubuka et al., 2012a). A recent study in mice also reported that central RFRP-3 infusion increased LH release and that this effect was diminished in GPR54 knockout mice, suggesting that RFRP-induced stimulation of LH release is in part GPR54-dependent (Ancel et al., 2017). RFRP cells can influence HPG axis activity through multiple pathways. RFRP cells influence GnRH release through projections to GnRH cell bodies (Ducret et al., 2009; Kriegsfeld et al., 2006; Poling et al., 2012; Smith et al., 2008), regulate LH release through direct actions on the anterior pituitary via projections to the external secretory zone of the median eminence (Clarke et al., 2008; Kriegsfeld et al., 2006; Murakami et al., 2008; Smith et al., 2012; Ubuka et al., 2009), and potentially regulate GnRH indirectly through projections to kisspeptin cells (Peragine et al., 2017; Poling et al., 2013; Rizwan et al., 2012).

1.2 External influences on the HPG axis

1.2.1 Social information

Social interaction is an unescapable requirement of life. As sexually reproducing organisms, animals must engage with an opposite-sex conspecific in order to procreate. Many species live in mixed-sex social groups for at least some portion of their adult lives. The makeup, patterning, and outcomes of resulting social interactions have a strong influence on reproductive ability. Succeeding or failing in social interactions may determine whether a potential mate engages or avoids, or determine access to the resources necessary to attract a mate in the first place. Thus, animals are exquisitely sensitive to their social context and will integrate this information when deciding to reproduce. General social context, such as place in a social hierarchy or the makeup of one's home social group, has long-lasting effects on circulating sex steroids and reproductive physiology (e.g., (Bruce, 1959; Vandenbergh, 1967; Whitten, 1957; Williamson et al., 2017a)). However, this section will focus on less discussed phenomena in which social interactions have acute consequences on HPG axis activity.

Not only does exposure to a potential mate elicit solicitation or appetitive sexual behaviors, in males of many species it also quickly stimulates a transient increase in LH leading to an acute increase in testosterone (T) (Coquelin and Bronson, 1980; Nyby, 2008). In rodents, this phenomenon is driven largely by exposure to female chemosensory cues (i.e., pheromones) (Liberles, 2014; Petrulis, 2013; Singer et al., 1988). The function of this anticipatory T release is not settled and likely differs across species, but hypotheses include facilitating sexual behavior

and penile reflexes, bolstering spermatogenesis, promoting production of androgen-dependent pheromones, acutely reducing anxiety, and reinforcement of mate-seeking behavior (Aikey et al., 2002; Alexander et al., 1994; Hart, 1983; James and Nyby, 2002; Nyby, 2008). A similar phenomenon occurs in anestrous female ungulates, in which exposure to male pheromones rapidly stimulates GnRH pulses and initiates out-of-season estrous cycling (Gelez and Fabre-Nys, 2004; Knight and Lynch, 1980; Martin et al., 1986). Recently, the arcuate kisspeptin system has been implicated in mediating this "male effect" in goats (De Bond et al., 2013; Murata et al., 2011; Sakamoto et al., 2013; Wakabayashi et al., 2010). However, the neural pathway(s) by which female chemosensory information elicits anticipatory T release in males remains unresolved.

Males also experience a transient increase in T in response to antagonistic encounters. This highly conserved phenomenon is often considered within the framework of the Challenge Hypothesis proposed by Wingfield and colleagues (Wingfield, 2012; Wingfield et al., 1990). This hypothesis, initially developed to describe patterns of T seen in avian species across breeding seasons, predicts that T is elevated during periods of social challenge. The Challenge Hypothesis proposes that animals exhibit a minimum basal T concentration, an elevated basal T concentration during extended periods of social challenge (e.g., a breeding season with mate and resource competition), and a maximal T concentration during acute periods of challenge. In this view, animals will increase T as necessary to facilitate responding to the social challenge, but will otherwise avoid the energetic cost of T production and the negative physiological consequences of long-term elevated T (e.g., suppressed immune function and paternal behavior) (Wingfield et al., 2001).

In *Peromyscus californicus*, this acute T increase is necessary for developing the Winner Effect – a phenomenon in which winners of an antagonistic encounter are more likely to win future encounters (Fuxjager et al., 2011a; Fuxjager et al., 2011b; Oyegbile and Marler, 2005). This relationship between acute T increase and winning likely extends to humans, in whom T increases after winning a sporting event or even just witnessing one's favorite team win (Carré and Olmstead, 2015; Edwards, 2006). Beyond facilitating the Winner Effect, this acute increase in T has also been hypothesized to aid territory formation (reinforcing return to an area in which one defeated the prior occupant) and social hierarchy formation (Gleason et al., 2009). Similar to reflexive T release in response to females and consistent with traditional HPG axis-driven increases in T, concentrations typically peak 30-45min after initiation of the antagonistic encounter (Buck and Barnes, 2003; Gleason et al., 2009; Oyegbile and Marler, 2005; Sachser and Prove, 1984). However, it remains unknown which neural elements of the HPG axis are acutely sensitive to antagonistic contexts and may drive this transient T increase.

1.2.2 Photoperiodic information

In order to successfully reproduce, animals need sufficient food and water, tolerable weather and temperature, and some modicum of safety from predators. All of these necessities vary seasonally for the vast majority of the planet. As a result, many species limit breeding to a specific time of year so that the most energetically expensive phase of reproduction (often lactation, but may be birth, weaning, etc. for some species) occurs when conditions are most favorable (Bronson, 1989). Often, these species rely on information about the prevailing

photoperiod to synchronize breeding to the optimal circannual time. Photoperiodic information is transduced into a nocturnal melatonin signal, where duration of melatonin release from the pineal gland encodes duration of night (Reiter, 1993; Tamarkin et al., 1985).

In hamsters, exposure to winter-like short-day photoperiod suppresses reproduction and initiates gonadal regression, characterized by a fall in gonadotropin release, circulating sex steroids, and gonadal size along with a suspension of spermatogenesis or ovulatory cyclicity (Carter and Goldman, 1983; Hoffman and Reiter, 1965; Hoffmann, 1973). Approximately four months later, hamsters become insensitive to the inhibitory effects of short-day photoperiod and their HPG axis reactivates, driving recrudescence of gonadal size and function (Bittman, 1978; Schlatt et al., 1995; Schlatt et al., 1993). This process enables hamsters to cease breeding as conditions become unfavorable, but also begin the months-long restoration process in time to take advantage of the earliest favorable conditions. In contrast, sheep are short-day breeders allowing for them to deliver young in early spring, and are therefore stimulated by exposure to long-duration melatonin (Malpaux et al., 1997). Comparative analysis across these species has been tremendously informative in dissociating the effects of melatonin from components essential for changes in reproductive state (Weems et al., 2015).

Understanding the neural substrates mediating photoperiodic control of the HPG axis has long been of interest to the field of neuroendocrinology. GnRH release is decreased in the non-breeding season (Barrell et al., 1992), but GnRH cells do not appear to respond directly to melatonin (Malpaux et al., 2001). Additionally, sensitivity to negative feedback by steroid hormones increases in the non-breeding season, to varying extents across species (Kriegsfeld and Bittman, 2009; Legan et al., 1977). Since the discovery of kisspeptin and RFRP, these peptides have received immense interest as potential mediators of photoperiodic control of GnRH release. As discussed in detail in chapter 4, there is abundant evidence suggesting that changes within kisspeptin and RFRP systems indeed drive changes in HPG axis activity in response to photoperiod (Clarke and Caraty, 2013; Dardente et al., 2016; Henningsen et al., 2016b; Simonneaux et al., 2013).

Upstream of RFRP and kisspeptin, recent work implicates changes in thyroid hormones in communicating changes in melatonin to the HPG axis (reviewed in (Dardente et al., 2014)). This model proposes that long-duration melatonin acts on the pars tuberalis to inhibit production of thyroid stimulating hormone (TSH) (Dardente et al., 2010; Dupre et al., 2010). TSH alters expression of the enzymes type 2 deiodinase (Dio2) in the mediobasal hypothalamus, which catalyzes the conversion of the T_4 prohormone into the more biologically active T_3 hormone, and type 3 deiodinase (Dio3), which converts T_3 into the bio-inactive T_2, (Hanon et al., 2008). Exposure to short-day photoperiod decreases the ratio of Dio2:Dio3 and decreases local concentrations of T_3, whereas transfer to long-day increases Dio2:Dio3 (Barrett et al., 2007; Revel et al., 2006b; Saenz de Miera et al., 2013; Watanabe et al., 2004; Yasuo et al., 2007, 2010; Yoshimura et al., 2003). Resulting changes in local T_3 then signal to other neural loci to modify GnRH secretion. The exact pathway between this system and Kisspeptin and RFRP remains a topic of inquiry, but exogenous TSH or T_3 treatment to reproductively regressed male hamsters does reverse short day photoperiod-induced changes in kisspeptin and RFRP labeling (Henson et al., 2013; Klosen et al., 2013).

1.3 Questions

The work presented in the following chapters investigates the social and photoperiodic regulation of neural elements of the HPG axis. In Chapter 2, I asked whether aggression was associated with changes in Kisspeptin or RFRP systems in male mice, a necessary first step to begin understanding the neural substrates driving acute T increases in response to social challenge. In Chapter 3, I investigated the photoperiodic gating of the male sexual behavior and reflexive T release in response to female chemosignals using Syrian hamsters. This study explores the integration of both social and photoperiodic information within specific elements of the chemosensory pathway and of the HPG axis. Finally, in Chapter 4, I examined changes in *kiss1* and *rfrp* expression across regression and recrudescence in male and female Siberian hamsters. These data provide basic insight into the photoperiodic regulation of upstream GnRH regulators. Finally, I conclude with a broad consideration of my work as a whole and propose avenues for future research.

Chapter 2: Aggressive Interactions are Associated with Reductions in RFamide-Related Peptide, but not Kisspeptin, Neuronal Activation in Mice

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2.1 Abstract

Aggressive interactions lead to changes in both future behavior and circulating testosterone (T) concentrations in animals across taxa. The specific neural circuitry and neurochemical systems by which these encounters alter neuroendocrine functioning are not well understood. Neurons expressing the inhibitory and stimulatory neuropeptides, RFamide-related peptide (RFRP) and kisspeptin, respectively, project to neural loci regulating aggression in addition to neuroendocrine cells controlling sex steroid production. Given these connections to both the reproductive axis and aggression circuitry, RFRP and kisspeptin are in unique positions to mediate post-encounter changes in both T and behavior. The present study examined the activational state of RFRP and kisspeptin neurons of male C57BL/6 mice following an aggressive encounter. Both winners and losers exhibited reduced RFRP/FOS co-localization relative to handling stress controls. Social exposure controls did not display reduced RFRP neuronal activation, indicating that this effect is due to aggressive interaction specifically rather than social interaction generally. RFRP neuronal activation positively correlated with latencies to display several offensive behaviors within winners. These effects were not observed in the anteroventral periventricular (AVPV) nucleus kisspeptin cell population. Together, these findings point to potential neuromodulatory role for RFRP in aggressive behavior and in disinhibiting the reproductive axis to facilitate an increase in T in response to social challenge.

2.2 Introduction

Aggressive encounters lead to alterations in reproductive axis activity and downstream testosterone (T) concentrations. Modulating T in response to social interactions enables individuals to rapidly adapt to a changing social context while avoiding the costs associated with chronically elevated T (e.g., immunosuppression, reductions in parental care). The Challenge Hypothesis predicts that T concentrations are elevated at times of social instability and competition, and increase in response to territorial or resource challenges to facilitate antagonistic responses (Wingfield et al., 1990). Originally proposed to explain variability in T responsiveness in birds, this hypothesis has found support across diverse taxa including fish, rodents, and humans (Almeida et al., 2014; Antunes and Oliveira, 2009; Archer, 2006; Carré and Olmstead, 2015; Fuxjager et al., 2011b; Goymann et al., 2007; Scotti et al., 2009; Tibbetts and Crocker, 2014).

The outcome of an antagonistic encounter differentially affects post-encounter T, with winners generally increasing (or remaining elevated) and losers generally decreasing T concentrations (Carré and Olmstead, 2015; Gleason et al., 2009; Huhman et al., 1991; Oliveira, 2005; Rose et al., 1972). In humans, the Biosocial Model of Status argues that win-driven increases in T reinforce dominant behaviors whereas loss-driven decreases in T inhibit status-seeking behaviors, including future aggression (Mazur, 1985; Mazur and Booth, 1998). These so-called Winner and Loser Effects have been described in a myriad of species whereby winning increases future aggressiveness and the probability of winning future encounters whereas losing

increases submissiveness and decreases the probability of winning (Brain and Poole, 1974; Frischknecht et al., 1982; Hsu et al., 2006; Huhman et al., 1991; Lehner et al., 2011; Oliveira et al., 2009). Whereas social modulation of T appears to mediate at least some portion of winner and loser effects (Carré and Olmstead, 2015; Hirschenhauser et al., 2013; Oliveira et al., 2009; Solomon et al., 2009; Trainor et al., 2004), changes to behavior still occur when T modulation is prevented (Albert et al., 1989; Fuxjager et al., 2011b; Hirschenhauser et al., 2013; Maruniak et al., 1977; Nock and Leshner, 1976; Oliveira et al., 2009), suggesting alterations in the neural substrates driving future behavior (e.g., (Fuxjager et al., 2010; Huhman and Jasnow, 2005).

Due to its well characterized projections to both the reproductive axis and neural loci implicated in the control of aggression (Kriegsfeld et al., 2006; Ubuka et al., 2012a; Ubuka et al., 2009), the hypothalamic neuropeptide RF-amide related peptide (RFRP) represents a promising candidate system mediating antagonistic encounter-induced changes in T and behavior. RFRP (the mammalian orthologue of avian gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000)) functions as a potent negative regulator of the mammalian reproductive axis (reviewed in (Kriegsfeld et al., 2014; Tsutsui and Ubuka, 2014; Ubuka et al., 2013b)). RFRP neurons localized to the dorsomedial hypothalamus (DMH) inhibit reproductive axis activity by suppressing release of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) (Ducret et al., 2009; Kriegsfeld et al., 2006; Tsutsui et al., 2000; Ubuka et al., 2012c).

In addition to its role in reproductive axis regulation, RFRP neurons also project to limbic structures associated with the control of social and motivated behaviors, including the amygdala, bed nucleus of the stria terminalis (BNST), lateral septum, anterior hypothalamus, and periaqueductal gray (Goodson, 2005; Kriegsfeld et al., 2006; Newman, 1999; Ubuka et al., 2012a; Ubuka et al., 2009). The function of these projections is not well studied, but their existence suggests a role for RFRP in the direct neuronal regulation of behavior in addition to downstream impact on the reproductive axis. In support of this possibility, RFRP was recently shown to suppress female sexual behavior independent of actions on circulating gonadal steroids (Piekarski et al., 2013). RFRP has been implicated in the regulation of rapid hormonal change in response to female stimuli in male birds (Tobari et al., 2014), male and female sexual behavior in birds and rodents (Bentley et al., 2006; Johnson et al., 2007; Klingerman et al., 2011; Ubuka et al., 2013a) and aggression in male birds (Ubuka et al., 2014; Ubuka et al., 2013a; Ubuka et al., 2012b) (for review see (Calisi, 2014)).

In contrast to RFRP, kisspeptin, the product of the *Kiss1* gene, potently stimulates the reproductive axis (Clarke and Caraty, 2013; de Roux et al., 2003; Han et al., 2005; Kriegsfeld, 2013b; Seminara et al., 2003; Terasawa et al., 2013). Despite the well-recognized role of kisspeptin in reproduction, its role as a behavioral modulator has only been minimally examined. Similar to the RFRP system, kisspeptin neurons project to brain regions outside the reproductive axis, including the amygdala and BNST (Lehman et al., 2013), pointing to a potential role in modulating social and motivated behaviors. In agreement with this possibility, kisspeptin receptor mRNA expression is positively associated with social status and territoriality in the cichlid fish *Astatotilapia burtoni* (Grone et al., 2010). Additionally, kisspeptin cells receive socially relevant information via the chemosensory system, the primary sensory modality for social communication in most rodents (Jouhanneau et al., 2013).

In the present study, we considered the possibility that RFRP and kisspeptin cells alter their activity, presumably to mediate alterations in T and behavior, in response to an antagonistic encounter. To explore whether RFRP and kisspeptin are sensitive to antagonistic contexts, we investigated the pattern of RFRP and kisspeptin cellular activation following an aggressive encounter in male mice. Additionally, these findings were considered in relation to the offensive and defensive behaviors displayed during the encounter. Because winning and losing frequently induce diametrically opposed changes in T concentrations, both winners and losers from each encounter were examined, and results are considered in the context of previous literature on antagonistic T modulation.

2.3 Materials and methods

2.3.1 Animals

Adult (> 60 days of age, n = 38) male C57BL/6J mice were obtained from The Jackson Laboratory (Sacramento, CA) and maintained on a 14:10 light:dark cycle (14 h light/day, lights off at 13:00 Pacific Standard Time). Upon arrival, mice were group-housed (4-5 per cage) at 23 ± 1°C in polypropylene cages (30 x 15.5 x 13 cm) furnished with Tek-Fresh Lab Animal Bedding (Harlan Laboratories, Madison, WI) and cotton nesting material. Tap water and rodent chow (Teklad Global Rodent Diet 2918, Harlan Laboratories) were available *ad libitum*. Mice were allowed to acclimate to local conditions for 1-2 weeks before surgery. All procedures were approved by the Animal Care and Use Committee of the University of California at Berkeley and conformed to principles and practices in the NIH guide for the use and care for laboratory animals.

2.3.2 Surgical procedures

Because aggressive interactions alter testosterone concentrations in opponents, and changes in gonadal steroid levels may affect upstream regulators of the reproductive axis, such as RFRP or kisspeptin, through positive or negative feedback, testosterone concentrations were clamped to basal concentrations via castration and testosterone propionate (TP) capsule implantation. Castrations were performed by anesthetizing mice with isoflurane vapors (Clipper Distributing Company, St. Joseph, MO) and excising the testes via two midline incisions in the lower abdominal cavity as previously described. Testosterone replacement was achieved through implantation of subcutaneous Silastic capsules (1.02 mm I.D. x 2.16 mm O.D.; Dow Corning, Midland, MI) filled with crystalline TP (10 mm TP; Sigma Aldrich, St. Louis, MO) through a small midline incision in the nape of neck at the time of castration. Silastic capsules were primed prior to implantation by submersion in 0.9% saline for 12-24 h and yield plasma T concentrations within physiological range for this species (Scordalakes and Rissman, 2003). Mice received buprenorphine (0.1 mg/kg, Hospira, Lake Forest, IL) subcutaneously for post-operative analgesia. Immediately following surgery, mice were returned to a clean cage and singly housed for the remainder of the study.

2.3.3 Aggressive encounter

A variation of the classic resident-intruder paradigm was used to elicit offensive aggression from experimental mice, and to provide winning and losing experiences. In the typical resident-intruder test, a smaller, group-housed intruder is introduced into the home cage of a larger, singly-housed resident and the resident's aggression is quantified (Kriegsfeld et al., 1997). Because this study was concerned with the role of RFRP and kisspeptin in mediating future behavior and physiology following a winning or losing encounter, we elected to provide a balanced version of the resident-intruder test where both mice were of similar size, weight, and housing history.

To facilitate territory formation and increase the probability of offensive attack, animals' cages were not changed or cleaned for 7 days preceding the resident-intruder test. Mice were weighed during the light phase approximately 4-5 h before the resident-intruder test (10-12 days after surgery) and assigned to one of three groups: Control (n = 6), Resident (n = 16), and Intruder (n = 16), keeping average body weight balanced across groups. In order to differentiate between resident and intruder mice during the resident-intruder test, a small patch of fur (roughly 2 cm x 2 cm) was shaved from the lower back of control and intruder animals. Resident animals received a sham experience in which the razor was turned on and brought near to the animal while the lower back was touched to mimic the auditory and tactile experiences of shaving.

All resident-intruder tests occurred between 1 h and 4 h after lights off (i.e., early part of the dark/active phase) and were performed under dim red light. For resident-intruder pairs, the intruder mouse was transferred to the resident's home cage for 10 min and then returned to its original home cage. To provide a handling stress control group, Control animals were transferred to a clean, empty cage for the 10 min period and then likewise returned to their original home cage. The resident-intruder test was video-recorded with a digital camera (Sony DCR-PC5, Sony Electronics, San Diego, CA) in night vision mode for later behavioral analysis. In no cases did the resident-intruder test result in noticeable physical injury to either mouse. To optimize detection of FOS expression, brains were collected one hour after the end of the resident-intruder test as described below.

2.3.4 Assessment of aggressive encounters

Videos of the resident-intruder test were analyzed by two independent observers blind to the experimental design using vCode version 1.2.1 (Hagedorn et al., 2008) (http://social.cs.uiuc.edu/projects/vcode.html) for offensive, defensive, and locomotor behaviors. The following offensive behaviors were quantified for both mice: frequency, latency, and duration of attacks (i.e., lunge toward opponent with attempt to pin or bite) and escalated attacks (i.e., a tumbling attack of markedly higher intensity), along with the frequency and latency of bites. The duration, frequency, and latency of mice adopting the defensive, upright position were also quantified as an example of defensive behavior. Display of submissive behavior (i.e., fleeing, freezing, defensive upright position, or flinching) after each attack-bout was also recorded on a qualitative presence/absence basis to determine winner and loser. A mouse was categorized as a winner if it displayed at least three consistent attacks eliciting submissive behaviors from its opponent while not being attacked itself before the end of the encounter (see

(Oyegbile and Marler, 2005)). Locomotor activity was also assayed by analyzing time spent stationary during the resident-intruder test. A locomotor score was computed by subtracting total stationary time from total time (10 min).

2.3.5 Perfusion and immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital (200 mg/kg, Med-Pharmex, Pomona, CA) and perfused transcardially with approximately 30 ml of 0.9% saline, followed by approximately 100 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.3). Brains were postfixed for 4 h in 4% paraformaldehyde followed by cryoprotection in 30% sucrose in 0.1 M PBS at 4°C until sectioned. Brains were sectioned in the coronal plane at 40 μm on a Leica 3050S cryostat and stored at -20°C in an ethylene glycol and sucrose based antifreeze until immunohistochemistry was performed. The AVPV region of one animal in the Losers group was accidentally destroyed during sectioning.

To visualize the colocalization of FOS with RFRP or kisspeptin, double-label immunofluorescence was performed on separate sets of every fourth 40 μm brain slice. Free floating sections were washed in PBS, incubated for 10 min in 0.5% hydrogen peroxide, washed in PBS again, and then blocked for 1 h in 2% normal goat serum suspended in 0.1% Triton X-100 (PBT). Sections were then incubated for 48 h at 4°C in either a rabbit polyclonal anti-GnIH antibody (1:40,000; PAC 123/124, a generous gift from Dr. George Bentley) or a rabbit polyclonal anti-kisspeptin-10 antibody (1:8,000; Millipore, Billerica, MA) with 1% normal goat serum in PBT. After incubation in the primary antibody, sections were washed in PBT, incubated for 1 h in biotinylated goat anti-rabbit IgG (1:250, Vector Laboratories, Burlingame, CA), washed in PBT, and incubated for 1 h in avidin-biotin-horseradish peroxidase complex (ABC Elite Kit, Vector Laboratories). Sections were then washed with PBT followed by 0.6% biotinylated tyramide solution for 30 min. After washing with PBS, cells were fluorescently labeled with Alexa Fluor 488 strepdavidin conjugate (1:150, Life Technologies, Grand Island, NY). Next, sections were washed with PBS and incubated for 48 h at 4°C with a rabbit anti-FOS primary antibody (1:5,000 for RFRP/FOS, 1:20,000 for kisspeptin/FOS; sc-52, Santa Cruz Biotechnology, Dallas, TX) and 1% normal donkey serum in PBT. Sections were then washed with PBT and labeled with the fluorophore CY-3 donkey-anti-rabbit (1:150, Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, sections then washed with PBS and mounted on gelatin-coated slides, dehydrated and cleared with xylene, and coverslipped.

2.3.6 Microscopy and quantification

To examine the percentage of RFRP and Kisspeptin cells expressing FOS, sections were examined at the conventional light microcopy level using the standard wavelengths for Alexa Fluor 488 (488 nm) and CY-3 (568 nm) with a Zeiss Z1 microscope (Thornwood, NY). Every fourth section through the dorsomedial hypothalamus was examined for colocalization of RFRP and FOS, whereas every fourth section through the AVPV was examined for colocalization of kisspeptin and FOS. A second population of kisspeptin cells is also found in the arcuate nucleus, but is not able to be visualized in mice without pretreatment with colchicine to reduce fiber density, and so was not examined in this study. Each label was captured as a single image at 200x magnification without adjusting the plane of focus between captures and then

superimposed digitally. Brain areas were examined by two observers blind to the experimental conditions using Photoshop software (Adobe Systems, Inc., San Jose, CA) to view the Alexa Fluor 488 and CY-3 channels independently or together. A cell was considered to be double labeled if FOS was expressed in the nucleus without extending beyond its predetermined borders. Cells without a clearly identifiable nucleus were not included in analysis.

To confirm that conventional light microscopy did not result in false positives, a subset of RFRP cells was also examined using a Zeiss Axiovert 100TV fluorescence microscope with a Zeiss LSM 510 laser-scanning confocal attachment. The sections were excited with an argon-krypton laser using the standard excitation wavelengths for Alexa Fluor 488 and CY-3. Stacked images were collected as 0.8μm multitract optical sections. Using the ZEN Black software (Zeiss), the channels were digitally superimposed and cells were categorized as single or double labeled using the definition described above. One section each from 2-3 animals per group was confirmed in this manner.

2.3.7 Statistics

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). FOS colocalization data were arcsine transformed prior to analysis to meet assumptions of normality. To simplify data presentation of FOS colocalization data, figures are presented using untransformed proportions. Body weight, cell count and FOS colocalization data were analyzed with one way analyses of variance (ANOVA). *A priori* planned comparisons to analyze group differences were also performed utilizing Fisher's Least Significant Difference test. Linear regression was used to examine correlations between behavioral data and arcsine transformed RFRP/FOS and kisspeptin/FOS colocalization data. A 2 x 3 ANOVA (residency x encounter-outcome) was performed to investigate the effect of residency on the interpretation of the results. Additionally, analyses which revealed statistically significant effects were repeated excluding resident-losers and intruder-winners to confirm that combining residents and intruders within Winner or Loser groups did not bias results. All results were considered statistically significant if $p < 0.05$. Effect size was calculated as eta squared (η^2) for ANOVAs and *Cohen's d* for pairwise comparisons (http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php).

2.4 Results

2.4.1 Resident-intruder: victory, defeat, and pacifism

Of the 16 resident-intruder pairs tested, 9 pairs resulted in a clear winner, 2 pairs fought but resulted in a tie (and were therefore excluded from the present study), and 5 pairs displayed no aggression (hereafter termed "NoFighters"). These non-fighting animals were employed as self-selected, pseudo-control group, engaging in unrestricted social interaction similar to fighting mice, without exhibiting or receiving attacks. These data permit differentiation of the role of RFRP and kisspeptin neuronal activity in aggression specifically, compared to non-aggressive social interaction.

Of the 9 resident-intruder pairs that yielded a clear victor, 6 resulted in the resident winning and 3 with the intruder winning. ANOVAs revealed no main effect of residency (resident or intruder) or residency x encounter-outcome (win, loss, or no-fight) interactions on FOS colocalization data (RFRP/FOS: $F_{1,22} = 0.06$, $p > .05$, $\eta^2 < 0.01$; $F_{2,22} = 2.25$, $p > 0.05$, $\eta^2 = 0.15$; Kiss/FOS: $F_{1,22} = 0.05$, $p > .05$, $\eta^2 < 0.01$; $F_{2,22} = 1.99$, $p > .05$, $\eta^2 = 0.15$). As a result, mice are grouped by behavioral experience (Control, Winner, Loser, NoFighter) in all of the following analyses, although data were re-analyzed excluding resident-winners and intruder-winners where appropriate to increase confidence in interpretation. The failure of all residents to win their encounter may be due to many factors, including similarity in body mass and housing history (single vs group-housed) for residents and intruders, as well as variable experiences in group hierarchies before the social isolation period. Body weights did not differ across groups ($F_{3,25} = 0.20$, $p > 0.05$, $\eta^2 = 0.02$), although the weights of five animals were not recorded (one Control, two Winners, two Losers).

2.4.2 RFRP

Engaging in an aggressive interaction was associated with decreased RFRP neuronal activation. A one-way ANOVA revealed a main effect of group on the proportion of RFRP-immunoreactive (-ir) cells co-expressing FOS ($F_{3,30} = 3.28$; $p = 0.03$, $\eta^2 = 0.25$) (**Figure 1a-b**). An *a priori* planned comparison revealed that both Winners and Losers displayed significantly less RFRP/FOS colocalization than Controls ($t_{30} = 2.62$, $p = 0.01$, $d = 1.43$ and $t_{30} = 2.34$, $p = 0.03$, $d = 1.54$ respectively). In contrast, the NoFighter group did not differ from the Control group ($t_{30} = 0.86$; $p > 0.05$, $d = 0.49$). This pattern of results remains unchanged after exclusion of resident-losers and intruder-winners ($F_{3,24} = 4.07$, $p = 0.02$, $\eta^2 = 0.34$; Winners: $t_{24} = 2.46$, $p = 0.02$, $d = 1.67$; Losers: $t_{24} = 2.98$, $p = 0.01$, $d = 2.21$; NoFighters: $t_{24} = 0.91$, $p > 0.05$, $d = 0.49$). This effect was not driven by differences in total number of RFRP-ir cells, as total cell counts did not differ among groups ($F_{3,30} = 1.67$; $p > 0.05$, $\eta^2 = 0.14$) (**Figure 1c**).

RFRP/FOS was positively correlated with latencies to attack, bite, and initiate an escalated attack within Winners ($r = 0.74$, $p = 0.02$; $r = 0.78$, $p = 0.01$; $r = 0.73$, $p = 0.03$, respectively) (**Figure 2a**). No such relationship was found between RFRP/FOS and durations or frequencies of offensives behaviors in Winners (**Table 1**). Losers displayed “floor” levels of offensive behaviors and RFRP/FOS was not significantly correlated with any offensive or defensive behavioral measures within this group (**Figure 2b, Table 1**). Importantly, locomotion was not correlated with RFRP/FOS within Winners (**Table 1**), suggesting that the correlation with offensive latency was not driven by increased locomotor activity. Similarly, locomotion was not correlated with RFRP/FOS within Losers (**Table 1**), although it was negatively correlated within NoFighters ($r = -0.64$, $p = 0.05$).

2.4.3 Kisspeptin

Kisspeptin neuronal activation within the AVPV was not found to vary with behavioral experience. A one-way ANOVA revealed no effect of group on the proportion of kisspeptin-ir cells expressing FOS ($F_{3,29} = 0.31$, $p > 0.05$, $\eta^2 = 0.03$), with *a priori* planned comparisons revealing no differences among groups ($p > 0.05$ in all cases) (**Figure 3a-b**). Analogous to the findings for RFRP, the total number of kisspeptin-ir cells counted in the AVPV did not vary

between groups ($F_{3,29} = 0.05$, $p > 0.05$, $\eta^2 < 0.01$) (**Figure 3c**). The AVPV kisspeptin population is sexually dimorphic and considerably larger in females. As a result, very few (overall mean = 7.45 ± 0.65 cells) AVPV kisspeptin cells per animal were observed in this study. Finally, kisspeptin/FOS colocalization was not significantly correlated with any offensive, defensive, or locomotor behaviors measured within any group (**Table 2**).

2.5 Discussion

The present study reveals an association between RFRP neuronal activation and aggressive interaction. Both winners and losers of a single aggressive encounter exhibited reduced RFRP cellular activation relative to controls. Animals that were exposed to a novel male but did not engage in aggressive interactions did not exhibit this reduction. These findings indicate that engagement in aggressive interactions specifically, rather than social interaction generally, is associated with a reduction in RFRP neuronal activation. The extent of RFRP cellular activation was also correlated with latencies to display several offensive behaviors, including attacking, biting, and initiating an escalated attack, suggesting a neuromodulatory role for RFRP within the aggression system. Finally, neither engagement in, nor the outcome of, an aggressive encounter was associated with changes in cellular activation within the AVPV kisspeptin population. Unfortunately, however, the arcuate nucleus kisspeptin population could not be analyzed under the current design due to the density of kisspeptin fibers in this brain region. Together, these findings point to a neuromodulatory role of RFRP, but not AVPV kisspeptin, in the mammalian aggression circuit, and to a novel pathway by which aggressive experience can acutely modulate the reproductive axis.

Alterations in RFRP neuronal activation during aggressive interactions may act to facilitate initiation of offensive behavior. In mice, as well as other rodents, RFRP neurons localized to the DMH project to neural loci implicated in the control of aggression, including the amygdala, BNST, anterior hypothalamus, lateral septum, and periaqueductal gray (Kriegsfeld et al., 2006; Nelson and Trainor, 2007; Ubuka et al., 2012a). The correlation between RFRP neuronal activation and offensive latencies, but not total durations or frequencies, suggest that RFRP is associated specifically with the initiation of aggression rather than overall expression or intensity. As indicated previously, a neuromodulatory role of RFRP in social behavior circuits has been shown in hamsters (Piekarski et al., 2013), as well as in aggression specifically in Japanese quail and white-crowned sparrows (Ubuka et al., 2013a; Ubuka et al., 2012b). It is possible that the correlations revealed in the present study arise due to aggressive interactions modulating the RFRP neuronal system rather than the converse. However, given RFRP projections to numerous neural loci implicated in aggression and RFRP's anti-aggressive effect in avian species (Kriegsfeld et al., 2006; Ubuka et al., 2013a; Ubuka et al., 2012b), it seems likely that the RFRP system acts directly on aggression circuitry. It is also possible that RFRP may modulate aggression circuitry by regulating local aromatase activity and local neurosteroid concentrations, as was recently found in Japanese quail (Ubuka et al., 2014; Ubuka and Tsutsui, 2014).

In agreement with the Challenge Hypothesis (Wingfield et al., 1990), the reduction in RFRP neuronal activation during aggressive interactions may function in intact animals to facilitate T release. RFRP is a potent negative regulator of the reproductive axis, suppressing

downstream production and release of T (Kriegsfeld et al., 2014; Ubuka et al., 2013b). Males of several rodent species elevate T within 45 minutes of an aggressive encounter (Buck and Barnes, 2003; Oyegbile and Marler, 2005; Sachser and Prove, 1984; Scotti et al., 2009). Although this acute T response has not been extensively examined in mice, dominant mice generally display elevated baseline T compared to subordinates or controls (Bronson, 1973; Bronson and Marsden, 1973; Machida et al., 1981), suggesting that mice may also acutely modulate T in response to challenges. This phenomenon is typically studied using animals in established territories or given multiple winning experiences (Oyegbile and Marler, 2005; Sachser and Prove, 1984), and is often considered in relation to winner effects (Fuxjager et al., 2011a; Gleason et al., 2009; Oliveira et al., 2009). Correspondingly, defeat, especially chronic or repeated defeat, is associated with reductions in baseline T (Bronson and Eleftheriou, 1964; Huhman et al., 1991). However, to our knowledge, the effects of defeat on T in mice have only been explored using repeated defeat paradigms or by examining subordinate mice in a chronic hierarchy, and so the effects of a single, acute loss on T are unknown. Given that the present study utilized naïve animals in a single aggressive encounter, both animals may have responded to this novel challenge with acute RFRP-mediated disinhibition of the reproductive axis, neither animal having been conditioned to lose yet. Indeed, repeated defeats are often necessary before observing defeat-associated behavioral changes (Huhman et al., 1991). Differential effects of winning and losing may not be evident in the RFRP system until repeated experiences have been gained, mirroring the timeline seen with effects on baseline T and aggression in this species (Bronson and Eleftheriou, 1964; Caramaschi et al., 2008). Characterizing long-term changes in the RFRP system following establishment of dominance or social defeat, and the role of RFRP in mediating associated changes to baseline T and reproductive function, represents an interesting opportunity for further inquiry

The baseline activational state of the RFRP system in male mice in the present study is considerably higher than that previously reported in other species and sexes. Control and NoFighter groups displayed RFRP/FOS colocalization proportions of 79-84%, whereas previously reported rates of *Rfrp*/*cfos* co-expression in female mice utilizing double-label in situ hybridization range from 30-60% (Semaan and Kauffman, 2015). Similarly, RFRP/FOS colocalization in female Syrian hamsters range from 15% to 60%, depending on estrous state (Gibson et al., 2008; Kriegsfeld et al., 2006; Russo et al., 2015). Furthermore, male Pekin ducks display low levels of GnIH/FOS colocalization, 3% in controls rising to 58% after 48hr of fasting (Fraley et al., 2013). One possible explanation for elevated rates reported in the present study is that the castration and testosterone replacement treatment may have potentially increased negative feedback on the reproductive axis via increased RFRP neuronal activation. Alternatively, these data may be due to the isolation period preceding the resident-intruder experience. Social isolation is considered stressful in mice (Brain, 1975), and stress has been shown to increase RFRP-ir cell number and upregulate RFRP mRNA expression in house sparrows and rats via a glucocorticoid-dependent process (Calisi et al., 2008; Geraghty et al., 2015; Kirby et al., 2009; Son et al., 2014). Resident-intruder testing and aggressive interactions increase glucocorticoids in several species, especially in defeated animals (Bronson and Eleftheriou, 1965; Huhman et al., 1991). Thus, it is therefore notable that both winning and losing mice exhibit reduced RFRP neuronal activation, despite presumably elevating corticosterone.

Absence of findings in the AVPV kisspeptin population in the present study does not preclude the involvement of the arcuate nucleus kisspeptin population in aggression or associated reproductive changes. The arcuate kisspeptin system plays a major role in generating daily pulses of GnRH, and consequently LH (Ezzat et al., 2015; Okamura et al., 2013a). Thus, if reductions in RFRP neuronal activation during aggression function to disinhibit GnRH or LH release, the arcuate kisspeptin population might be expected to concomitantly increase activation and stimulate GnRH release, together driving downstream T release. Similar temporal coordination of RFRP disinhibition with kisspeptin excitation of the GnRH system is critical for optimal preovulatory LH surge functioning (Khan and Kauffman, 2012; Russo et al., 2015). Work in goats has also established the arcuate kisspeptin system as a major target of the olfactory system, conveying socially relevant chemosensory information and triggering generation of LH pulses in response to female stimuli (Jouhanneau et al., 2013; Kendrick, 2014). Whether a similar mechanism underlies antagonistic modulation of T is a question for future research.

Together, the present findings point to a role for the RFRP system in modulating the expression of offensive aggression and potentially behaviorally-modified sex steroid concentrations. To our knowledge, this is the first report implicating RFRP in the regulation of aggression in a mammalian species, and provides further support for the role of RFRP as a neuromodulator in social behavior circuits. The present findings, in combination with previous studies pointing to a role for RFRP in modulating motivated behavior, underscore the potential for further investigation of this neuropeptide as locus for the interpretation and propagation of socially relevant information.

2.6 Acknowledgments

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2.7 Tables

Table 1. Correlations between behavior and RFRP/FOS colocalization

Behavior	Frequency		Duration		Latency	
	r	*p*	*r*	*p*	*r*	*p*
Winners						
Escalated Attack	-0.158	0.685	-0.215	0.579	0.726	0.027*
Attack	-0.466	0.207	-0.213	0.583	0.739	0.023*
Bite	-0.365	0.334	-	-	0.782	0.013*
Locomotion	-	-	0.024	0.951	-	-
Losers						
Escalated Attack	0.530	0.142	0.454	0.220	-0.605	0.085
Attack	0.057	0.885	0.141	0.718	-0.052	0.895
Bite	0.055	0.889	-	-	-0.077	0.845
Defensive Upright	-0.072	0.853	0.059	0.880	0.260	0.500
Locomotion	-	-	-0.010	0.979	-	-
NoFighters						
Locomotion	-	-	-0.635	0.049*	-	-

Table 2. Correlations between behavior and kisspeptin/FOS colocalization

Behavior	Frequency		Duration		Latency	
	r	*p*	*r*	*p*	*r*	*p*
Winners						
Escalated Attack	-0.263	0.495	-0.026	0.948	0.019	0.961
Attack	-0.242	0.530	0.078	0.841	-0.113	0.773
Bite	-0.091	0.816	-	-	0.053	0.892
Locomotion	-	-	-0.535	0.138	-	-
Losers						
Escalated Attack	-0.358	0.384	0.052	0.902	0.087	0.839
Attack	0.239	0.570	0.360	0.381	-0.184	0.663
Bite	0.581	0.131	-	-	-0.530	0.177
Defensive Upright	0.265	0.527	0.019	0.964	0.071	0.867
Locomotion	-	-	0.078	0.854	-	-
NoFighters						
Locomotion	-	-	0.151	0.678	-	-

2.8 Figures

Figure 1 (A) Representative photomicrographs depicting RFRP-ir (green) and FOS-ir (red) neurons from each experimental group. Open arrows indicate single-label RFRP-ir cells whereas closed arrows indicate RFRP/FOS-ir cells. (B) Mean (± SEM) percent of RFRP cells co-expressing FOS. (C) Mean (± SEM) number of RFRP cells observed on every fourth 40μm section spanning the DMH. * denotes $p < .05$ compared to Controls.

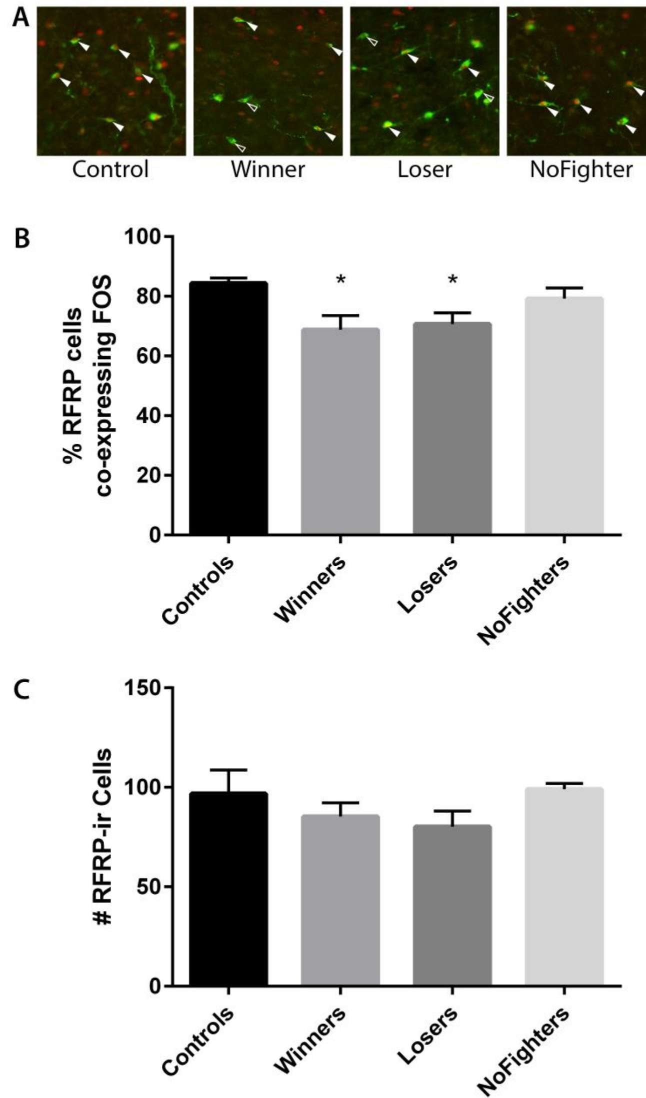


Figure 2. Regression between latencies to initiate offensive behaviors (attack, bite, escalated attack) and arcsine transformed percent RFRP/FOS colocalization within (A) Winners and (B) Losers. * denotes p < .05.

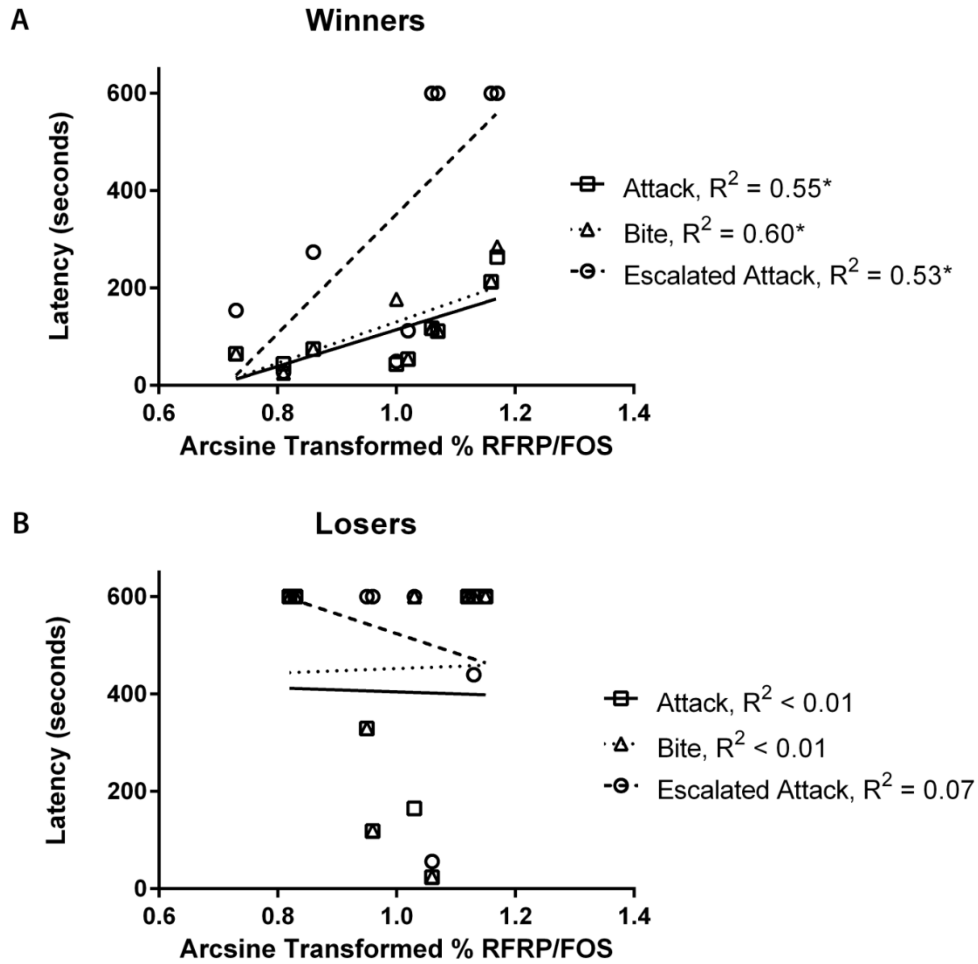
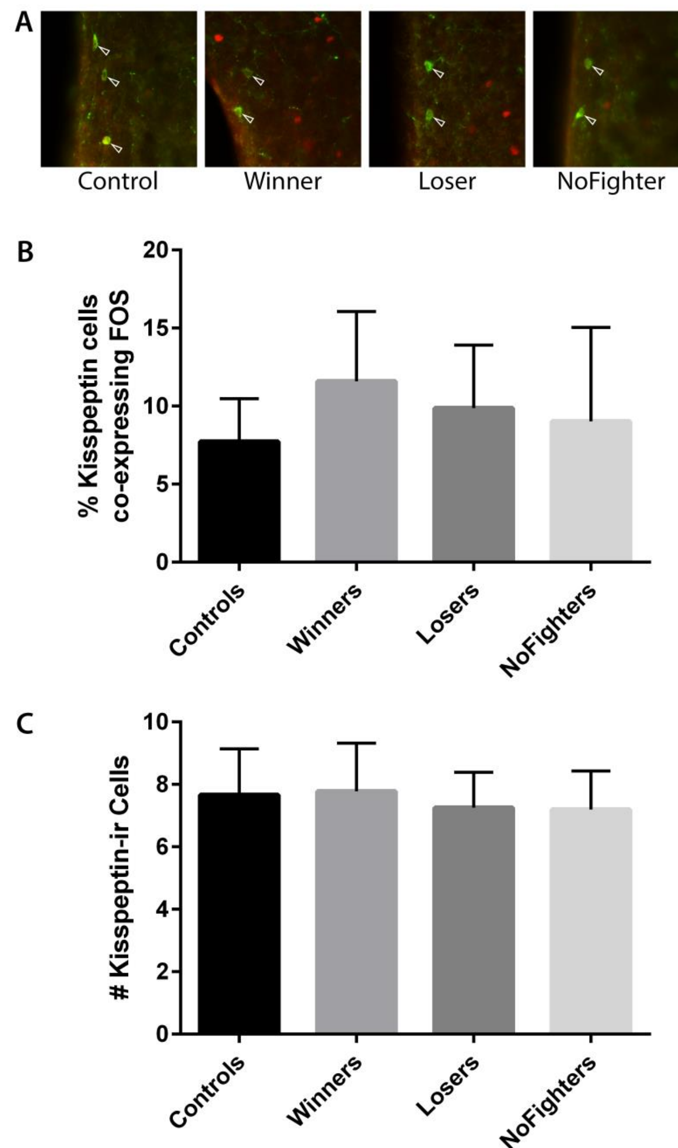


Figure 3. (A) Representative photomicrographs depicting kisspeptin-ir (green) and FOS-ir (red) neurons within the AVPV from each experimental group. Open arrows indicate single-label kisspeptin-ir cells. (B) Mean (± SEM) percent of AVPV kisspeptin cells co-expressing FOS. (C) Mean (± SEM) number of kisspeptin cells observed on every fourth 40μm section spanning the AVPV.



Chapter 3: The Preoptic Area and the RFamide-Related Peptide Neuronal System Gate Seasonal Changes in Chemosensory Processing

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3.1 Abstract

Males of many species rely on chemosensory information for social communication. In male Syrian hamsters (*Mesocricetus auratus*), as in many species, female chemosignals potently stimulate sexual behavior and a concurrent, rapid increase in circulating luteinizing hormone (LH) and testosterone (T). However, under winter-like, short-day photoperiods, when Syrian hamsters are reproductively quiescent, these same female chemosignals fail to elicit behavioral or hormonal responses, even after T replacement. It is currently unknown where in the brain chemosensory processing is gated in a seasonally dependent manner such that reproductive responses are only displayed during the appropriate breeding season. The goal of the present study was to determine where this gating occurred by identifying neural loci that respond differentially to female chemosignals across photoperiods, independent of circulating T concentrations. Adult male Syrian hamsters were housed under either long-day (reproductively active) or short-day (reproductively inactive) photoperiods with half of the short-day animals receiving T replacement. Animals were exposed to either female hamster vaginal secretions (FHVS) diluted in mineral oil or to vehicle, and the activational state of chemosensory processing centers and elements of the neuroendocrine reproductive axis were examined. Components of the chemosensory pathway upstream of hypothalamic centers increased expression of FOS, an indirect marker of neuronal activation, similarly across photoperiods. In contrast, the preoptic area of the hypothalamus (POA) responded to FHVS only in long-day animals, consistent with its role in promoting expression of male sexual behavior. Within the neuroendocrine axis, the RF-amide related peptide (RFRP), but not the kisspeptin neuronal system responded to FHVS only in long-day animals. Neither response within the POA or the RFRP neuronal system was rescued by T replacement in short-day animals, mirroring photoperiodic regulation of reproductive responses. Considering the POA and the RFRP neuronal system promote reproductive behavior and function in male Syrian hamsters, differential activation of these systems represents a potential means by which photoperiod limits expression of reproduction to the appropriate environmental context.

3.2 Introduction

Species inhabiting temperate or boreal climates have adapted to survive winter through strict temporal regulation of reproduction, with the most energetically expensive phases of reproduction (e.g. lactation) restricted to times of year when resources are most abundant (i.e. spring). For Syrian hamsters (*Mesocricetus auratus*), reproduction is limited to long-day (LD) photoperiods of spring and summer and exposure to winter-like, short-day (SD) photoperiods drives reproductive quiescence (Gaston and Menaker, 1967). Quiescence can be observed through gonadal regression (Zucker and Morin, 1977), but also through alterations in the responsiveness to sexually-relevant chemosensory cues (Anand et al., 2002; Morin and Zucker, 1978). The neural mechanisms by which photoperiod regulates chemosensory processing to limit reproduction to the appropriate season are currently unknown.

Female chemosignals (e.g., pheromones) elicit reproductive responses in males of many species (Liberles, 2014; Nyby, 2008), but male Syrian hamsters are especially reliant on chemosensory cues for social signaling. In the natural environment, solitary dwelling females scent mark using vaginal secretions to attract males to her burrow on the day of ovulation (Lisk et al., 1983). Female hamster vaginal secretions (FHVS) are an "attractive" stimulus and exposure promotes expression of male sexual behavior (Darby et al., 1975; Johnston, 1974; Murphy, 1973). Lesions to either the main or accessory olfactory systems impair male sexual behavior (Murphy and Schneider, 1970; Pfeiffer and Johnston, 1994). In parallel to its behavior-promoting qualities, exposure to FHVS also stimulates a robust neuroendocrine response in the form of rapid release of luteinizing hormone (LH) and testosterone (T) (Macrides et al., 1974; Richardson et al., 2004).

In contrast to reproductively active males, hamsters housed under SD photoperiods fail to show either behavioral or neuroendocrine responses to these same chemosensory cues (Anand et al., 2002; Morin and Zucker, 1978). Differences in responses to female chemosignals is not solely attributable to differences in circulating T. Expression of male sexual behavior is T-dependent (Powers and Bergondy, 1983; Whalen and Debold, 1974), and hamsters housed under SD photoperiods undergo gonadal regression and have correspondingly low concentrations of circulating T (Sisk and Turek, 1983). However, T replacement is much less effective at restoring male sexual behavior in SD hamsters than in LD hamsters (Campbell et al., 1978; Miernicki et al., 1990; Morin and Zucker, 1978; Pospichal et al., 1991). Thus, it is likely that photoperiod induces changes in the underlying neural substrates processing chemosignals to limit expression of reproductive responses to the appropriate season. The goal of the present study was to reveal these regulation centers by identifying neural loci that respond to female chemosignals differentially across photoperiods, and whose response in SD hamsters is not rescued by T replacement.

Photoperiod could impact chemosensory processing at any point in the chemosensory pathway, from early sensory processing by the main and accessory olfactory systems through integration by the medial amygdala (Coolen and Wood, 1998; Petrulis, 2013). Alternatively, early processing may remain consistent across photoperiods, but differences in downstream hypothalamic target structures may gate behavioral or neuroendocrine output. To test these hypotheses, the present study examined induction of FOS, the protein product of the immediate early gene *cfos* and indirect marker of neuronal activation, along the chemosensory pathway from sensory input to hypothalamic targets (Fiber et al., 1993; Petrulis, 2013), in response to FHVS.

Considering the robust neuroendocrine component of male responses to female chemosignals, gating of chemosensory processing may also occur within elements of the neuroendocrine reproductive axis. Although chemosignal-induced increases in LH are presumed to be driven by upstream release of gonadotropin releasing hormone (GnRH), neither expression of GnRH mRNA nor rates of GnRH/FOS coexpression are altered by acute conspecific pheromonal stimulation (Gore et al., 2000; Richardson et al., 2004; Taziaux and Bakker, 2015). The neuropeptide kisspeptin is expressed within neurons of the anteroventral periventricular nucleus (AVPV) and arcuate nucleus of the hypothalamus and potently stimulates GnRH release (de Roux et al., 2003; Irwig et al., 2004; Lehman et al., 2013). Whereas the AVPV kisspeptin

population projects directly to GnRH cell bodies (Yeo and Herbison, 2011), the arcuate kisspeptin cell population acts on GnRH terminals in the mediobasal hypothalamus to facilitate GnRH release without necessitating changes in GnRH cell firing rate (or FOS coexpression) (d'Anglemont de Tassigny et al., 2008). The arcuate kisspeptin system has also been implicated in mediating male chemosignal-induced increases in LH in female goats (De Bond et al., 2013; Jouhanneau et al., 2013; Sakamoto et al., 2013). Thus, we also examined these populations of kisspeptin cells as possible loci at which female chemosignals act to increase LH release.

In addition to kisspeptin, the neuropeptide RFamide-related peptide (RFRP; the mammalian ortholog of avian gonadotropin inhibitory hormone (GnIH)) also potently regulates release of GnRH and is sensitive to social context (Calisi et al., 2011; Jennings et al., 2016; Kriegsfeld et al., 2006; Tobari et al., 2014). In male Syrian hamsters, RFRP stimulates release of LH in both LD and SD photoperiods (Ancel et al., 2012), although the stimulatory nature of this effect is sex (Kriegsfeld et al., 2006) and species specific (Ubuka et al., 2012a). Interestingly, RFRP may also directly regulate release of LH by the anterior pituitary through projections to the median eminence (Kriegsfeld et al., 2006; Smith et al., 2012; Tsutsui et al., 2000), suggesting an alternative pathway for chemosensory regulation of neuroendocrine function. Finally, expression of RFRP is also strongly regulated by photoperiod, independent of changes in gonadal steroids (Mason et al., 2010; Revel et al., 2008), pointing to a potential role in integrating social and photoperiodic information to gate chemosensory responses.

3.3 Materials and methods

3.3.1 Animals

Adult (56 days of age, n = 37) male Syrian hamsters (*Mesocricetus auratus*; LVG (SYR)) obtained from Charles River (Wilmington, MA) were maintained on a 14:10 h light:dark cycle (long-day, LD, lights off 22:00 Pacific Standard Time (PST)) upon arrival. After a 10 day acclimation period, 23 hamsters were transferred to a 10:14 h light:dark cycle (short-day, SD, lights off 20:00 PST) and 14 remained in the LD photoperiod. Five adult (> 60 days of age) female Syrian hamsters were housed under a 14:10 h light:dark cycle to supply FHVS. Hamsters were singly housed at 23 ± 1 °C in polypropylene cages (48 × 25 × 21 cm) furnished with Tek-Fresh Lab Animal Bedding (Harlan Teklab, Madison, WI). Tap water and Lab Diet Prolab 5P00 were available *ad libitum*. All procedures were approved by the Animal Care and Use Committee of the University of California at Berkeley and conformed to principles enumerated in the NIH guide for the use and care for laboratory animals

Twelve weeks after transfer into SD photoperiod, twelve SD animals received subcutaneous Silastic capsules (1.98 mm I.D., 3.18 O.D.; Dow Corning, Midland, MI) containing crystalline testosterone propionate (TP) (20mm TP bounded by 3mm silicone sealant at each end, Sigma Aldrich, St. Louis, MO) whereas remaining animals (n=11 SD, n=14 LD) received empty capsules, yielding three groups (LD, SD, SD+T). To implant capsules, hamsters were anesthetized with isoflurane vapors (3%; Clipper Distributing Company, St. Joseph, MO) and capsules were inserted through a small midline incision in the nape of the neck. Silastic capsules were primed prior to implantation by submersion in 0.9% saline for 24 h, and yield plasma T concentrations within the physiological range for this species (Campbell et al., 1978).

Hamsters received buprenorphine (0.1 mg/kg; Hospira, Lake Forest, IL) subcutaneously for post-operative analgesia.

3.3.2 FHVS exposure

Nine to eleven days after implantation of Silastic capsules, half of the animals in each group (n = 7 LD, 6 SD, 6 SD+T) were exposed to FHVS whereas the other half (n = 7 LD, 5 SD, 6 SD+T) were exposed to vehicle. FHVS were collected from intact, cycling females on the morning of estrus over the week preceding exposure and stored at -20°C. FHVS were thawed shortly before exposure, diluted 1:2 with mineral oil, and kept on ice until use. Hamsters were weighed during the light phase approximately 12 h before exposure and assigned to a stimulus groups. Exposure was achieved by applying approximately 20µl of diluted FHVS or vehicle directly to the snout using a standard laboratory spatula. Separate spatulas were used for FHVS and vehicle, and tools were cleaned with 70% ethanol between animals. All exposures occurred between one and four hours after lights off (i.e., early part of the dark/active phase) under dim red light. Hamsters were then left undisturbed for 1 h before being transferred to another room for perfusion.

3.3.3 Perfusion and immunohistochemistry

Hamsters were deeply anesthetized with sodium pentobarbital solution (200 mg/kg) and perfused transcardially with 100 ml 0.9% saline followed by 300 ml 4% paraformaldehyde in 0.1M PBS (pH 7.3). Paired testes, epididymis, brown adipose tissue (BAT), and epididymal white adipose tissue (EWAT) were collected and weighed to confirm animals had responded to SD photoperiod with gonadal regression appropriately. Brains were postfixed for 3 h in 4% paraformaldehyde followed by cryoprotection in 30% sucrose in 0.1 M PBS for 48 h. Brains were then frozen at −80°C until processed. 40 µm coronal brain sections were collected on a Leica 2050S cryostat at −20°C. Slices were stored at −20°C in an ethylene glycol and sucrose based antifreeze until immunohistochemistry was performed.

To visualize expression of FOS as well as the colocalization of FOS with kisspeptin or RFRP, double-label immunofluorescence was performed on separate sets of every fourth 40 µm brain section. Free floating sections were washed in PBS, incubated for 10 min in 0.5% hydrogen peroxide, washed in PBS again, and then blocked for 1 h in 2% normal goat serum suspended in 0.1% Triton X-100 (PBT). Sections were then incubated for 48 h at 4 °C in either a rabbit polyclonal anti-GnIH antibody (1:120,000; PAC 123/ 124, Dr. George Bentley) or a rabbit polyclonal anti-kisspeptin antibody (1:2000; Dr. Jens Mikkelsen) with 1% normal goat serum in PBT. After incubation in the primary antibody, sections were washed in PBT, incubated for 1 h in biotinylated goat anti-rabbit IgG (1:250, Vector Laboratories, Burlingame, CA), 1 h in avidin-biotin-horseradish peroxidase complex (ABC Elite Kit, Vector Laboratories), and 30min in 0.6% biotinylated tyramide solution, with PBT washes in between each step. After washing with PBS, cells were fluorescently labeled with CY-2 strepdavidin conjugate (1:150, Jackson ImmunoResearch Laboratories, West Grove, PA) and washed with PBS again. Next, sections incubated for 48 h at 4 °C with a rabbit anti-FOS primary antibody (1:10,000; sc-52, Santa Cruz Biotechnology, Dallas, TX) and 1% normal donkey serum in PBT. Sections were then washed with PBT and labeled with the fluorophore CY-3 donkey-anti-rabbit (1:150, Jackson

ImmunoResearch Laboratories, West Grove, PA). Finally, sections were washed with PBS and counterstained with Hoechst (for Kisspeptin/FOS) or DAPI (for RFRP/FOS).

3.3.4 Microscopy and quantification

Sections were examined at the conventional light microcopy level using the standard wavelengths for CY-2 (488 nm), CY-3 (568 nm), and DAPI/Hoechst (358 nm) with a Zeiss Z1 microscope (Thornwood, NY). Each label was captured as a single image at 200× magnification without adjusting the plane of focus between captures and then superimposed digitally. Brain areas were examined by observers blind to the experimental conditions using Image J (NIH) to view the three channels independently or together.

Expression of FOS was manually quantified within the piriform cortex (anterior, median, posterior), septum, ventromedial hypothalamus (VMH), medial amygdala (postero-dorsal, MeApd, and postero-ventral, MeApv) anterior cortical amygdala (ACo), preoptic area (POA), bed nucleus of the stria terminalis (BNST), the accessory olfactory bulb (AOB) (granular and mitral layers), and the main olfactory bulb (MOB) granular layer using sections double labeled for Kisspeptin and FOS. Every fourth section through the dorsomedial hypothalamus was examined for colocalization of RFRP and FOS, and every fourth section through the anteroventral periventricular nucleus (AVPV) was examined for colocalization of kisspeptin and FOS. Neuroanatomical regions were defined by reference to a published Syrian hamster atlas (Morin and Wood, 2001). A cell was considered to be double labeled if FOS was expressed in the nucleus without extending beyond its predetermined borders. Cells without a clearly identifiable nucleus were not included in analysis.

A second population of kisspeptin cells is also found in the arcuate nucleus, but cell bodies are not able to be visualized without pretreatment with colchicine to reduce fiber density. As a result, arcuate kisspeptin/FOS coexpression was not examined in this study. Instead, kisspeptin-immunoreactive (-ir) fiber density was quantified using a previously published approach (Losa et al., 2011). Briefly, images of kisspeptin-ir fibers were taken at 400x magnification from one section each corresponding to the anterior, median, and posterior arcuate nucleus. Images were then binarized and depixelated to minimize background, and fibers were skeletonized to a thickness of 1 pixel to control for variation in fiber thickness and brightness. The resulting pixels were quantified using the Image J Voxel Counter plug-in (NIH) and are reported as mean volume, or the proportion of voxels identified to contain kisspeptin-ir fibers.

3.3.5 Statistics

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). FOS colocalization data were arcsine transformed prior to analysis to meet assumptions of normality. Data (regional FOS expression; RFRP and kisspeptin cell counts and FOS co-localization) were analyzed using two-way Analyses of Variance (ANOVAs) for each variable with stimulus (FHVS or vehicle) and photoperiod (LD, SD, or SD+T) as factors. If the ANOVA revealed a main effect of photoperiod, Tukey's tests were used to probe group differences. If the ANOVA revealed a significant interaction effect, planned comparisons using

Fisher's LSD test were performed to examine the effect of FHVS compared to vehicle within each photoperiodic group. All results were considered statistically significant if $p < 0.05$.

3.4 Results

3.4.1 Confirmation of responsiveness to photoperiod exposure

As expected, animals exposed to SD photoperiods experienced gonadal regression and presented with smaller reproductive tissues than their LD counterparts. Body and tissue weights on the day of chemosensory exposure are listed in Table 1. Two-way ANOVAs revealed main effects of photoperiod for paired testes ($F_{(2, 29)} = 29.80$, $p < 0.001$), paired epididymis ($F_{(2, 29)} = 19.68$, $p < 0.001$), and EWAT ($F_{(2, 29)} = 6.45$, $p < 0.01$) weights. In all cases, both SD and SD+T animals has lower tissue weights than LD animals (Tukey's test; LD vs SD and LD vs SD+T $p < 0.05$, SD vs SD+T $p > 0.05$). There were no effects of photoperiod on body mass or BAT weights ($p > 0.05$ in both cases). There were no effects of stimulus or interactions between stimulus and photoperiod for any somatic weights ($p > 0.05$ in all cases).

3.4.2 FOS induction along the chemosensory pathway

Regional expression of FOS, an indirect protein marker of recent neuronal activation, in only two regions, the granular layer of the AOB and the POA, revealed a statistically significant interaction effect (AOB granular layer $F_{(2, 23)} = 6.43$, $p < 0.01$; POA $F_{(2, 30)} = 3.52$, $p = 0.04$) (Table 2). Planned comparisons revealed that FHVS exposure increased the number of FOS-ir cells in this region only within LD animals ($p = 0.04$), whereas no difference between FHVS and vehicle groups was detected within SD or SD+T animals ($p > 0.05$) (Figure 1). In contrast, no differences between vehicle and FHVS were found within LD and SD groups for the AOB granular layer ($p > 0.05$), but FHVS exposure increased FOS expression in this region within the SD+T cohort ($p < 0.01$) (Table 2). For all other regions along the chemosensory pathway, the magnitude of FOS induction following FHVS exposure was grossly similar across photoperiods. ANOVAs revealed either a main effect of stimulus (ACo $F_{(1, 27)} = 9.756$, $p < .01$; MeApd $F_{(1, 27)} = 7.227$, $p = .01$; MeApv $F_{(1, 26)} = 10.47$, $p < .01$; VMH $F_{(1, 30)} = 5.201$, $p = .03$) or no effects on the number of FOS-ir cells. In no cases was a main effect of photoperiod discovered ($p > 0.05$ in all cases).

3.4.3. Responses within the reproductive axis

Total cell number and the proportion of cells colocalizing FOS was quantified for RFRP (within the dorsomedial hypothalamus (DMH)), and kisspeptin (within the AVPV) immunoreactive (-ir) cells. Additionally, kisspeptin-ir fiber density was quantified at three levels of the arcuate nucleus (anterior, median, and posterior). Of these metrics, an ANOVA revealed a significant interaction between stimulus and photoperiod only for the total number of RFRP-ir cells ($F_{(2, 31)} = 4.790$, $p = 0.02$), with the number of cells reduced following FHVS exposure compared to vehicle exposure only within LD animals (Fisher's LSD $p < 0.001$) but not within either SD or SD+T animals ($p > 0.05$ in both cases) (Figure 2A). In contrast, the proportion of RFRP-ir cells co-expressing FOS did not vary across groups ($p > 0.05$ for all ANOVA terms) (Figure 2B).

Photoperiod-driven changes in RFRP immunoreactivity have been reported previously (Mason et al., 2010) and were replicated in the present study. A main effect of photoperiod was found for RFRP-ir cell number ($F_{(2, 31)} = 22.60$, $p < 0.001$), with LD animals overall displaying more RFRP-ir cells than both SD and SD+T animals, with SD+T animals similarly displaying more cells than SD animals (Tukey's test, $p < 0.05$ in each case) (Figure 2A). Kisspeptin immunoreactivity has also been reported to vary across photoperiods in this species (Clarke and Caraty, 2013), but in the present study, effects of both photoperiod and stimulus failed to reach statistical significance (Figure 3). Only two AVPV kisspeptin-ir cells were found to co-express FOS (one cell each in a LD/FHVS and SD+T/Vehicle animal), so statistical differences in this measure were not analyzed.

3.5 Discussion

The present study sought to identify regions or cell types in the brain that restrict expression of reproductive responses to female chemosignals to the appropriate season, identified by their differential responses to FHVS across photoperiods. Analysis of neuronal activation in early chemosensory processing centers indicates that early sensory processing remains consistent across photoperiods, suggesting that photoperiodic regulation of downstream structures is critical for differences seen in reproductive output. Indeed, downstream chemosensory targets of the preoptic area of the hypothalamus (POA) and RFRP-ir cells within the dorsomedial hypothalamus both respond to female chemosignals within LD but not SD hamsters. The POA and RFRP neuronal system promote reproductive behavior and function respectively in male Syrian hamsters (Ancel et al., 2012; Hull and Dominguez, 2007). Importantly, responses within SD hamsters are not rescued by T replacement, mirroring previous reports of the inability of T replacement to rescue male sexual behavior under SD photoperiod (Campbell et al., 1978; Miernicki et al., 1990; Morin and Zucker, 1978; Pospichal et al., 1991). Taken together, these data suggest that photoperiod acts on the POA and RFRP neuronal system in a T-independent manner, contributing to the gating of chemosensory responsiveness in SD photoperiods (Figure 4).

Despite differences in behavioral and neuroendocrine output in response to FHVS, neuronal activation of early chemosensory processing centers (e.g., MOB, ACo, BNST, MeA), as indirectly measured by FOS expression, does not vary across photoperiods and concentrations of circulating T. The significant interaction between stimulus and photoperiod seen for the AOB granular layer presents a notable exception to this statement. It is unclear why animals of the SD+T group would have a more robust response to FHVS than their SD or LD counterparts. However, it is unlikely this finding has strong biological significance in the present context, given that SD+T animals are similar to SD animals in their behavioral responses to the stimulus (Campbell et al., 1978; Miernicki et al., 1990; Morin and Zucker, 1978; Pospichal et al., 1991). Overall, these findings are consistent with previous reports that detection of, and neural responses to, FHVS do not require high circulating T (Fiber and Swann, 1996; Peters et al., 2004; Romeo et al., 1998; Swann, 1997). However, exposure to SD photoperiod is independently associated with changes in sensitivity to steroid hormones and with morphological changes in many chemosensory processing centers (see (Kriegsfeld and Bittman, 2009) for review). Thus, it is possible that photoperiod-driven alterations to these structures might contribute to differential responsiveness to female chemosignals. Results of the current study suggest that any such

photoperiod-driven changes within chemosensory centers do not interfere with neuronal activation by female chemosignals.

The POA is a major target of the chemosensory system and is essential for the expression of male sexual behavior ((Coolen and Wood, 1998); see (Hull and Dominguez, 2007) for review). The POA is a critical integration center of chemosensory and hormonal information, with removal of either input type to this region abolishing copulation (Wood and Newman, 1995). In the present study, exposure to FHVS increases neuronal activation in this region in LD but not SD animals, consistent with a role in gating reproductive behavior. Induction of FOS after FHVS exposure within the POA has been reported to require T (Swann, 1997). However, our findings indicate that chemosensory activation of this region is not rescued by T replacement in SD animals, further paralleling regulation of behavior across photoperiods (Morin and Zucker, 1978). Future research will be necessary to explore the specific mechanisms by which photoperiod regulates POA function to gate responsiveness to female chemosignals.

As mentioned previously, exposure to female chemosignals elicits a rapid increase in LH and T in LD hamsters, presumably to facilitate reproductive behavior and function upon encountering a potential mate (Anand et al., 2002; Nyby, 2008; Richardson et al., 2004). Investigation of the kisspeptin and RFRP systems, both potent upstream regulators of GnRH and LH release in male Syrian hamsters, revealed RFRP to be differentially responsive to female chemosignals across photoperiods. The number of RFRP-ir cells decreased one hour after FHVS exposure in LD but not SD animals, and this response was not rescued by T replacement. To our knowledge, this is the first report that the RFRP neuronal system is sensitive to chemosensory information. These data are consistent with building evidence that RFRP mediates social modulation of reproduction (Calisi et al., 2011; Jennings et al., 2016; Peragine et al., 2017; Tobari et al., 2014). In male Syrian hamsters, unlike many other mammalian species, RFRP acts to stimulate release of LH under both LD and SD photoperiods (Ancel et al., 2012). Thus, the acute decrease in RFRP-ir cell number observed in the present study may reflect acute release of the RFRP peptide, facilitating LH release in LD males in response to female chemosignals. A rapid release of the RFRP peptide would reduce the amount of peptide detectable with immunohistochemistry, leading to a reduction in total cell numbers. Changes in post-translational regulation cannot be excluded, but seem unlikely considering the rapid time course (1 h) and robust decrease (~50%). If true, the reduced detection of activated RFRP-ir neurons might also explain the absence of statistically significant changes in the proportion of RFRP-ir cells coexpressing FOS in response to FHVS. Regardless, these data point to the RFRP neuronal system as an important integration center for chemosensory and photoperiodic information. Expression of RFRP mRNA and peptide is regulated by photoperiod (Mason et al., 2010; Revel et al., 2008), and the high density of melatonin receptors in the DMH suggests RFRP neurons may be directly sensitive to photoperiod-driven changes in melatonin secretion (Maywood et al., 1996; Ubuka et al., 2005). Input onto RFRP neurons has not been extensively mapped, so it is unclear whether RFRP neurons receive direct input from chemosensory loci and whether any such afferents are modulated by photoperiod.

Photoperiodic modulation of RFRP neuronal responses to female chemosignals may also contribute to differences in evoked behavior. RFRP's actions on LH release are species and sex specific (Ancel et al., 2012; Kriegsfeld et al., 2006; Ubuka et al., 2012a). RFRP inhibits sexual

behavior in species/sexes in which RFRP also inhibits LH release (Bentley et al., 2006; Johnson et al., 2007; Piekarski et al., 2013; Ubuka et al., 2014). RFRP-ir neurons project to brain regions outside the neuroendocrine reproductive axis, suggesting a wider neuromodulatory role (Kriegsfeld et al., 2006). Relevant to the present investigation, RFRP neurons project to the POA, and chronic infusion of RFRP in female Syrian hamsters both reduced sexual behavior and altered expression of FOS in the POA, independent of downstream effects on sex steroids (Piekarski et al., 2013).

Findings in ungulates have implicated the kisspeptin population of the arcuate nucleus in mediating male pheromone-induced increases in LH within females. Exposure to male chemosignals causes an increase in multiunit activity recorded in close proximity to arcuate kisspeptin cells coupled to increases in LH, and the male chemosignal-induced rise in LH is blocked by infusion of a kisspeptin antagonist (De Bond et al., 2013; Murata et al., 2011). We therefore hypothesized that the arcuate kisspeptin system may also respond to female chemosignals in male hamsters. Considering expression of kisspeptin in this region is regulated by photoperiod at least somewhat independently from changes in steroid hormones (Ansel et al., 2010; Revel et al., 2006a), this population also seemed a promising candidate for photoperiodic gating of neuroendocrine responses. FHVS exposure did not cause changes in kisspeptin-ir fiber density in this region, but we were unfortunately unable to quantify the activational state of this population due to intense fiber staining obscuring cell bodies. Not unexpectedly, the current study detected no differences within the AVPV kisspeptin population after FHVS exposure. This population is implicated in sex steroid positive feedback and the control of ovulation in females (see (Kriegsfeld, 2013a) for review), but is much smaller in males and has previously been found to be unresponsive to same- and opposite-sex exposure in mice (Jennings et al., 2016; Szymanski and Keller, 2014; Taziaux and Bakker, 2015).

Taken together, the present study identifies the POA and the RFRP neuronal system as important centers for the integration of photoperiodic and chemosensory information. Responses within these systems to female chemosignals are gated seasonally, and responsiveness is not rescued by T replacement in SD animals. These results mirror photoperiodic regulation of behavioral and neuroendocrine responses to female chemosignals (Anand et al., 2002; Miernicki et al., 1990; Morin and Zucker, 1978). Thus, changes within the POA and RFRP neuronal system present a likely means by which photoperiod regulates processing of chemosensory cues to restrict reproduction to the appropriate season.

3.6 Acknowledgments

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3.7 Tables

Table 1. Body and tissue weights on day of chemosensory exposure.

	LD						SD						SD+T					
	Vehicle			FHVS			Vehicle			FHVS			Vehicle			FHVS		
Tissue Weight (g)	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
Body Weight	194.1	8.5	7	197	7.5	7	186.6	9.9	5	164.9	12.8	6	191.3	5.2	6	176.5	15.6	6
Paired Testes*	3.1	0.3	7	2.3	0.4	7	0.5	0.1	5	0.4	0.0	6	1.1	0.5	5	0.7	0.2	5
Paired Epididymis*	1.2	0.1	7	1.0	0.2	7	0.4	0.1	5	0.3	0.0	6	0.6	0.2	5	0.4	0.1	5
BAT	0.5	0.0	7	0.5	0.1	7	0.5	0.0	5	0.4	0.0	6	0.4	0.0	5	0.5	0.1	5
EWAT*	5.9	0.7	7	5.7	0.5	7	4.1	0.7	5	3.2	0.7	6	4.1	0.5	5	4.2	0.9	5

Table 2. Number of FOS-ir cells in regions of the chemosensory pathway following stimulus exposure.

	LD					
	Vehicle			FHVS		
Region	Mean	SEM	n	Mean	SEM	n
MOB granular layer	99.9	13.2	6	83.9	16.2	7
AOB granular layer‡	115.1	25.1	5	100.8	12.8	7
AOB mitral layer	28.6	5.1	5	29.9	2.3	7
Pyriform Cortex - Anterior	21.3	8.2	7	54.4	25.3	5
Pyriform Cortex - Median	29.4	14.2	7	71.6	24.8	7
Pyriform Cortex - Posterior	22.1	7.0	7	58.6	19.0	7
ACo†	7.7	3.8	6	16.4	7.5	7
BNST	15.7	3.6	7	23.9	6.7	7
MeApd†	6.7	2.6	6	24.3	7.1	7
MeApv†	4.5	1.2	6	17.3	5.1	6
POA‡	25.6	4.8	7	55.3*	17.5	7
Septum	2.6	0.9	7	6.6	3.9	7
VMH†	5.1	2.3	7	6.6	2.6	7

	SD						SD+T					
	Vehicle			FHVS			Vehicle			FHVS		
Region	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
MOB granular layer	62.5	7.8	3	99.4	16.7	5	74.4	7.4	4	97.9	12.2	6
AOB granular layer‡	132.8	22.8	2	93.0	8.2	5	67.0	16.2	4	150.2*	15.3	6
AOB mitral layer	14.5	3.5	2	22.3	3.6	5	21.8	5.2	4	30.9	4.7	6
Pyriform Cortex - Anterior	27.4	11.4	5	63.5	26.2	6	34.4	12.8	5	43.7	19.5	6
Pyriform Cortex - Median	33.4	10.7	5	69.3	29.4	6	61.2	28.2	6	29.7	8.8	6
Pyriform Cortex - Posterior	41.8	13.9	5	62.7	15.4	6	39.8	11.0	5	33.0	12.5	6
ACo†	7.6	3.3	5	40.5	12.7	6	20.2	10.6	5	51.5	15.8	4
BNST	16.8	8.1	5	25.7	5.6	6	18.0	5.0	6	14.2	4.8	6
MeApd†	8.6	2.9	5	25.0	8.9	5	17.0	9.2	5	35.6	13.3	5
MeApv†	15.0	5.9	5	37.2	10.8	6	7.8	3.0	4	22.4	3.3	5
POA‡	24.0	7.2	5	37.2	3.7	5	56.2	8.7	6	33.0	9.0	6
Septum	2.4	1.7	5	5.2	2.2	6	5.7	2.7	6	2.5	0.8	6
VMH†	5.2	1.6	5	13.5	3.6	6	3.6	1.0	5	10.2	4.2	6

3.8 Figures

Figure 1. Mean (± SEM) number of FOS-ir cells in the POA. * denotes $p < 0.05$ comparing vehicle to FHVS exposed animals.

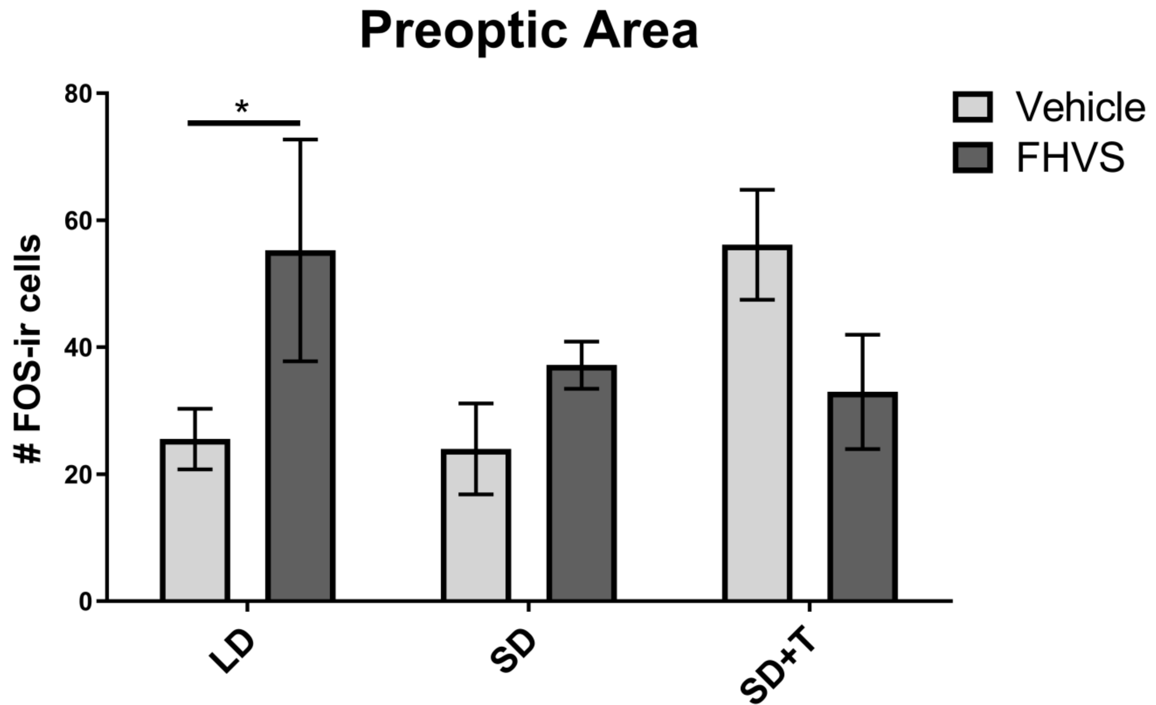


Figure 2. (A) Mean (± SEM) number of RFRP-ir cells in the DMH. (B) Mean (± SEM) percentage of RFRP-ir cells co-expressing FOS. (C) Representative photomicrographs depicting RFRP-ir (green) and FOS-ir (red) cells in LD animals exposed to vehicle (left) or FHVS (right). Scale bar represents 100μm. * denotes $p < 0.05$ comparing vehicle to FHVS exposed animals.

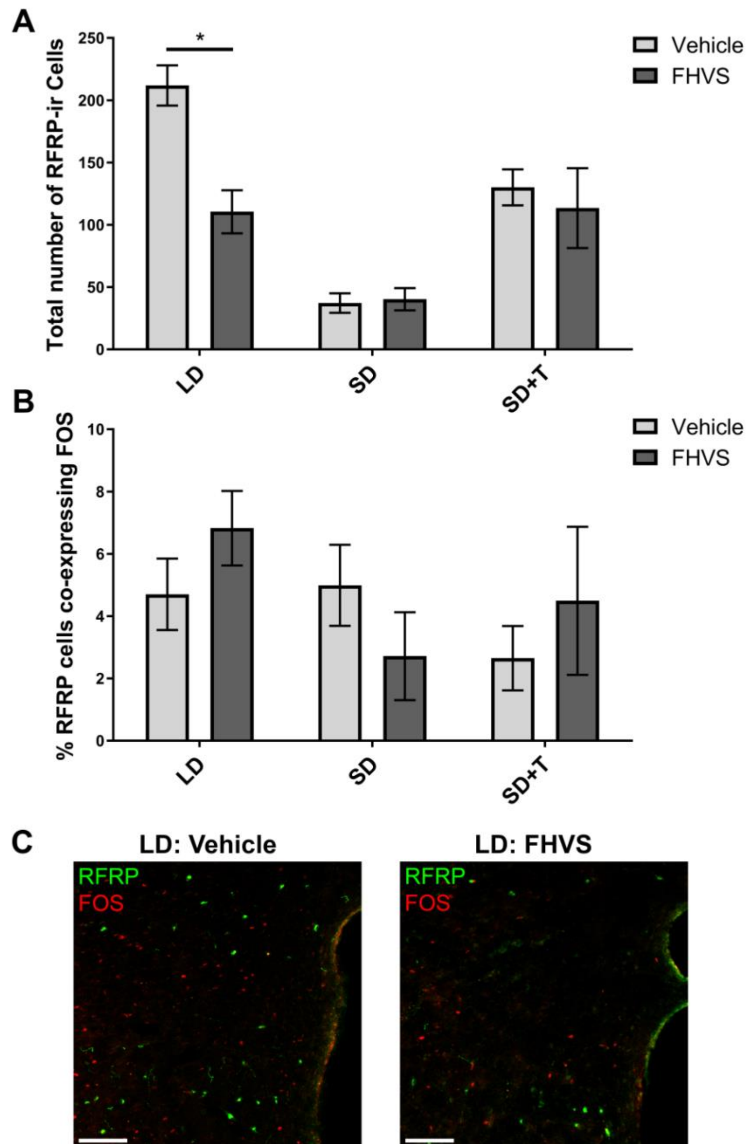


Figure 3. (A) Mean (± SEM) number of kisspeptin-ir cells in the AVPV. (B) Mean (± SEM) volume of kisspeptin-ir fibers in the anterior arcuate nucleus. (C) Mean (± SEM) volume of kisspeptin-ir fibers in the median arcuate nucleus. (D) Mean (± SEM) volume of kisspeptin-ir fibers in the posterior arcuate nucleus.

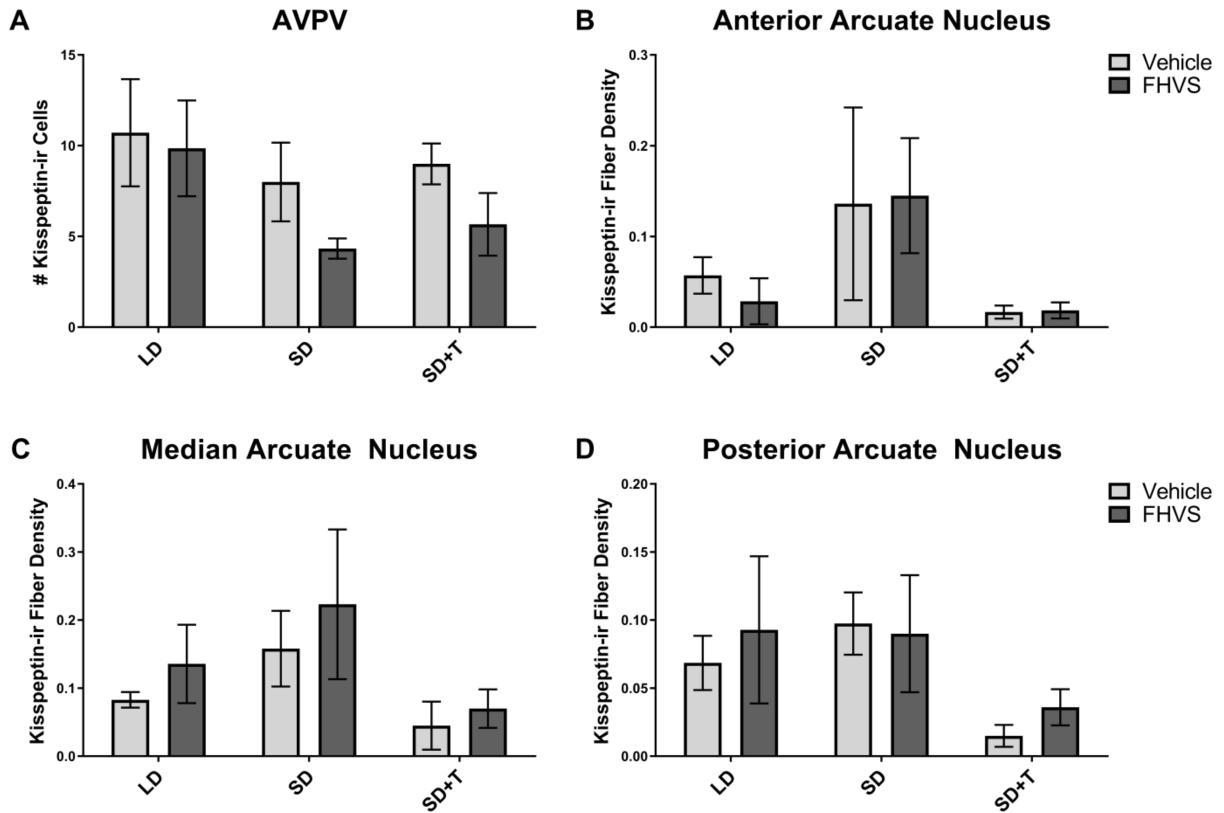
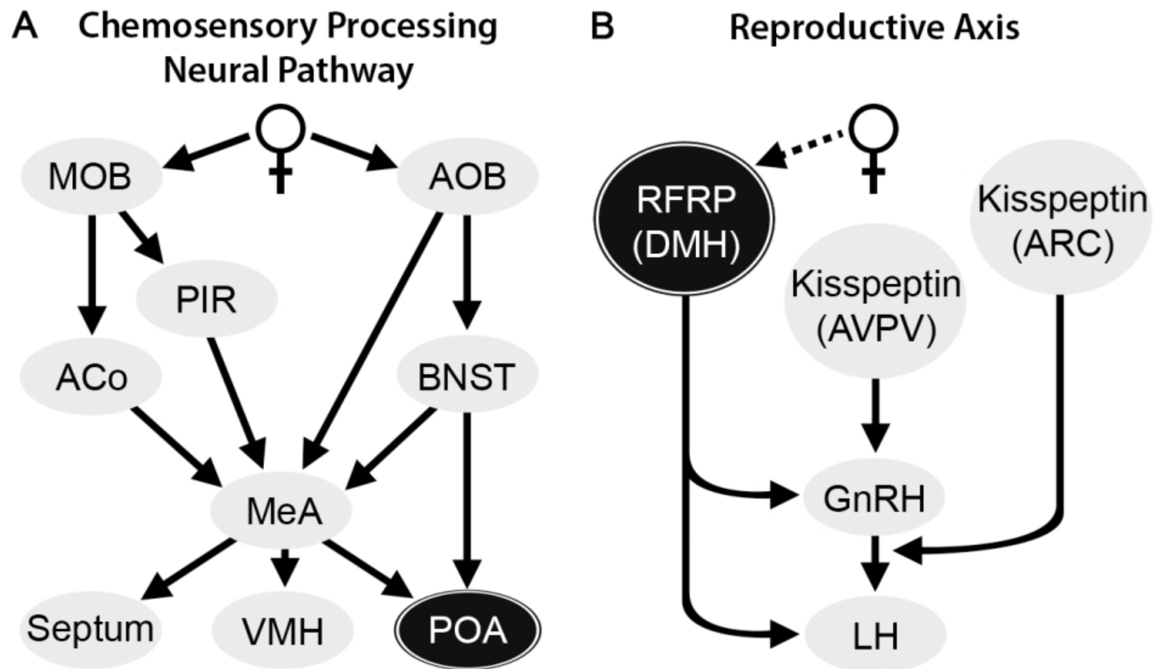


Figure 4. Summary of present findings. (A) Diagram of neural loci through which chemosensory information is processed. Unlike upstream chemosensory processing loci, the response of the POA to chemosensory information is modulated by photoperiod, represented by dark shading. ♀ represents chemosensory information, i.e., FHVS. (B) Diagram of the neuroendocrine reproductive axis. The RFRP neuronal system is sensitive to chemosensory information, and this response is modulated by photoperiod. The dotted line indicates that the pathway by which chemosensory information is transmitted to the RFRP neuronal system remains to be established.



Chapter 4: Changes in *Kiss1* and *Rfrp* Expression across Photoperiodic Transitions in the Siberian Hamster

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4.1 Abstract

Like many species, Siberian hamsters experience annual cycles of reproductive activity and quiescence. Exposure to short-day (SD) photoperiod suppresses gonadotropin release and causes regression of the gonads. Approximately 4 months later, hamsters become insensitive to the effects of SD photoperiod, gonadotropin release reactivates, and gonads redevelop in a process called spontaneous recrudescence. The hypothalamic neuropeptides kisspeptin and RFamide related peptide (RFRP) have been implicated in driving these changes in reproductive activity. Both peptides potently regulate release of gonadotropins, and their expression differs between reproductively active and inactive hamsters. The present studied used real time quantitative PCR to examine changes in expression of *kiss1* and *rfrp* (the genes encoding kisspeptin and RFRP, respectively) at multiple time-points throughout regression and recrudescence in both male and female Siberian hamsters. Overall, the results of the present study suggests that changes in *kiss1* expression in the anteroventral periventricular nucleus (AVPV) are not essential for initiating regression or recrudescence, that photoperiodic regulation of *kiss1* in the arcuate nucleus is mediated primarily by post-translational processes, and that changes in *rfrp* expression may contribute to regression but are not necessary for recrudescence. These findings highlight the importance of investigating endogenous patterns of change during transitional periods in physiology and inform our understanding of the photoperiodic control of reproduction.

4.2 Introduction

In order to maximize reproductive success, many animals utilize information about the prevailing photoperiod (proportion of light:dark within a 24h day) to synchronize breeding activity with seasonal changes in resource availability (Bronson, 1989). The Siberian hamster (*Phodopus sungorus*) is reproductively active under summer-like, long-day (LD) photoperiod but ceases breeding when exposed to a winter-like, short-day (SD) photoperiod (Hoffmann, 1973). Photoperiodic information is transduced into a nocturnal melatonin signal, with long-duration melatonin release from the pineal gland encoding the longer duration of night/dark-phase under a SD photoperiod. This melatonin signal acts to suppress activity of the hypothalamic-pituitary-gonadal (HPG) axis and initiate gonadal regression (Carter and Goldman, 1983; Hoffman and Reiter, 1965). After approximately 4 months exposure to SD photoperiod, hamsters become photorefractory (i.e., resistant to the inhibitory effects of photoperiod), the HPG axis reactivates, and the gonads regenerate even if the animal is still housed under SD photoperiod in a phenomenon called spontaneous recrudescence (Bittman, 1978; Schlatt et al., 1995; Schlatt et al., 1993). Exposure to LD photoperiod restores sensitivity to the inhibitory effects of SD photoperiod (Stetson et al., 1977), and thus hamsters experience photoperiod-driven cycles of reproductive activity and quiescence.

Although great progress has been made in understanding the neural mechanisms linking melatonin to the HPG axis (Hanon et al., 2008; Henson et al., 2013; Watanabe et al., 2004;

Yoshimura et al., 2003), questions yet remain about how upstream elements of the HPG axis drive changes in reproductive activity during regression and recrudescence. Since their discoveries at the turn of the century, the hypothalamic neuropeptides kisspeptin and RFamide-related peptide-3 (RFRP-3; the mammalian ortholog to avian gonadotropin inhibitory hormone) have received immense interest as potential neural substrates underlying these reproductive changes.

Initially discovered as a puberty regulator (de Roux et al., 2003; Seminara et al., 2003), the neuropeptide kisspeptin has since emerged as a prominent regulator of the HPG axis, potently stimulating release of gonadotropin releasing hormone (GnRH) which in turn elicits release of gonadotropins (e.g., luteinizing hormone (LH)) and downstream sex steroids (Gottsch et al., 2004; Irwig et al., 2004; Messager et al., 2005). Kisspeptin is produced from the *kiss1* gene within two hypothalamic nuclei, the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) (Lehman et al., 2010b). Kisspeptin expressed in the AVPV is critical for the pre-ovulatory surge of LH (Kinoshita et al., 2005; Smith et al., 2006; Williams et al., 2011), and is sexually dimorphic with higher expression in females (Clarkson and Herbison, 2006). In the AVPV, kisspeptin expression is reduced by exposure to inhibitory SD photoperiods in Syrian, Siberian, and Turkish hamsters (Ansel et al., 2010; Greives et al., 2007; Mason et al., 2007; Piekarski et al., 2014). However, expression of AVPV kisspeptin is associated more with reproductive state than with photoperiod directly. Unlike hamsters, ewes are reproductively active under SD and inactive under LD photoperiod. Mirroring this, kisspeptin expression in the AVPV is relatively higher in ewes housed in SD relative to LD photoperiod (Chalivoix et al., 2010; Smith et al., 2008). Despite this correlation with reproductive state, it is unclear whether changes in AVPV kisspeptin contribute to the inhibition and reactivation of the HPG axis across photoperiods.

On the other hand, kisspeptin produced in the ARC is thought to regulate the GnRH pulse generator and thus promote basal concentrations of LH (Okamura et al., 2013b). ARC kisspeptin displays photoperiod-induced changes in expression, but the direction of effect is species specific even amongst LD-breeding rodents. Whereas ARC kisspeptin expression is decreased under SD in Syrian hamsters (Ansel et al., 2010; Revel et al., 2006a), expression is counterintuitively increased under SD in Siberian and European hamsters (Greives et al., 2007; Mason et al., 2007; Saenz de Miera et al., 2014). The majority of studies showing this SD increase in ARC kisspeptin have analyzed total kisspeptin-immunoreactive cell numbers (Greives et al., 2008; Greives et al., 2007; Henson et al., 2013; Klosen et al., 2013; Mason et al., 2007), leaving open the possibility for alternate patterns of regulation at the mRNA level (but see (Rasri-Klosen et al., 2017)).

RFRP-3 is produced exclusively within the dorosomedial nucleus of the hypothalamus (DMH) by the gene *rfrp*, and has traditionally been thought to inhibit release of GnRH and LH (Hinuma et al., 2000; Kriegsfeld et al., 2006; Tsutsui et al., 2000). However, recent studies have revealed additional species, sex, and environment specific complexities. RFRP-3 inhibits LH release in LD male Siberian hamsters, but stimulates LH release in fully regressed SD males (Ubuka et al., 2012a). Further studies in Syrian hamsters and mice indicate additional species/sex/estrous differences (Ancel et al., 2012; Ancel et al., 2017). Expression of RFRP-3 is decreased by exposure to SD or melatonin in various hamster species and in sheep, despite

opposing photoperiodic regulation of these species' reproduction (Mason et al., 2010; Piekarski et al., 2014; Revel et al., 2008; Saenz de Miera et al., 2014; Smith et al., 2008; Smith et al., 2012). Thus, RFRP-3 expression appears to be much more closely associated with photoperiod, and has been suggested to be a critical upstream HPG axis element initiating gonadal regression in response to SD (Henningsen et al., 2016b; Simonneaux and Ancel, 2012).

Whereas numerous studies have examined differences in the kisspeptin and RFRP-3 systems between fully reproductively active animals and fully regressed animals, relatively few studies have examined expression when HPG axis activity is in flux during regression and recrudescence. Investigating patterns of expression across these transitional periods in relation to known patterns of change in gonadotropin release and gonadal function could identify potential causal relationships for future investigation. Therefore, the present study used quantitative real-time PCR (qRT-PCR) to examine relative changes in *kiss1* and *rfrp* mRNA in male and female Siberian hamsters at various time-points throughout gonadal regression and spontaneous recrudescence.

4.3 Materials and methods

4.3.1 Animals

Adult (>60 days of age) male and female Siberian hamsters were obtained from a breeding colony maintained at Indiana University, Bloomington. All animals were initially group-housed (2–4 per cage with same sex siblings upon weaning at 18–20 days of age), held on a LD photoperiod (light:dark 16:8). At the start of the experiment animals were randomly assigned to experimental groups and individually housed in polypropylene cages (27.8 × 17.5 × 13.0 cm) at 20 ± 2°C with relative humidity maintained at 50 ± 5%. Tap water and food (PMI LabDiet 5012, Rat Diet, St. Louis, Mo) were available *ad libitum*. All experimental procedures were approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) and conformed to principles enumerated in the NIH guide for the use and care for laboratory animals.

In this species, some animals are not responsive to SD exposure and fail to initiate gonadal regression. Such non-responders can be identified by a failure to decrease body weight during regression. Animals were considered non-responders if their body weight had not decreased at least 5% at 4 wk or 10% thereafter. Due to insufficient numbers for statistical analysis as independent groups, all non-responders were excluded from analysis.

4.3.2 Experimental design

At the beginning of the experiment, 34 males and 52 females were transferred to SD photoperiod (light:dark 8:16), whereas 23 males and 41 females were maintained on the LD photoperiod to provide age-matched LD comparison groups. After transfer to SD, it takes approximately 8wk for Siberian hamsters to reach the fully regressed state. Recrudescence begins after approximately 16 wk of SD exposure, with increasing LH and sex steroid concentrations that reach LD concentrations by 20-24 wk. In contrast, gonadal size and function are still impaired at this point and full reproductive capability is not regained until approximately

30 wk (Gorman and Zucker, 1995; Prendergast et al., 2002; Schlatt et al., 1995; Schlatt et al., 1993). Therefore, to examine *kiss1* and *rfrp* expression during these key physiological periods, animals were randomly assigned to be sacrificed and tissue collected after 4 wk (mid-regression), 12 wk (fully regressed), 24 wk (mid-recrudescence), or 30 wk (late recrudescence) of photoperiod exposure, with an additional female group collected at 2 wk (early regression) (**Table 1**). Body weights of each animal were recorded at the start of experiment and at regular intervals thereafter.

4.3.3 Tissue collection

Animals were sacrificed by rapid decapitation during the light phase. Brains were quickly extracted, immediately frozen with liquid nitrogen, shipped to the University of California Berkeley on dry ice, and stored at -80°C until use. To separate tissue containing the AVPV from tissue containing the ARC and allow examination of these distinct *kiss1* populations, brains were warmed to -16 °C in a cryostat (Leica 2050S) and sectioned on the coronal plane at 100µm. The AVPV was collected within 7 2mm diameter midline cryopunches using a clean, RNAse-free biopsy tool (Harris Uni-Core), beginning at the emergence of the 3rd ventricle and ending at the suprachiasmatic nucleus (SCN). The ARC and DMH were collected together within 13 3mm diameter midline punches, beginning at the posterior periventricular nucleus and ending with the disappearance of the 3rd ventricle, with the edge of the biopsy tool aligned to the ventral surface of the brain (Morin and Wood, 2001). Any cryostat surfaces in contact with the tissue were thoroughly cleaned with 95% ethanol and treated with RNAse-away (Thermo Fisher Scientific) between samples, or covered in RNAse-free aluminum foil if cleaning was impractical.

Total RNA was extracted with PureZOL extraction methods (BioRad) for male samples and RNAqueous micro kits (Ambion) for female samples followed by DNAse treatment (DNA-free, Invitrogen). RNA concentration and purity were assessed by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific). Samples resulted in an average A260/A260 ratio 2.06 with a range of 1.99-2.13. One sample per group was randomly selected and RNA integrity assessed on an Agilent Bioanalyzer 2100; all provided RIN values above 7.6. Reverse transcription was accomplished with iScript cDNA synthesis kits (BioRad). Due to experimenter error during punch collection or RNA extraction, the following samples were lost or yielded no RNA: 6 male ARC/DMH samples (2 SD-4wk, 1 LD-12wk, 1 SD-24wk, 1 LD-30wk, 1 SD-30wk), 8 male AVPV samples (3 LD-4wk, 1 SD-4wk, 2 LD-12wk, 1 SD-12wk 1 SD-24wk), 3 female AVPV samples (1 SD-2wk, 1 SD-24wk, 1 LD-30wk).

4.3.4 qRT-PCR

Analysis of relative gene expression via qRT-PCR was carried out by following manufacturer's instructions for SSOAdvanced SYBR Green supermix (BioRad). Male samples were run on an Applied Biosystems StepOne machine with 15µL reaction volumes whereas female samples were run on a BioRad CFX384 machine in 10µL reaction volumes. Primers were designed from published sequences for Siberian hamsters using the NCBI Primer BLAST software (**Table 2**). Primer sets were validated for specificity prior to use with positive, negative, and no template controls, and melt curve analysis was performed on each sample after the PCR. Efficiency of each primer set was determined by standard curve; primers were 94.6-109.0%

efficient under conditions used with male samples and 94.9-104.3% under conditions used with female samples with R^2 values above 0.998. All male samples were run in duplicate and female *rfrp*, *gapdh*, and *hsp90* samples were run in triplicate. Female *kiss1* samples were run in five technical replicates with the highest and lowest C_q excluded to improve C_q variance. For all genes, replicate sets in which C_q values varied beyond 0.5 cycles were excluded from analysis (male AVPV *kiss1*: 1 LD-4wk, 3 SD-12wk, 3 SD-24wk, 2 LD-30wk; male ARC *kiss1*: 1 SD-4wk, 1 LD-12wk, LD-30wk; male *rfrp*: 1 SD-4wk, 1 SD-24wk; female AVPV *kiss1*: 3 SD-4wk, 1 LD-12wk, 1 SD-12wk, 1 SD-30wk; female ARC *kiss1*: 1 LD-2wk, 1 SD-30wk; female *rfrp*: 1 LD-30wk). Resulting data were analyzed following the delta delta C_q method outlined in (Pfaffl, 2001). Because *hsp90* expression was found to vary with photoperiod in female ARC/DMH samples, female *rfrp* and ARC *kiss1* data use only *gapdh* as reference gene, whereas all other analyses use the geometric mean of both *gapdh* and *hsp90* expression values as reference.

4.3.5 Statistics

All statistical analyses were performed using Prism software (version 7.02, GraphPad). Data were analyzed with Two-Way ANOVA (factors: photoperiod, time). *A priori* planned investigation of the effects of photoperiod at each time point was performed using Fisher's LSD tests. P-values below 0.05 were considered statistically significant. Sex was not analyzed as a statistical factor because male and female samples were processed separately using different reagents and equipment and thus the sample sets are deemed not directly comparable. To satisfy assumptions of normality and to improve understanding of biological meaningfulness, relative gene expression values (fold change relative to the age-matched LD animals collected at the same time-point) were $\log_2$ transformed prior to statistical analysis. Log_2 transformed expression values more than two standard deviations from the group mean were excluded as outliers (one each: male SD-12wk, SD-24wk *rfrp*; female LD-4wk *rfrp;* female SD-30wk AVPV *kiss1*; female SD-2wk, LD-4wk, SD-12wk, and SD-30wk ARC *kiss1*).

4.4 Results

4.4.1 AVPV kiss1

Expression of *kiss1* within the AVPV was downregulated by exposure to SD photoperiod in both males ($F_{1, 32} = 7.10$, $p = 0.01$) and females ($F_{1, 73} = 6.59$, $p = 0.01$) (**Figure 1**). Results of two-way ANOVA did not reveal main effects of time (males $F_{3, 32} = 1.43$, $p = 0.25$; females $F_{4, 73} = 1.27$, $p = 0.29$) or a significant interaction between photoperiod and time (males $F_{3, 32} = 0.86$, $p = 0.47$; females $F_{4, 73} = 1.65$, $p = 0.17$). Further examination with Fisher's LSD tests indicated that expression of AVPV *kiss1* within both sexes was decreased by SD at 12 wk (males $t_{32} = 2.31$, $p = 0.03$; females $t_{73} = 2.43$, $p = 0.02$) and 24 wk (males $t_{32} = 2.11$, $p = 0.04$; females $t_{73} = 2.06$, $p = 0.04$) but not at 2 or 4 wk of SD exposure ($p > 0.05$ in all cases). Furthermore, expression of *kiss1* in the AVPV no longer differed between LD and SD animals by 30 wk of photoperiod exposure ($p > 0.05$ in both sexes).

4.4.2 ARC kiss1

Kiss1 expression within the ARC was not impacted by photoperiod exposure in either sex (males $F_{1, 41} = 0.46$, $p = 0.50$; females $F_{1, 78} = 1.09$, $p = 0.30$) (**Figure 2**). There were also no main effects of time (males $F_{3, 41} = 0.28$, $p = 84$; females $F_{4, 78} = 1.87$, $p = 0.12$) or interactions between photoperiod and time (males $F_{3, 41} = 0.17$, $p = 0.92$; females $F_{4, 78} = 1.14$, $p = 0.34$). Additional examination of the effects of photoperiod at each time-point likewise revealed no differences between LD and SD animals at any time-point ($p > 0.05$ in all cases).

4.4.3 Rfrp

Expression of *rfrp* in males was decreased by exposure to SD photoperiod ($F_{1, 41} = 42.64$, $p < 0.001$) (**Figure 3A**). However, two-way ANOVA did not reveal a main effect of time ($F_{3, 41} = 2.51$, $p = 0.07$) or an interaction effect between photoperiod and time ($F_{3, 41} = 1.45$, $p = 0.24$) in males. Expression of *rfrp* was decreased in SD males at all time-points (4 wk $t_{41} = 2.17$, $p = 0.04$; 12 wk $t_{41} = 2.69$, $p = 0.01$; 24 wk $t_{41} = 4.94$, $p < 0.0001$; 30 wk $t_{41} = 3.27$, $p < 0.01$). In contrast, expression of *rfrp* in females was regulated by photoperiod ($F_{1, 81} = 39.79$, $p < 0.0001$) and time ($F_{4, 81} = 9.57$, $p < 0.0001$), and the effects of photoperiod varied by time (interaction $F_{4, 81} = 8.05$, $p < 0.0001$) (**Figure 3B**). Expression of *rfrp* did not differ between LD and SD females at 2 wk or 4 wk ($p > 0.05$), but was significantly decreased by SD exposure at all following time-points (12 wk $t_{81} = 5.56$, $p < 0.0001$; 24 wk $t_{81} = 3.75$, $p < 0.001$; 30 wk $t_{81} = 4.75$, $p < 0.0001$).

4.5 Discussion

The present study investigated patterns of change in AVPV *kiss1*, ARC *kiss1*, and *rfrp* expression across gonadal regression and recrudescence in both male and female Siberian hamsters. These data reveal varying relationships between important upstream HPG axis regulators, photoperiod, and reproductive state. AVPV *kiss1* expression tracked changes in reproductive status overall, but data collected mid-regression and mid-recrudescence reveal a delay between changes in HPG axis activity and changes in AVPV *kiss1* expression. ARC *kiss1* displayed an unanticipated stable pattern of expression across changes in reproductive state, providing novel insight into the photoperiodic regulation of this system. Finally, *rfrp* expression reflected photoperiod but not reproductive state, confirming that changes in *rfrp* expression are not necessary for spontaneous recrudescence but may be important for gonadal regression. Each of these findings is discussed in more detail below.

Kiss1 expression in the AVPV fell in regressed animals (12 wk) and returned to LD levels in late recrudescence (30 wk), but data at mid-regression (4 wk) and mid-recrudescence (24 wk) suggest changes in AVPV *kiss1* expression may lag behind changes in HPG axis activity. Four wk of exposure to SD photoperiod is sufficient to greatly inhibit gonadotropin release and gonadal function in both sexes of Siberian hamster (Gorman and Zucker, 1995; Prendergast et al., 2002; Schlatt et al., 1995; Schlatt et al., 1993), yet AVPV *kiss1* expression did not differ from LD animals at this time-point. Furthermore, circulating gonadotropin and sex steroid concentrations return to LD levels by 24 wk of SD (Schlatt et al., 1995; Schlatt et al., 1993), yet AVPV *kiss1* expression at this time-point remains downregulated. Thus, changes in AVPV *kiss1* expression across regression and recrudescence are delayed relative to known

patterns of change in HPG axis activity. Considering that AVPV kisspeptin expression is strongly regulated by sex steroid positive feedback (Ansel et al., 2010; Greives et al., 2008; Rasri-Klosen et al., 2017; Smith et al., 2008), this system likely responds to changes in circulating sex steroids during regression/recrudescence and that the resultant changes in *kiss1* expression take time to manifest. Therefore, it seems unlikely that changes in AVPV *kiss1* expression are important for initiating regression or recrudescence, although this system could still play a role in maintaining the newly established regressed or reactivated state. It is also worth considering that expression of the kisspeptin receptor GPR54 has been reported to vary across photoperiods (Simonneaux et al., 2013; Xu et al., 2017). Additional research will be necessary to confirm that changes in kisspeptin sensitivity (rather than in kisspeptin production or release) in AVPV-targets do not play a role in driving early gonadal regression or recrudescence.

The present study found no changes in ARC *kiss1* expression across regression or recrudescence in either males or females, in contrast to previous reports of increased kisspeptin labeling in the ARC of regressed Siberian hamsters (Greives et al., 2008; Greives et al., 2007; Henson et al., 2013; Klosen et al., 2013; Mason et al., 2007; Rasri-Klosen et al., 2017). This combination of stable mRNA expression and increased peptide labeling has also been described in the GnRH system of many seasonally breeding rodents (Bittman et al., 1996; Kriegsfeld et al., 2000; Porkka-Heiskanen et al., 1997; Urbanski et al., 1991; Yellon, 1994). Those data have been interpreted to indicate stable production of GnRH across photoperiods with decreased release under the non-breeding photoperiod resulting in the 'building up' of the GnRH peptide and increased immunohistochemical labeling (Kriegsfeld and Bittman, 2009). Considering ARC kisspeptin strongly promotes GnRH release (Okamura et al., 2013b; Yip et al., 2015), the present findings suggest that the ARC kisspeptin system exhibits a similar pattern of stable production and inhibited release under SD, although alternate changes in post-translational regulation cannot be excluded. It was recently reported that the number of *kiss1* mRNA positive cells in the ARC was increased in regressed male Siberian hamsters ((Rasri-Klosen et al., 2017), see also (Simonneaux et al., 2009)). The absence of similar changes in *kiss1* mRNA in the present study likely reflect differences in methodological approaches between the in situ hybridization used in that study and qRT-PCR used in the present. Unfortunately, because changes in the ARC kisspeptin system likely primarily occur post-translationally, the present study was unable to investigate how such changes relate to the progression of gonadal regression and recrudescence. Although exogenous kisspeptin treatment increases gonadal size in SD males (Bailey et al., 2017; Rasri-Klosen et al., 2017; Revel et al., 2006a), it remains unclear how endogenous ARC kisspeptin might contribute to the surge in HPG axis activity seen during early-mid recrudescence.

Rfrp expression was decreased by exposure to SD and remained downregulated thereafter, indicating that *rfrp* is under strong photoperiodic inhibition and that increased *rfrp* expression is not necessary for spontaneous gonadal recrudescence. These data are consistent with reports that melatonin treatment reduces *rfrp* expression (Revel et al., 2008; Ubuka et al., 2012a), possibly through actions on melatonin receptors in the DMH or SCN (which projects directly to RFRP-3 cells (Gibson et al., 2008)) or through melatonin-induced changes in thyroid hormone signaling (Klosen et al., 2013; Revel et al., 2006b; Saenz de Miera et al., 2014). Indeed, in male Syrian hamsters the number of *rfrp* positive cells remains decreased in late

recrudescence (28 wk of SD) (Revel et al., 2008). It has been suggested that changes in the RFRP-3 system occur early in the cascade of gonadal regression and serve to drive decreases in HPG axis activity (Henningsen et al., 2016b). The present male data may be consistent with this view, as *rfrp* expression is reduced at the earliest time-point examined (4 wk), unlike *kiss1*. However, *rfrp* expression in females was not downregulated by 2 wk or 4 wk of SD, despite this duration of SD being sufficient to cause reductions in estrous cyclicity and gonadal function (Schlatt et al., 1993). Additional studies are needed to investigate whether this is a true sex or species difference or whether other aspects of RFRP-3 signaling change during early-mid regression in females, such as the changes in RFRP-3 fiber distribution seen at 3 wk SD in male Syrian hamsters (Mason et al., 2010). Additionally, with the increasing evidence that RFRP-3's effect on HPG axis activity is highly dependent on context (Ancel et al., 2012; Ancel et al., 2017; Henningsen et al., 2016a; Ubuka et al., 2012a), it will be essential to determine the physiological effects of RFRP-3 throughout regression and recrudescence in order to fully understand the potential roles this peptide plays in mediating these critical reproductive changes.

Taken together, these findings highlight the importance of investigating endogenous patterns of change during transitional periods in physiology. Such data can inform hypotheses regarding the driving factors underlying these transitions. Overall, the present study suggests that changes in AVPV *kiss1* expression are not essential for initiating regression or recrudescence, that photoperiodic regulation of ARC *kiss1* is mediated primarily by post-translational processes, and that changes in *rfrp* expression may contribute to regression but are not necessary for recrudescence.

4.6 Acknowledgements

This work was performed with the assistance of Drs. Gregory Demas and Allison Bailey at Indiana University (experimental housing, collection of tissue), and of Marisa Yang and Kathy Zhang at UC Berkeley (RNA extraction), with oversight by Dr. Lance Kriegsfeld.

4.7 Tables

Table 1. Group n and mean ± SEM percent change in body weight (BW).

		Males			Females		
Time-point	Photoperiod	n	% BW Change (Mean)	% BW Change (SEM)	n	% BW Change (Mean)	% BW Change (SEM)
2 wk	LD	-	-	-	6	-0.67%	1.93%
	SD	-	-	-	12	1.18%	2.35%
4 wk	LD	6	1.08%	2.28%	13	-2.77%	1.54%
	SD	7	-8.67%	1.95%	10	-11.49%	2.49%
12 wk	LD	6	4.28%	3.31%	9	5.44%	3.10%
	SD	9	-24.60%	3.53%	13	-25.52%	2.02%
24 wk	LD	5	2.67%	1.56%	6	3.57%	8.28%
	SD	10	-27.96%	2.32%	3	-30.06%	4.72%
30 wk	LD	6	8.02%	3.93%	7	11.21%	7.90%
	SD	8	-26.63%	3.39%	14	-24.75%	3.39%

Table 2. Primer sets used for qRT-PCR.

Primer	Forward	Reverse	Temp	Product Size
kiss1	ACGAAGGCAAA CCCTAACC	CAGTCCTCCAG GTTTCCTCTCT	60	105
rfrp	GCCATTTGCTTC AGAGGTTCTTG	GCTTGTCTCCT T GGTTGCTTTC	60	115
gapdh	TTCTTGTGCAGT GCCAGCCTCG	CTGTGCCGTTG AACTTGCCGTG	60	207
hsp90	TAGGCTATCCCA TCACCCTCT	CCTCCTCATCT GAGCCTACG	60	150

4.8 Figures

Figure 1. Mean ± SEM relative expression of *kiss1* within the AVPV of male (A) and female (B) hamsters after various durations of exposure to photoperiod. * denotes $p < 0.05$ difference between LD and SD animals at the designated time-point.

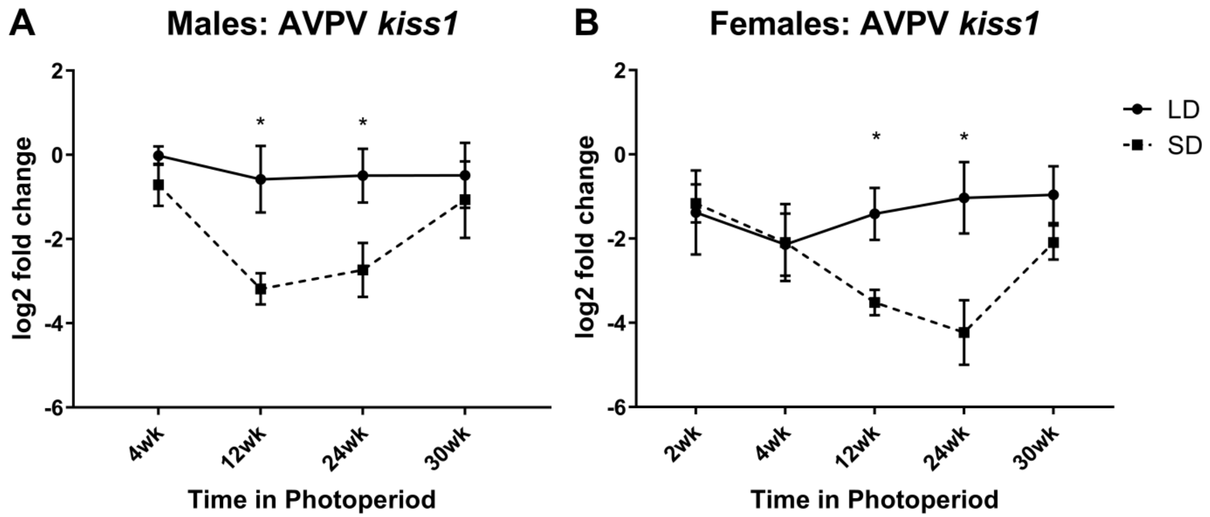


Figure 2. Mean ± SEM relative expression of *kiss1* within the ARC of male (A) and female (B) hamsters after various durations of exposure to photoperiod.

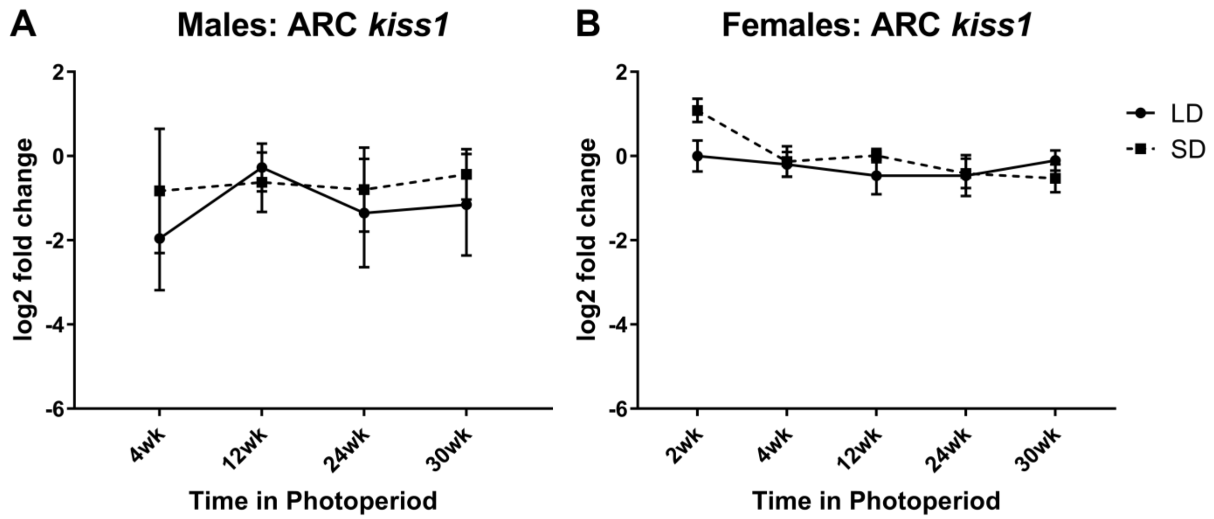
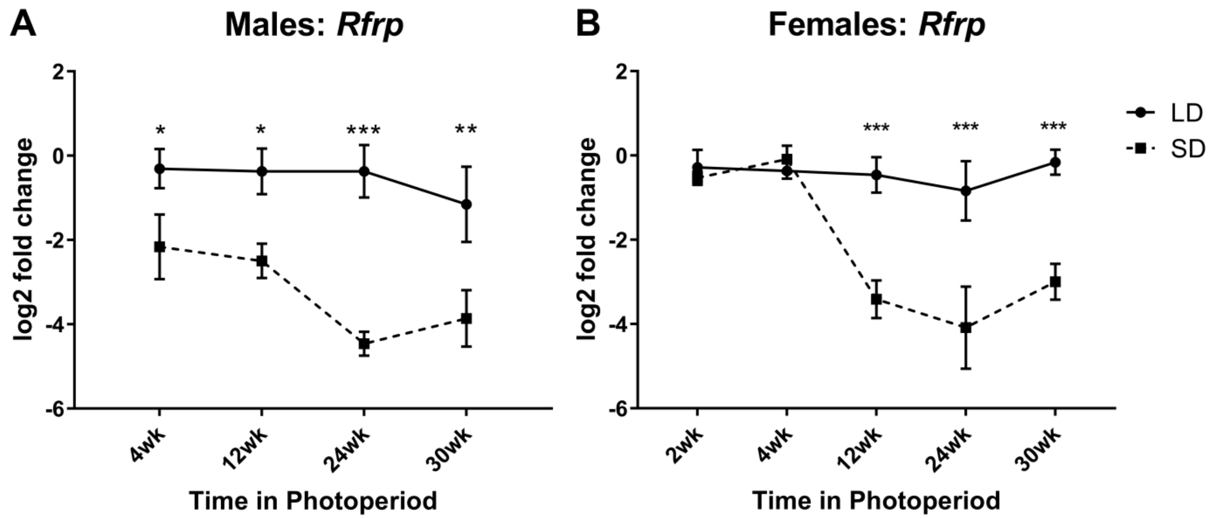


Figure 3. Mean ± SEM relative expression of *rfrp* after various durations of exposure to photoperiod in male (A) and female (B) hamsters. * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$ difference between LD and SD animals at the designated time-point.



Chapter 5: Conclusions

The work presented in the previous chapters explored social and photoperiodic control of the HPG axis, focusing on the upstream regulators kisspeptin and RFRP. Chapter 2 revealed that neuronal activation in RFRP, but not kisspeptin, cells was associated with participating in an aggressive encounter in mice (Jennings et al., 2016). This study was the first to link RFRP to mammalian aggression, and informs our understanding of how HPG axis activity quickly changes during dominance hierarchy formation in this species (Williamson et al., 2017b). Chapter 4 described patterns of kiss1 and rfrp expression across photoperiod-driven changes in reproductive state using the Siberian hamster. This project provides basic insight into a phenomenon at the heart of many seasonally breeding species' lives. Chapter 3 explored both social and photoperiodic control of the HPG axis, by asking where in the brain is social chemosensory information gated across photoperiods. This study once again indicated the RFRP, but not kisspeptin, neuronal system to be an important target of social information, and to further integrate this information with the photoperiodic context (Jennings et al., 2017).

Taken together, the studies presented herein highlight the importance of the RFRP system in integrating information about the external environment, especially social information. Although kisspeptin has potent stimulatory effects on GnRH release and is regulated by photoperiod, changes in kisspeptin-expressing cells were not associated with either aggressive or sexual social contexts. In contrast, RFRP was sensitive to aggressive, sexual, and photoperiodic information (Jennings et al., 2016; Jennings et al., 2017). To my knowledge, the work presented in Chapter 3 is the first study to implicate RFRP as a target of chemosensory information. Additionally, whereas GnIH (the avian ortholog to RFRP) has been associated with competition for nesting opportunities and reported to alter aggressive behavior in birds (Calisi et al., 2011; Ubuka et al., 2013a), the work presented in Chapter 2 is the first to reveal an association between RFRP and aggression in a mammal. Therefore, the present data add to a growing body of evidence implicating RFRP as an important integrator of environmental information for the control of reproduction.

The RFRP system is also sensitive to the effects of stress. Acute and chronic stress has been shown to increase RFRP/GnIH expression and increase RFRP neuronal activation (Calisi et al., 2008; Clarke et al., 2016; Kaewwongse et al., 2011; Kirby et al., 2009). This upregulation of RFRP appears to be driven by elevated glucocorticoids. RFRP cells express glucocorticoid receptors (GR), GR activation increases RFRP synthesis, and adrenalectomy blocks stress-induced increases in RFRP (Gojska and Belsham, 2014; Kirby et al., 2009; Soga et al., 2012; Son et al., 2014). RFRP also stimulates the hypothalamic-pituitary-adrenal axis (Kim et al., 2015; Lin et al., 2016; Ullah et al., 2017), suggesting that the relationship between glucocorticoids and RFRP is bidirectional. RFRP's response to stress is likely of great biological relevance, as preventing the stress-induced increase in RFRP rescues pregnancy success in a rat model (Geraghty et al., 2015).

Additionally, RFRP expressing cells are responsive to metabolic information. Food restriction increases RFRP expression and neuronal activation (Fraley et al., 2013; Li et al., 2014). On the other hand, obesity is also associated with increased RFRP expression in mice

(Poling et al., 2014), and this appears not be leptin-driven (Rizwan et al., 2014). Furthermore, RFRP treatment stimulates feeding in many species (Clarke et al., 2012; Johnson et al., 2007; Tachibana et al., 2005). This has led some to argue that RFRP may act to balance reproductive and metabolic needs (Clarke et al., 2012; Klingerman et al., 2011; Schneider et al., 2017). Indeed, changes in RFRP neuronal activation are associated with changes in sexual motivation and food hoarding under food-restriction (Benton et al., 2017; Klingerman et al., 2011).

Thus, RFRP cells integrate information from a wide variety of sources relevant to reproduction, and may function to modulate GnRH/LH release in response. Another exciting possible function of RFRP is as a behavioral modulator. RFRP expressing cells project widely throughout the brain, including to regions associated with the expression of social behaviors (such as the lateral septum, amygdala, bed nucleus of the stria terminalis, and periaqueductal grey), in addition to GnRH-containing regions also implicated in behavior (such as the preoptic area or anterior hypothalamus) (Goodson, 2005; Kriegsfeld et al., 2006; Newman, 1999; Peragine et al., 2017; Ubuka et al., 2009). Expression of RFRP receptors mirrors this wide distribution (Dardente et al., 2008; Henningsen et al., 2016c; Poling et al., 2012). Exogenous RFRP/GnIH inhibits male and female sexual behavior in white crown sparrow, Japanese quail, rats, Syrian hamsters, and naked mole rats (Bentley et al., 2006; Johnson et al., 2007; Peragine et al., 2017; Piekarski et al., 2013; Ubuka et al., 2013a), although not in sheep, mice, or cynomolgus macaques (Clarke et al., 2012). RNAi-mediated knockdown of GnIH increased antagonistic behaviors in male white crown sparrow and Japanese quail (Ubuka et al., 2013a; Ubuka et al., 2012b), possibly through regulation of neuroestrogen synthesis (Ubuka et al., 2014; Ubuka and Tsutsui, 2014). RFRP/GnIH also stimulates feeding or food hoarding in chicks, ducks, mice, rats, Syrian hamsters, sheep, and macaques (Benton et al., 2017; Clarke et al., 2012; Fraley et al., 2013; Johnson et al., 2007; McConn et al., 2016; Tachibana et al., 2005), but not in white crown sparrow or Japanese quail (Ubuka et al., 2014; Ubuka et al., 2013a; Ubuka et al., 2012b). Micro-infusion data indicates that this effect may be site-specific or differ between RFRP-1 and RFRP-3 (Kovacs et al., 2014; Kovacs et al., 2012). Finally, central infusion of RFRP or overexpression of RFRP receptors is anxiogenic in rats and mice (Kaewwongse et al., 2011; Kim et al., 2015; Lin et al., 2016). Exploring the role of RFRP expressing cells within behavior circuits will be an exciting avenue for future research.

However, understanding of the functional role of RFRP is hindered by a lack of some basic but essential information. RFRP-3 is considered the most homologous RFRP to avian GnIH, but there is no RFRP-3 specific antibody available at present. This creates ambiguity when discussing the contributions of RFRP-expressing cells or the release of RFRP-1 and RFRP-3 peptides specifically. Additionally, the contributions of specific RFRP receptors have only recently begun to be dissected through use of a GPR147 (NPFFR1) knock out mouse (Leon et al., 2014). Indeed, RFRP receptor specific antagonists have been an issue in the past, with a formerly popular RFRP receptor antagonist uncovered to be a kisspeptin receptor agonist (Liu and Herbison, 2014). Recent validation of a new RFRP receptor specific antagonist should be helpful in future investigations (Kim et al., 2015).

One of the most pressing issues at the moment in the study of RFRP is determining when and why RFRP inhibits LH release in some contexts and stimulates it in others. Numerous studies have reported that RFRP infusions decrease circulating LH (Anderson et al., 2009;

Clarke et al., 2008; Clarke et al., 2012; Kriegsfeld et al., 2006), although some failed to observe an effect of RFRP on LH (Murakami et al., 2008; Rizwan et al., 2009). In 2012, it was reported that RFRP stimulate LH release in male Syrian and Siberian hamsters, although the effect in Siberian hamsters depended on whether the animal was housed under long-day or short-day photoperiod (Ancel et al., 2012; Ubuka et al., 2012a). Recently, a study in mice reported that RFRP increased LH release in males, inhibited LH in peri-ovulatory females, and had no effect on LH in females at other points in the estrous cycle (Ancel et al., 2017). What mechanisms underlie the opposing effects of RFRP on LH release across different species, sexes, and contexts? Without an understanding of the physiological effect of the RFRP peptides, we cannot make accurate predictions about the function of RFRP-expressing cells in regulating reproductive physiology or behavior

Finally, the studies undertaken in this dissertation highlight the value of comparative biology. Each of these studies approached a different question using a different model species which was most appropriate to the question asked. Mice have become a popular model for studying aggression and there is a great history of examining HPG axis activity in mice dominance hierarchies, hence their selection for the study presented in Chapter 2. But most lab mice strains are not photoperiodic, and have been under such strong selection pressure against seasonal breeding in commercial breeding facilities that the melatonin system has become highly dysregulated or nonfunctional. Syrian and Siberian hamsters are excellent lab species and have historically been two of the most common model species for the study of biological rhythms. The study presented in Chapter 3 also took advantage of decades of previous research investigating neural responses to chemosensory cues in the Syrian hamster, providing a solid foundation of comparison between present and past findings. Siberian hamsters presented a unique pattern of RFRP and kisspeptin expression begging to be further examined in Chapter 4. The questions asked in this dissertation could not have been answered using the same model species across experiments. Furthermore, as exemplified in the discussion above, comparisons across species inform our understanding of conserved mechanisms. When mechanisms appear to be conserved across mammals or across taxa, it supports the potential translational value to humans. Despite the overwhelming popularity of the lab mouse in modern neuroscience, we must be open to studying other species in order to progress toward a true understanding of the brain and behavior.

Chapter 6: References

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