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Seasonal Regulation of Reproduction: Mechanisms Regulating the RFamides, Kisspeptin and Gonadotropin Inhibitory Hormone in Siberian Hamsters

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SEASONAL REGULATION OF REPRODUCTION: MECHANISMS REGULATING THE RFAMIDES, KISSPEPTIN AND GONADOTROPIN INHIBITORY HORMONE IN SIBERIAN HAMSTERS

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ABSTRACT

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Siberian hamsters exhibit seasonal rhythms in reproductive driven by melatonin secretion. Tissues that mediate Mel-dependent alterations in reproduction include the suprachiasmatic nucleus (SCN) and the nucleus reuniens (NRe). Several neuroendocrine factors are implicated in the control of reproduction by melatonin, including two RFamides, kisspeptin and gonadotropin-inhibitory hormone (GnIH), as well as triiodothyronine (T_3). Melatonin implants localized to the SCN or NRe tested the hypothesis that separate melatonin target tissues regulate RFamide expression. We also hypothesized that T_3 and the RFamides are part of a single circuit. The results of chapter 2 indicate that melatonin acting at the SCN reduces the number of kisspeptin neurons in the anteroventral periventricular nucleus and melatonin acting at the NRe reduced GnIH expression, supporting the hypothesis that RFamides are regulated by separate melatonin targets. Results from chapter 3 indicate that T_3 up-regulates RFamide expression, thus T_3 and the RFamides are parts of a single pathway.

PREFACE

The research goal of my lab is to understand the neuroendocrine mechanism underlying seasonal reproduction. Siberian hamsters, *Phodopus sungorus*, are excellent model organisms for this research since they exhibit robust seasonal changes in physiology and behavior. Siberian hamsters inhabit temperate latitudes in areas of Kazakhstan, Siberia, and Mongolia. These regions experience changes in day length from 7 hours of light to 17 hours of light, and changes in temperature from an average of -40º C during the winter months to an average of 25º C during the summer months. These fluctuations in environmental resources lead to alterations in physiology and behavior. These include seasonal changes in reproduction, pelage color and quality, immune function, thermoregulation, body weight, aggressive behavior, and sex behavior. The focus of my thesis research was on examining the effects on neuroendocrine molecules that mediate the effects of melatonin on the reproductive axis. I am the primary author on both of these co-authored manuscripts. Chapter 1 (Introduction) has been formatted according to the *Journal of Biological Rhythms*. Chapter 2 (Different neural target tissues mediate melatonin-dependent regulation of the RFamides, kisspeptin and gonadotropin-inhibitory hormone, in Siberian hamsters) has been formatted for submission to the *Journal of Neuroendocrinology*. Chapter 3 (Exogenous T_3 increases expression of the RFamides, kisspeptin and gonadotropin-inhibitory hormone, in short-day Siberian hamsters (*Phodopus sungorus*) has been formatted for submission to the *Journal of Biological Rhythms.* Chapter 4 (Summary of Conclusions) has been formatted according to the *Journal of Biological Rhythms*.

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CHAPTER 1: INTRODUCTION

Many animals that inhabit temperate climates time reproduction so that offspring are born when the environment is most conducive to survival. Siberian hamsters (*Phodopus sungorus*) exhibit robust seasonal rhythms in physiology and behavior driven by changes in day length, enabling them to synchronize the birth of offspring with the onset of favorable environmental conditions. The primary cue used by many animals to anticipate seasonal environmental changes is day length. Changes in day length are encoded by the duration of melatonin (Mel) secretion from the pineal gland. Mel acts at multiple neural target tissues to drive seasonal alterations in physiological functions including, but not limited to, reproduction, pelage color and quality, immune function, thermoregulation, body weight, aggressive behavior, and sex behavior. For example, long durations of Mel inhibit, whereas short durations of Mel stimulate, the reproductive axis. One target of Mel action involved in the regulation of reproduction is the release of hypothalamic gonadotropin- releasing hormone (GnRH; Paul et al., 2008). The mechanism mediating Mel-dependent regulation of GnRH, and thus the reproductive axis, has yet to be established. This stems in part from the observation that GnRH neurons do not express Mel receptors, suggesting that regulation of GnRH by Mel is indirect, perhaps involving additional upstream elements. Because many environmental cues act through GnRH neurons to impact reproduction in vertebrates, it is important to understand the pathways and mechanisms through which environmental cues access this molecule. The Siberian hamster serves as an excellent model to address this issue since they exhibit such robust, well-characterized seasonal rhythms in reproduction.

Two possible upstream regulators of GnRH are the RF-amides, kisspeptin (Kiss1) and gonadotropin inhibitory hormone (GnIH). Recent findings implicate these neuropeptides in the Mel-dependent regulation of reproduction. First, the expression of the RF-amides varies seasonally in a Mel-dependent fashion (for review: Parhar et al., 2012), and second, administration of exogenous Kiss1 stimulates, whereas GnIH inhibits, the reproductive axis (Oakley et al., 2009; Bentley et al., 2010). The specific target tissue(s) mediating Mel effects on RF-amide expression remain unknown. It is important for our complete understanding of environmental regulation of reproduction to characterize the pathway through which Mel acts to impact the RF-amides. In the future, the results of the current experiments could aid in the independent regulation of these factors in the treatment of reproductive disorders in humans and animals (de Roux et al., 2003; Seminara et al., 2003).

A third putative upstream regulator of the reproductive axis is the thyroid-derived hormone triiodothyronine (T_3). Exposure to SD results in a decrease of type 2 iodothyronine deiodinase (Dio2), an enzyme that catalyzes the conversion of the thyroid hormone thyroxine to T_3, presumably resulting in reduced T_3 availability (Watanabe et al., 2004). Further, T_3 injections stimulate testicular growth in SD-housed Siberian hamsters, thus mimicking exposure to LD lengths (Freeman et al., 2007). Thus, we hypothesize that decreased T_3 results in the SD reproductive phenotype, whereas increased T_3 availability stimulates the LD phenotype. Potential interactions among these three neurohormones have yet to be addressed. We hypothesized that these three molecules form parts of a single pathway by which photoperiod impacts the reproductive system.

In Experiment 1, we used Mel-containing cannulas implanted into two Mel target tissues, the suprachaismatic nucleus (SCN) and the nucleus reunions (NRe) in LD animals to test the hypothesis that Mel acting at separate target tissues independently regulates Kiss1 and GnIH. In Experiment 2 we employed daily injections of T_3 to test the hypothesis that T_3 and the RF-amides make up parts of a single neuroendocrine circuit involved in the regulation of reproduction. This hypothesis predicts that injections of T_3 sufficient to elicit gonadal growth will also result in increased RF-amide expression in SD male Siberian hamsters.

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CHAPTER 2: DIFFERENT NEURAL TARGET TISSUES MEDIATE MELATONIN-DEPENDENT REGULATION OF THE RFAMIDES, KISSPEPTIN AND GONADOTROPIN-INHIBITORY HORMONE, IN SIBERIAN HAMSTERS

Introduction

Many animals that experience predictable seasonal changes in their environment time reproduction so that offspring are born when environmental conditions, including food availability and ambient temperature, are the most conducive to offspring survival (1). Siberian hamsters (*Phodopus sungorus*) exhibit robust seasonal rhythms in physiology and behaviour and use exogenous cues to coordinate these rhythms with the external environment. The main exogenous cue used is photoperiod, or day length. Day length is encoded by the duration of nocturnal melatonin (Mel) secretion from the pineal gland (2, for review see 3). Thus, during exposure to short day (SD) lengths, animals generate a long-duration Mel rhythm, whereas exposure to long day (LD) lengths results in a short-duration Mel rhythm. Seasonal changes in Mel duration drive alterations in many physiological functions including, but not limited to, reproduction, pelage colour and quality, immune function, thermoregulation, body weight, aggressive behaviour, and sexual behaviour (for review: 3-5). Whereas the Mel target tissues necessary to drive many of these physiological rhythms, including reproduction, have been identified and include the hypothalamic Suprachiasmatic nucleus (SCN) and the thalamic periventricular thalamus (PVt) and nucleus reuniens (NRe), among others (6-12). The mechanism by which Mel binding to these targets alters activity of the hypothalamic-pituitary-gonadal (HPG) axis remains unknown.

The HPG axis regulates reproductive function and integrates environmental cues that influence the reproductive system. GnRH stimulates the release of the gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH), from the anterior pituitary (for review see; 13). LH and FSH stimulate gametogenesis and the production of the gonadal sex steroids, including testosterone, oestrogen, and progesterone. The sex steroids act in a classical feedback loop to, in part, regulate GnRH release (for review: 14, and references therein). Long-duration Mel inhibits the release of GnRH by two mechanisms: first, is a steroid-independent pathway in which Mel acts to inhibit the release of GnRH, even in the absence of steroid feedback; and second, Mel acts via a steroid-dependent pathway to increase the sensitivity of the HPG axis to steroid negative feedback (14, and references therein).

Interestingly, GnRH neurones do not express Mel receptors, indicating that Mel does not act directly on these neurones (15). This led to the hypothesis that neuroendocrine intermediate elements exist between reception of the Mel rhythm and GnRH regulation. Several candidate molecules, including thyroid hormones (16- 20) and a novel class of neuropeptides termed RFamides, have been identified. The RFamides include kisspeptin (Kiss1) and gonadotrophin-inhibitory hormone (GnIH) which is also known as RFamide-related peptide (RFRP; 15, 21, 22). Both of these peptides appear to be important in the regulation of the HPG axis (for review see 13) and are sensitive to changes in photoperiod and Mel. Additionally, Kiss1 neurones express steroid receptors (estrogen receptor alpha - ERα), suggesting that these neurones may mediate seasonal changes in steroid-feedback sensitivity (23, 24).

Kiss1 is a neuropeptide that is encoded by the Kiss-1 gene. Its receptor, GPR54, is a G-protein coupled receptor. Kiss1 expression has been localised to neurones located in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) of the hypothalamus of Siberian and Syrian hamsters (*Mesocricetus auratus*). In Siberian hamsters, Kiss1 expression in the AVPV is elevated in long days and inhibited in short days; whereas Kiss1 expression in the ARC shows the opposite pattern (17, 25- 27). Gonadal function and Kiss1 expression in Syrian hamsters exhibits a tight temporal coupling; exposure to SD elicits gonadal regression and a decrease in Kiss1 expression in the AVPV. The onset of photorefractoriness, which occurs in hamsters following ~20 weeks of exposure to SD, results in gonadal recrudescence and is accompanied by the return of Kiss1 expression to levels typically observed in LD (17). Seasonal changes in Kiss1 expression are pineal-dependent, thus, ablation of the pineal gland prevents down-regulation of Kiss1 expression in the AVPV of Syrian hamsters transferred to SD (17). Further, cell cultures derived from rat Kiss1-positive hypothalamic neurones exhibit decreased Kiss-1 gene expression when treated with exogenous Mel. Multiple lines of evidence support a role for Kiss1 in the regulation of GnRH. For example, GnRH neurones express the Kiss1 receptor, Gpr54 (13), and the Kiss1 peptide directly stimulates GnRH secretion in mammals (28). Additionally, Kiss1may also act at the level of the pituitary gland to directly stimulate the secretion of the gonadotrophins, LH and FSH (reviewed by 29). Despite mounting evidence that Kiss1 expression is Mel-dependent and that it can stimulate GnRH and gonadotrophin secretion, there is little direct evidence to suggest that changes in Kiss1 activity drive Mel-dependent changes in gonadal function. In Syrian, but not Siberian, hamsters, chronic exogenous Kiss1 results

in recrudescence of the testes in animals housed in SD (17, 25, 30). Taken together these data indicate that Kiss1 is likely involved in the seasonal regulation of reproduction in Siberian and Syrian hamsters, although species-specific differences exist.

Another RFamide family member that is implicated in the seasonal control of reproduction is GnIH which, in birds increases in SD and suppresses GnRH secretion from the hypothalamus (31). GnIH expression is restricted to the dorsomedial hypothalamus (DMH) of Siberian hamsters and its expression is dependent on photoperiod (21, 32). Experiments conducted *in vitro* indicate that when Mel is administered to a rat cell line that expresses GnIH, GnIH gene expression increases (24). This is consistent with a role for GnIH in the inhibition of reproduction. In Siberian hamsters, it appears that the actions of GnIH are photoperiod-dependent; thus, central GnIH administration suppressed LH secretion in LD housed hamsters while the same treatment stimulated LH secretion in short-day housed hamsters (32). Importantly, injecting Syrian hamsters with exogenous GnIH reduced circulating LH levels (33, but see 34), consistent with an inhibitory role on the HPG axis. In contrast, Revel et al. (2009) found that expression of GnIH was altered seasonally, but *increased* in long-day housed, as compared to short-day housed hamsters, in contrast to the above noted *in vitro* findings (24).

Mounting evidence suggests that the RFamides are involved in photoperiodic regulation of reproduction, although it remains unclear whether the seasonal changes observed in Kiss1 and GnIH are necessary for the expression of Mel-induced gonadal regression. It remains possible that gonadal regression can occur in the absence of changes in the expression of one, or both, of these neuropeptides. In addition, the

pathways by which Mel target tissues communicate photoperiod information to RFamide neurones remain uncharacterised. The present experiments are designed to identify the target tissues through which Mel acts to alter RFamide expression in the AVPV and DMH. It is hypothesised that the Mel target tissues sufficient to elicit gonadal regression in Siberian hamsters will also alter Kiss1 and GnIH expression. Alternatively, it is possible that gonadal regression will occur in the absence of changes in one, or both RFamides. Additionally, these experiments test the hypothesis that the two RFamides are regulated independently by separate Mel target tissues.

Materials and Methods

Subjects

Subjects were adult (~ 60 days of age) male Siberian hamsters from breeding pairs in our colony at the University of Memphis. The colony was derived from animals originally supplied by Dr. Irv Zucker at the University of California, Berkeley. Offspring were weaned at postnatal day 17–19 and housed in same-sex groups of two or three in polypropylene cages (27.8×7.5×13.0 cm). All animals were housed from birth under long day lengths (16 hours of light/day [16L]; light offset at1800 h CST). Adult male hamsters were housed individually in polypropylene cages following surgery. Hamsters had *ad libitum* access to food (8640 Teklad 22/5 Rodent Diet, Teklad Diets, Madison, WI) and water.

Surgery and site specific delivery of melatonin

At ~60 days of age each hamster underwent stereotaxic surgery to implant Mel-cannulas. Hamsters were divided into two groups of 15 animals per group. Stainless steel guide cannula (Plastics One, Inc., Roanoke, VA, USA) were aimed at the SCN and the

NRe (*cf*., 35). Only males with estimated testicular volumes (ETV; width^2*length) greater than 600 mm^3, were included (see 36). ETV was determined using analogue calipers while subjects were under light anaesthesia induced with isoflurane vapours (Isothesia, Butler Animal Health Supply, Dublin, OH, USA). After an injection of ketamine anaesthetic (0.34 ml/100grams body weight), the animals were placed into a stereotaxic device to hold them in place for the surgeries. Guide cannulas were inserted into the brains using predetermined coordinates (SCN; + 0.1 mm anterior from bregma, 0.0 mm lateral from midline, and 6.1 mm below dura: NRe; + 0.1 mm anterior to bregma, 0.0 mm lateral from midline, and -5.0 mm below dura). The guide cannula for the SCN consisted of bilateral 26-g stainless steel tubes extending below a threaded plastic pedestal. The guide cannula for the NRe consisted of 22-g stainless steel tubing extending below a threaded plastic pedestal. Guide cannulas were affixed to the skull using dental acrylic and anchored using three stainless steel screws. After recovery from surgery, internal cannulas (28-g for the NRe, 30-g for the SCN) were inserted into the threaded plastic pedestal and extended 0.5 mm below the guide cannula; internal cannulas were filled with either Mel mixed in beeswax (Mel:beeswax 1:4 ;SCN n = 9 and NRe n = 7) or beeswax as controls (SCN n = 3 and NRe n = 3). Subsequently, all hamsters were returned to 16L for five weeks. The internal cannulas were inserted every evening just before 5pm and removed at 10am CST, thereby extending the nocturnal Mel signal to 17 h to mimic the SD-like Mel rhythm (*cf.*, 12). Sterile dummy cannulas were inserted into the guide cannulas when the internal cannulas were removed. This allowed SD Mel durations to be restricted to the specific Mel target tissues while the rest of the system was exposed to the endogenous LD Mel rhythm. The responses to the implants were

determined by obtaining ETV and body mass at weeks 0, 3, and 5 post treatment, as well as paired testis weights (PTW) at the termination of the experiment.

Histology

After week five, brains were removed and prepared for histology by spinning immersion (*cf.*, 35). The brains were then cut into 30 □M sections on a freezing microtome for immunocytochemical staining (*cf.*, 35). Brains were stained for Kiss1- and GnIH-immunoreactivity (FMRFamide, RA2002, Neuromics Antibodies; diluted in KPBS 1:5000) using a nickel-DAB stain. The FMRFamide antibody recognises both Kiss1 and GnIH. However, because the two peptides are restricted to specific, non-overlapping sites within the brain, we were able to distinguish Kiss1-ir neurones from GnIH-ir neurones (17). The slides were observed under a light microscope to validate the location of the cannulas and to quantify expression of the neuropeptides. Briefly, the RFamides were quantified by counting immunoreactivity in a single representative section from each animal. Sections scored corresponded to rat bregma: -0.26 mm (AVPV) and -3.14 mm (DMH; *cf.*, 35). Because Teubner and Freeman (2006) found that cannulas > 200 μm from the target tissue failed to elicit a short-day response, hamsters with misplaced cannulas were dropped from the experiment. Animals with beeswax implants were combined into a single group (Vehicle, n = 6) as no significant differences existed between these groups with respect to ETV, body mass, Kiss1 expression, or GnIH expression ($p > 0.12$; for each measure).

Statistical analysis

Both body mass and ETV were analyzed using Repeated Measures ANOVA. PTW and RFamide expression were analyzed using two-way ANOVA followed by a

Fishers PLSD post hoc test to further examine the source of between-treatment differences. Differences were considered significant if $p \leq 0.05$.

Results

Localization of brain implants

Figure 1 depicts representative cannula placements. One SCN cannula missed the target tissue by > 200 µm and this subject was excluded from the results. The resulting number of animals per group were as follows; SCN: n = 13, one misplaced and three beeswax vehicle controls; NRe: n = 10, zero misses and three beeswax vehicle controls.

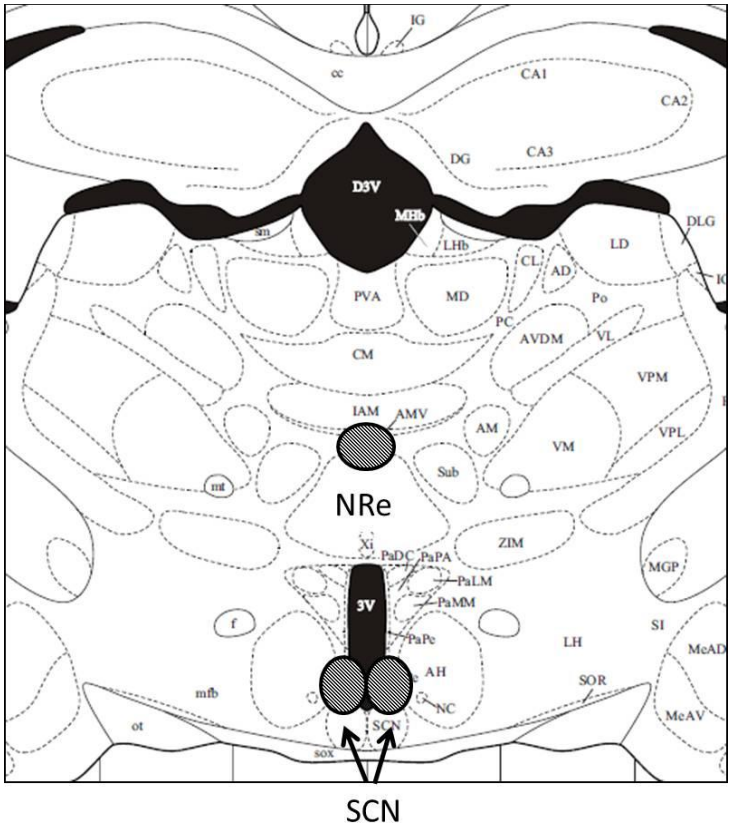
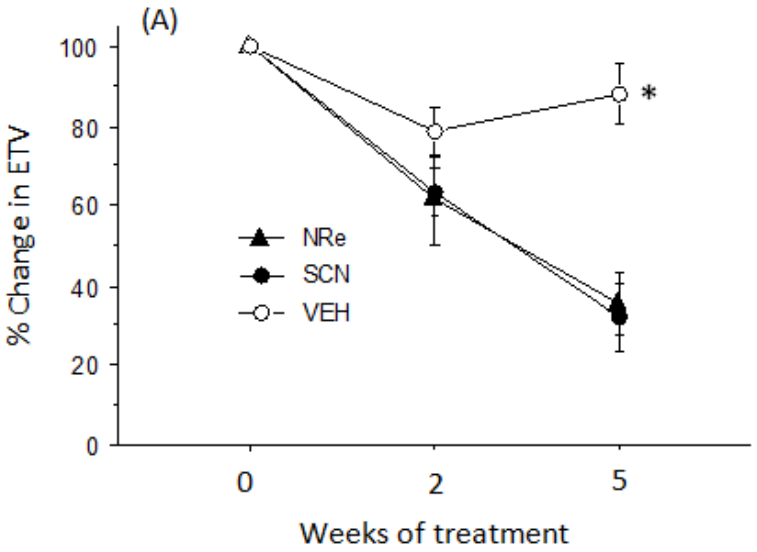


Figure 1. Representative schematic drawing of cannula placement in the SCN (n=13), and the NRe (n=10). *Hatched dots*, correct placement depicted bilaterally (SCN) or as midline structure (NRe).

Testis and body mass responses

There was a significant effect of Mel on ETV (Repeated measures ANOVA; $F_{2,17}$ = 6.38, p = 0.0087; Fig. 2A) and PTW (ANOVA; $F_{2,17}$ = 7.30, p = 0.0052); Fig. 2B). Mel delivery to both the SCN and NRe resulted in significant reduction in the percent change in ETV over the duration of the experiment compared to controls (Fishers PLSD, $p \leq$ 0.006 for both). Mel delivered to the NRe and SCN resulted in significantly lower PTW as compared to controls (Fishers PLSD, p = 0.01 and p = 0.001, respectively; Fig. 2B). Body mass did not differ significantly among groups (Repeated measures ANOVA; $F_{2,19}$ = 0.85, p > 0.4; data not shown).



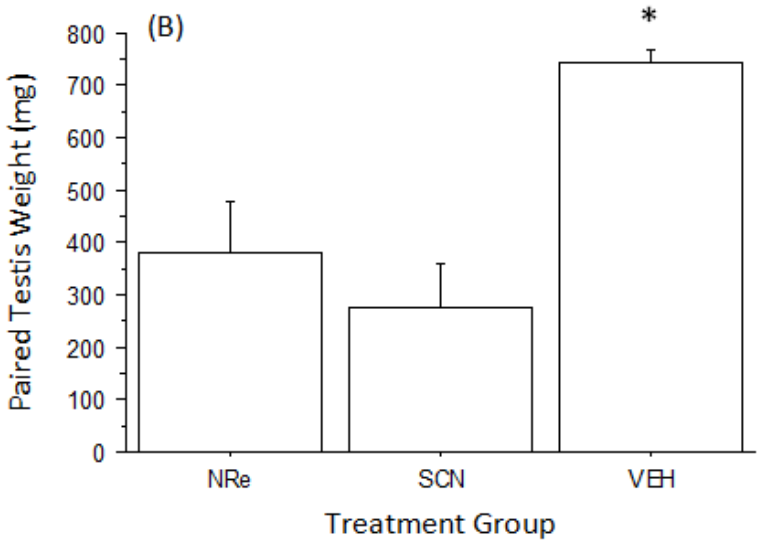


Figure 2. (A) Mean (± SE) percent change from initial value (Week 0 = initial) in estimated testis volumes (ETV). ETVs were obtained at weeks 0, 2, and 5 of the experiment to determine the reproductive status of each individual. (B) Mean (±SE) paired testis weight (PTW) taken at the termination of the experiment. (*****) Vehicle control hamsters exhibited significantly larger testes than either Mel treatment group ($P < 0.03$ for both measures). NRe and SCN refer to Mel cannula location; VEH designates the combined beeswax vehicle control group.

RFamide expression

Kisspeptin: There was a significant effect of Mel on Kiss1 expression (ANOVA; $F_{2,19}$ = 4.29, ; p = 0.029; Fig. 4A). The number of Kiss1-immunoreactive cells in the AVPV was significantly lower in hamsters with Mel cannulas located in the SCN as compared to controls (Fishers PLSD, p = 0.013). The number of Kiss1-immunoreactive (-ir) cells in the AVPV did not differ between controls and hamsters bearing Mel cannulas located in the NRe (Fishers PLSD, $p > 0.8$). There was no significant difference in Kiss1-ir between the NRe and SCN treatment group (Fishers PLSD, $p > 0.05$).

GnIH: There was no significant effect of Mel on GnIH expression overall (ANOVA; $F_{2,19} = 2.65$, $p = 0.093$; Fig. 4B). We used planned comparisons of the treatment groups vs. controls. Mel cannulas localised to the NRe resulted in significantly lower numbers of GnIH-ir cells in the DMH as compared to controls (Fishers PLSD, $p = 0.035$). Whereas Mel cannulas localised to the SCN did not result in significant differences in the number of GnIH-ir cells as compared to controls (Fishers PLSD, $p > 0.3$). No significant difference existed between Mel treatment groups with respect to the number of GnIH-ir cells in the DMH (Fishers PLSD, $p > 0.15$).

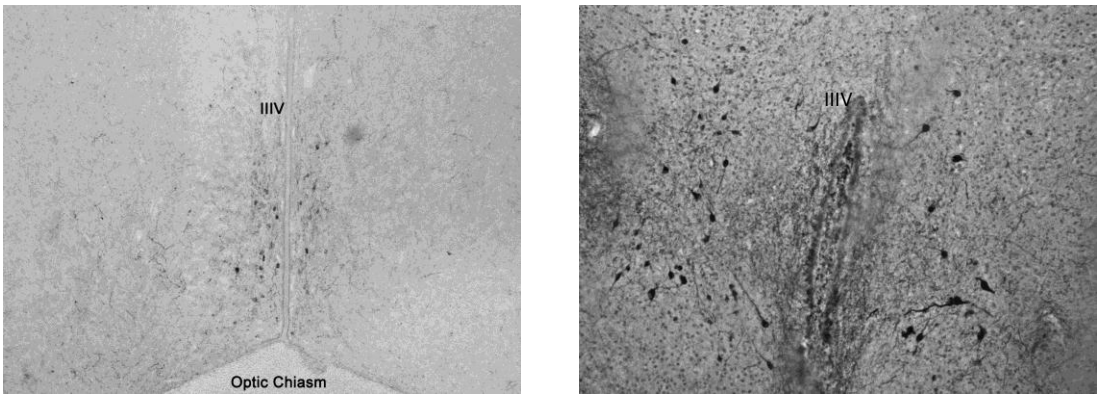


Figure 3. Representative photomicrographs illustrating RFamide-ir, (Left) Kiss1-ir cells in the AVPV and (Right) GnIH-ir cells in the DMH at 100x. (IIIV) refers to the third ventricle. Adobe Photoshop was used to rotate, crop and adjust the brightness of images.

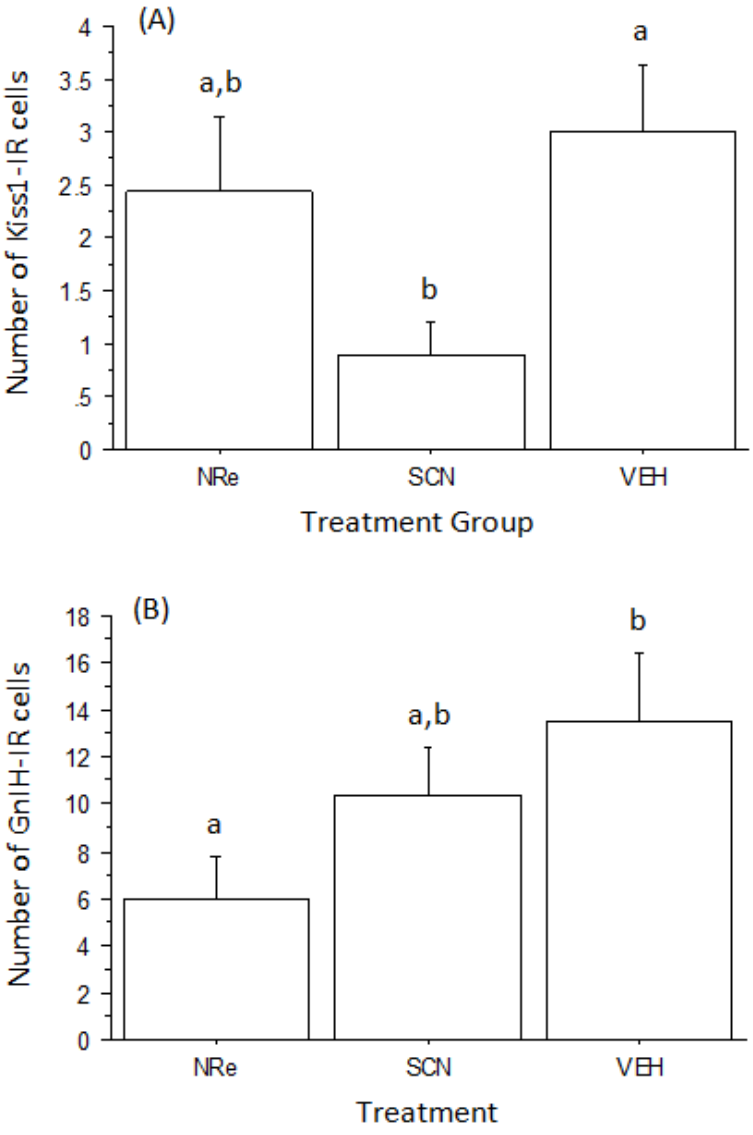


Figure 4. Number of RFamide-ir cells (A) Number of Kiss1-ir cells in the AVPV and (B) The number of GnIH-ir cells in the DMH. Bars with different letters differ significantly from one another ($P < 0.02$ for each comparison), whereas bars bearing the same letter do not differ significantly. Group designations the same as Fig. 2.

Discussion

The results of this study indicate that separate Mel target tissues mediate the effects of Mel on Kiss1 and GnIH in Siberian hamsters. Extending Mel exposure to mimic a SD-pattern to either the NRe or SCN resulted in significant reductions in testis size as compared to controls (*cf.*, 9 and 10), but did not have equivalent effects on RFamide expression. Thus, Mel implants that extended the Mel rhythm in the SCN resulted in gonadal regression and a reduction in the number of Kiss1-ir cells in the AVPV, whereas extending the Mel rhythm only in the NRe resulted in gonadal regression coupled with a reduction in the number of GnIH-ir cells in the DMH. This result suggests that gonadal regression in response to a SD-pattern of Mel does not require the concomitant changes in both RFamides that are typically observed after transfer to short photoperiods (30). In contrast, gonadal regression occurred with a decrease in either neuropeptide, independent of the other. This indicates that the changes in the pattern of Kiss1 and GnIH expression normally associated with a transfer from long- to short-photoperiods are not necessary for gonadal regression to occur, although decreases in the expression of either neuropeptide may be sufficient. These results also support and extend the hypothesis that multiple Mel target-tissues in Siberian hamsters serve distinct roles in the photoperiodic mechanism (9, 10, 12).

Both Kiss1 and GnIH regulate the reproductive axis. Kiss1 stimulates the release of LH and FSH (27, 37, 38), whereas GnIH generally exerts inhibitory effects on LH secretion and sexual behaviour (39, 40, 41, but see 32, 34). It appears that Kiss1 alters gonadotrophin secretion through stimulation of GnRH secretion as GnRH neurones express GPR54 receptors and exogenous Kiss1 results in increased secretion of GnRH

(37, 38, 42). The results from the present experiment indicate that gonadal regression in response to extending the Mel duration in the NRe elicit gonadal regression in the absence of decreases in Kiss1-ir in the AVPV. This suggests that maintaining a LD-like pattern of Kiss1expression in the AVPV alone is not sufficient to maintain LD-like testis size.

In both hamsters and rats, GnIH inhibits LH secretion from the pituitary. This can be attributed to either direct actions of GnIH on GnRH neurones, or through GnIH acting at the level of the anteriour pituitary gland (22, 32). Despite these actions of GnIH on gonadotrophin release, the results of the present experiment suggest that alterations in GnIH expression in the DMH are not necessary for the induction of gonadal involution, since extending Mel duration in the SCN of LD-housed hamsters resulted in gonadal regression in the absence of changes in GnIH expression.

Our findings indicate that Mel implants localised to the SCN or NRe result in testicular regression in Siberian hamsters and independently alter the expression of Kiss1 and GnIH. The action of Mel at the SCN inhibits the upstream positive regulator of GnRH, Kiss1; and Mel's action at the NRe results in decreased GnIH in the DMH. Together these results mimic the effects of transfer to a short photoperiod (21, 32). Despite evidence that expression of both RFamides typically decreases following transfer to SD in Siberian hamsters (13), our data indicate that the simultaneous inhibition of both is not necessary for gonadal regression. Our results are not the first to call into question the exact role of Kiss1 or GnIH in the photoperiodic regulation of the reproductive axis. Previous experiments in Siberian hamsters found that infusions or injections of Kiss1 administered to SD-housed males failed to elicit testicular growth (43).

Paradoxically, in Syrian hamsters, the administration of exogenous RFRP-3 (GnIH) *stimulated* Kiss1 expression and gonadal growth in SD-housed males (34). In addition, central administration of GnIH to Siberian hamsters housed in an inhibitory short photoperiod resulted in stimulation of LH secretion (32). These findings taken together with the current results suggest that the role of RFamides in the control of reproduction remains to be fully characterised and that their actions may vary across species.

The magnitude of gonadal regression observed in response to Mel cannulas localised to a single target tissue was less than that observed after transfer of a pineal-intact hamster to a short photoperiod or in response to peripheral Mel infusions (10); both treatments that result in all Mel target tissues being exposed to a long duration Mel rhythm. It is possible that to elicit complete gonadal regression, Mel must act at multiple target tissues simultaneously, resulting in a decrease in both Kiss1expression in the AVPV, and GnIH expression in the DMH. The intermediate reproductive responses observed in the present experiment may also be related to the fact that the hamsters implanted with Mel cannulas received Mel rhythms of different durations at different target tissues. Whereas all Mel targets were exposed to the endogenous short-duration (i. e., long-day [16L:8D] pattern) Mel rhythm, only one target tissue (either the SCN or NRe) was exposed to the inhibitory, long-duration implant. Thus, the intermediate physiological outcome might represent the sum of both excitatory and inhibitory Mel durations acting concurrently at individual target tissues. Lastly, exposure to SD may also modify Kiss1 and GnIH receptor numbers, thus, altering sensitivity to these neuropeptides (32).

The interactions between Kiss1 and GnIH in the regulation of reproduction remain unclear (13). One model postulates that Kiss1 and GnIH are parts of a circuit in which reciprocal neural connections allow each neuropeptide to suppress the other, thereby fine-tuning the regulation of the HPG axis (32). This model is consistent with the observation that in hamsters, expression of both neuropeptides is decreased following a transfer to inhibitory short photoperiods (17, 21, 25, 26, 32, 42), suggesting that the entire circuit may be down-regulated in response to short photoperiod exposure.

The results of this experiment indicate that the regulation of reproduction by photoperiod and Mel involves complex interactions of Mel acting at multiple target tissues to affect changes in the expression and activity of multiple neuroendocrine factors, including Kiss1 and GnIH among others (e.g., thyroid hormones; 19; Henson and Freeman, in review). This complexity is underscored by results from previous experiments in which administration of Kiss1 alone did not stimulate testis growth in SD-housed Siberian hamsters (25), and the demonstration that exogenous RFRP-3, or GnIH, stimulates the reproductive axis of SD-housed Syrian and Siberian hamsters (32, 34). These findings are consistent with our demonstration that gonadal regression can occur in the presence of either decreased Kiss1, or decreased GnIH expression but that changes in both simultaneously are not necessary for reproductive inhibition.

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CHAPTER 3: EXOGENOUS T_3 INCREASES EXPRESSION OF THE RFAMIDES, KISSPEPTIN AND GONADOTROPIN-INHIBITORY HORMONE, IN SHORT-DAY SIBERIAN HAMSTERS (*PHODOPUS SUNGORUS*)

Introduction

Animals that inhabit temperate latitudes experience predictable seasonal changes in resource availability that impact the likelihood of offspring survival (Bronson, 1988). Many animals, including Siberian hamsters (*Phodopus sungorus*), have evolved endogenous timing mechanisms that enable them to anticipate such changes and to make seasonal adjustments in physiology and behavior that may help them cope with seasonal environmental changes (for review see Prendergast et al., 2002). Thus, hamsters synchronize reproductive effort with the environment so that young are born when conditions are most favorable for offspring survival. Changes in day length are often used as an exogenous cue to track the seasons and serve to set the endogenous rhythm (i. e., serve as a *zeitgeber,*for review see Paul et al., 2008). Day length is encoded by the rhythm of pineal melatonin (Mel) secretion, the duration of which is directly proportional to the duration of the dark period in the light:dark cycle (Carter and Goldman, 1983). Mel secretion is driven by the mammalian circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Stephan and Zucker, 1972). In Siberian hamsters, exposure to short days (SD) results in a long duration of Mel secretion that is inhibitory to the reproductive axis, whereas the short duration Mel rhythm exhibited under long days (LD) is stimulatory to the reproductive axis (Carter and Goldman, 1983). The actions of Mel are mediated by binding to its receptors in several brain nuclei, including the paraventricular thalamus (PVt), the thalamic nucleus reunions (NRe), the

hypothalamic SCN, the subzona incerta, and the dorsomedial hypothalamic nucleus, as well as the pars tuberalis (Weaver et al., 1989; Badura and Goldman, 1992; Song and Bartness, 2001; Teubner and Freeman 2006; Leitner and Bartness, 2010). Long duration Mel signals acting at any of these nuclei are capable of inducing SD-like inhibition of the reproductive axis (Badura and Goldman, 1992; Freeman and Zucker, 2001; Leitner and Bartness, 2010; Henson and Freeman, in review).

Environmental cues impact reproduction through alterations in the hypothalamic-pituitary-gonadal (HPG) axis by altering the secretion of gonadotropin-releasing hormone (GnRH). GnRH acts upon the anterior pituitary to stimulate the secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) which act at the level of the gonads to stimulate the production of sex steroid hormones and gametogenesis. The sex steroids, testosterone, estradiol, and progesterone, in addition to gonadal protein hormones, exert feedback at the level of the hypothalamus and pituitary to regulate this axis (for review see Prendergast et al., 2002, and references therein). SD-patterns of Mel secretion inhibit the HPG axis, although the exact mechanism that mediates these effects remains unclear because GnRH-secreting neurons do not express Mel or sex steroid receptors (Clarke et al., 2009). This suggests that there are neuroendocrine intermediates between the Mel rhythm and the HPG axis.

Previous experiments have indicated that thyroid hormones may serve as a link between the Mel rhythm and Mel-dependent seasonal changes in the HPG axis in Siberian hamsters (Watanabe et al., 2004; Freeman et al., 2007), Syrian hamsters (*Mesocricetus auratus;* Saita et al., 2005; Revel et al., 2006), Japanese quail (*Coturnix coturnix japonica;* Yoshimura et al., 2003; Yasou et al., 2005), and domestic goats

(*Capra aegagrus hircus*; Yasou et al., 2006; Viguie et al., 2009). Siberian hamsters administered exogenous triiodothyronine (T_3) under SDs exhibited increased testicular volume similar to the response after transfer to LD (Freeman et al., 2007). Hamsters exposed to SD exhibit reductions in type-2 deiodinase (Dio2) gene expression in the ependymal cell layer surrounding the third ventricle (Watanabe et al., 2004; Revel et al., 2006), potentially decreasing local availability of T_3. Dio2 catalyzes the conversion of thyroxine (T_4) to T_3 by outer ring deiodination of T_4. Thus, a reduction in the Dio2 gene results in a tissue-level reduction of T_3 (Yoshimura et al., 2003). In domestic goats, which are SD breeders, Dio2 expression decreases in response to LD exposure (Yasuo et al., 2006).

A second enzyme involved in the availability of T_3, is type 3 deiodinase (Dio3). Dio3 inactivates both T_3 and T_4 by inner ring deiodination. The expression of Dio3 is increased in Japanese quail exposed to SD (Yasuo et al., 2005), likely leading to a reduction in T_3 at target tissues. Further, Japanese quail administered exogenous T_3 exhibit physiological responses consistent with exposure to LDs, including growth of the gonads. When Dio2 activity in LD quails was blocked by administration of propanoic acid, testis size was reduced (Yoshimura et al., 2003). Thus, Mel may impact the HPG axis through its actions on the enzymes Dio2 and Dio3, which in turn determine the availability of T_3 in key brain nuclei. A previous study in this lab indicated that increases in T_3 availability in the brain lead to testicular growth in Siberian hamsters housed under SDs (Freeman et al., 2007), although the specific mechanism remains unknown.

Another family of molecules that serve as neuroendocrine intermediates in the regulation of reproduction across taxa are the RFamides, including kisspeptin (Kiss1) and

gonadotropin-inhibitory hormone (GnIH; also known as RFamide related proteins or RFRP; see Bentley et al., 2006, 2010; Kriegsfeld et al., 2006; Johnson et al., 2007; Murakami et al., 2008). Both of these peptides appear to be important in the regulation of the HPG axis and their expression is altered in response to environmental stimuli, including changes in photoperiod (Revel et al., 2006, 2009; Greives et al., 2007; Mason et al., 2007; Roa et al., 2011). Kiss1 and GnIH neurons also express steroid receptors (estrogen receptor alpha [ERα] and androgen receptor), suggesting that these neurons may be important in determining steroid-feedback sensitivity of the HPG axis (reviews in Oakley et al., 2009; Bentley et al., 2010).

Kiss1, a neuropeptide that is encoded by the Kiss-1 gene, has been observed across taxa including mammals, birds, amphibians, and fish (see Oakley et al., 2009). Expression in mammals is limited to the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV), both of which are in close proximity to the third ventricle (Revel et al., 2006; Greives et al., 2007; Mason et al., 2007; Roa et al., 2011). Multiple lines of evidence suggest Kiss1 acts directly to stimulate GnRH secretion. First, Kiss1 receptors (also known as GPR54) are expressed by most GnRH neurons (Irwig et al., 2004). Second, Kiss1 neuronal projections are in close proximity to GnRH neurons (Smith et al., 2008). Third, Kiss1 acts directly to depolarize GnRH neurons in vitro (Oakley et al., 2009). Along with these direct effects, Kiss1 may also act indirectly on intermediary neurons, such as GABAergic cells, to regulate GnRH secretion (Oakley et al., 2009). GnIH expression is restricted to the dorsomedial hypothalamus (DMH) of Siberian hamsters and rats (Bentley et al., 2010). GnIH neurons also express ERα receptors suggesting that they also serve as sites of steroid feedback (Gingerich et al.,

2009). Importantly, injecting Syrian hamsters and LD Siberian hamsters with GnIH reduces circulating LH levels and the expression of sexual behaviors (Kriegsfeld et al., 2006; Ubuka et al., 2012), consistent with an inhibitory role upon the HPG axis.

It remains to be determined whether T_3 and the RFamides serve as distinct, parallel pathways through which environmental cues impact reproduction, or if they are part of a single pathway. We tested the hypothesis that exogenous T_3 will elicit changes in the expression of Kiss1 and GnIH, in addition to eliciting testicular growth in SD male Siberian hamsters.

Materials and methods

Subjects

Male Siberian hamsters (originally supplied by Irving Zucker at the University of California, Berkeley) were gestated and raised in 16L:8D (16L; light offset at 1700 CST), weaned and separated by sex at 17-19 days of age, and housed individually in polypropylene cages (29 x 18 x 13 cm) at 22ºC ± 1 for the duration of the experiment. Animals had access to *ad libitum* food (8640 Teklad 22/5 Rodent Diet, Teklad Diets, Madison, WI) and water.

T_3 Injections

T_3 injections consisted of 0.5 μg of 3, 3', 5 triiodo-L-thyronine (Sigma-Aldrich, Product # 564605) dissolved into 0.1ml of physiological saline, while control injections were 0.1ml of saline. Injections were administered between 1500 and 1600 CST. Adult Siberian hamsters (n = 19) were divided into three groups; one of nine, one of six, and one of four. The nine and six male groups were housed in a SD photoperiod of 10L:14D (10L; light offset at 1700 CST) until they showed responsiveness to short day

lengths (i. e., an estimated testis volume [ETV; width^2*length of left testis] under 200 mm^3). The 4 male group remained under 16L:8D. One group of SD animals received daily T_3 injections (n=9), while the other two groups were subject to saline injections to serve as LD (N=4) and SD (n=6) controls. Based on the response of SD hamsters to identical T_3 injections in a previous experiment in which the maximal testicular response to T_3 was observed after 3 weeks of injections (Freeman et al., 2007), treatments in the present experiment were administered for 3 weeks. Body mass and ETV were determined weekly during the treatment period.

Reproductive Response

ETV was used to assess the gonadal response to changes in photoperiod (Gorman and Zucker, 1995) and in response to T_3 injections. ETV was determined with analog calipers to the nearest 0.1mm while animals were under light anesthesia with isoflurane vapors (IsoSol, Vedco INC., St. Joseph, MO). All procedures were approved by the Animal Care and Use Committee at the University of Memphis.

Histology

At the termination of the experiment, brains were removed and prepared for histology by spinning immersion and subsequently sectioned (30µm in thickness)for immunocytochemical staining (*cf.* Kramer et al., 2008). The brains were stained using anti-Kiss1 antibody (1:2000, Kisspeptin, T4771, lot # 040380-5, Bachem) with 3,3'-diaminobenzidine (DAB) as the chromagen. The Kisspeptin antibody has previously been verified for use in Siberian hamsters (Grieves et al., 2007) and recognizes both Kiss1 and GnIH, but due to the specific and non-overlapping localization of cell bodies that express each peptide in the brain, we were able to distinguish Kiss1 from GnIH (Revel et al.,

2006). Sections were observed under a light microscope to quantify the number of cell bodies positive for each neuropeptide. RFamides were quantified by counting the number of immunopositive neurons in a single representative section from each animal. Sections scored corresponded to rat bregma: -0.26 mm (AVPV) and -3.14 mm (DMH; *cf.*, Kramer et al., 2008).

Statistical Analyses

Both body mass and ETV were analyzed using Repeated Measures ANOVA. RFamide expression was analyzed using two-way ANOVA. Post hoc analyses consisted of Fisher's PLSD test. Differences were considered significant if $p \leq 0.05$.

Results

Testis and Body Mass Response

There was a significant effect of treatment on ETV (Repeated Measures ANOVA; $F_{2,16} = 31.7$, $p < 0.0001$, Fig. 1). Hamsters administered exogenous T_3 exhibited significantly larger testis than SD control hamsters administered saline (Fisher's PLSD, $p = 0.03$). The LD saline control group exhibited significantly larger testes than either SD group (Fisher's PLSD, $p < 0.0005$ for both comparisons). Body mass did not differ significantly among groups (Repeated Measures ANOVA; $F_{2,16} = 0.89$, $p = 0.43$; mean average body mass ranged from 29.5 to 32.9g among treatments).

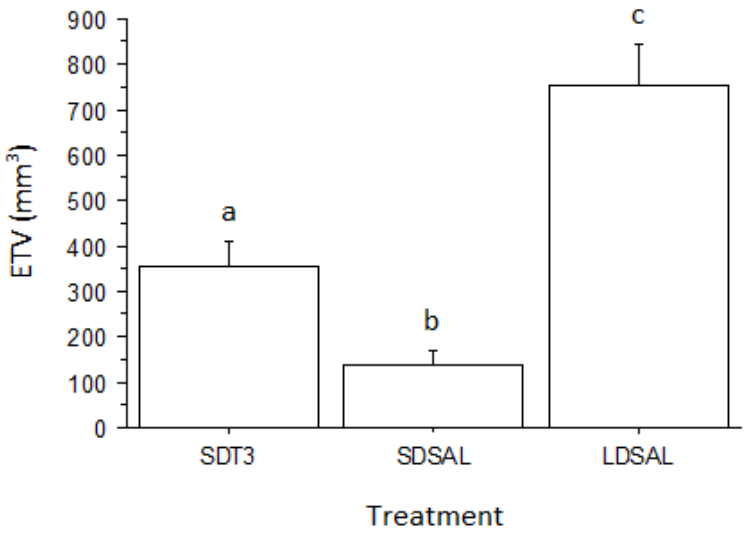


Figure 1. Mean (± 1 SE) ETV following three weeks of treatment. Different letters indicate significant difference between treatments ($p < 0.0001$). (SDT3) refers to short day hamsters treated with T_3, (SDSAL) and (LDSAL) refer to the short and long day saline injected control groups, respectively.

RFamide Expression

Kisspeptin. There was a significant effect of treatment on Kiss1 expression (ANOVA; $F_{2,16} = 9.64$, $p = 0.0018$, Fig. 3A). SD-housed control hamsters exhibited significantly fewer Kiss1-positive cells in the AVPV than the LD controls (Fisher's PLSD, $p = 0.0005$). SD hamsters injected with T_3 exhibited significantly more Kiss1-positive cells than the SD saline control group (Fisher's PLSD, $p = 0.027$), but significantly fewer than the LD saline control group (Fisher's PLSD, $p = 0.021$).

GnIH. There was no significant difference in GnIH expression overall (ANOVA; $F_{2,16} = 2.7$, $p = 0.095$, Fig. 3B). Although the number of GnIH-ir cells in the DMH was significantly lower in the SD control group than in the LD control group (Planned comparison Fisher's PLSD, $p = 0.03$). Administration of T_3 to SD-housed hamsters

resulted in GnIH expression that was intermediate to the SD and LD saline control groups (Planned comparison Fisher's PLSD, $p > 0.1$ for each comparison).

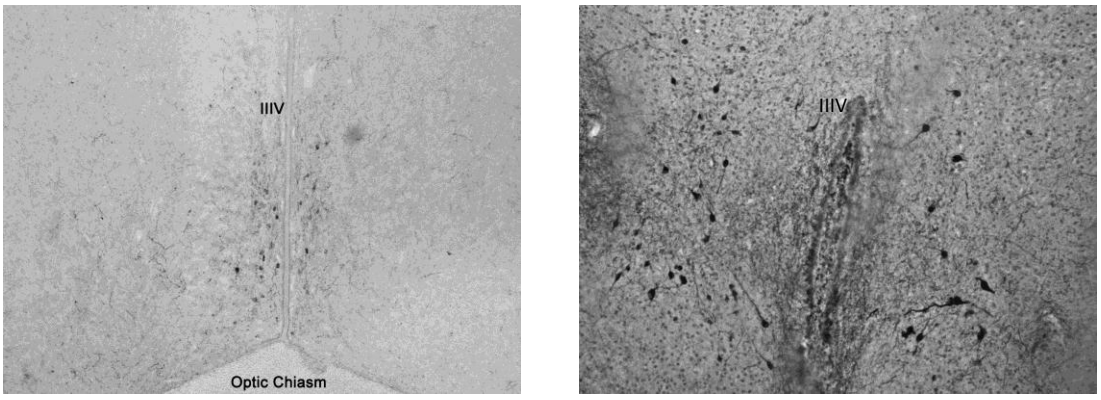
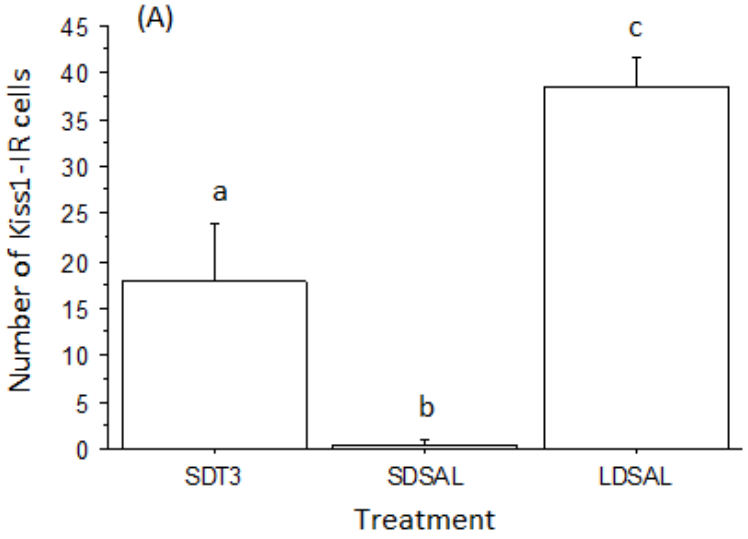


Figure 2. Representative photomicrographs illustrating RF-amide-ir, (Left) Kiss1-ir cells in the AVPV and (Right) GnIH-ir cells in the DMH at 100x. (IIIV) refers to the third ventricle. Adobe Photoshop was used to rotate, crop and adjust the brightness of images.



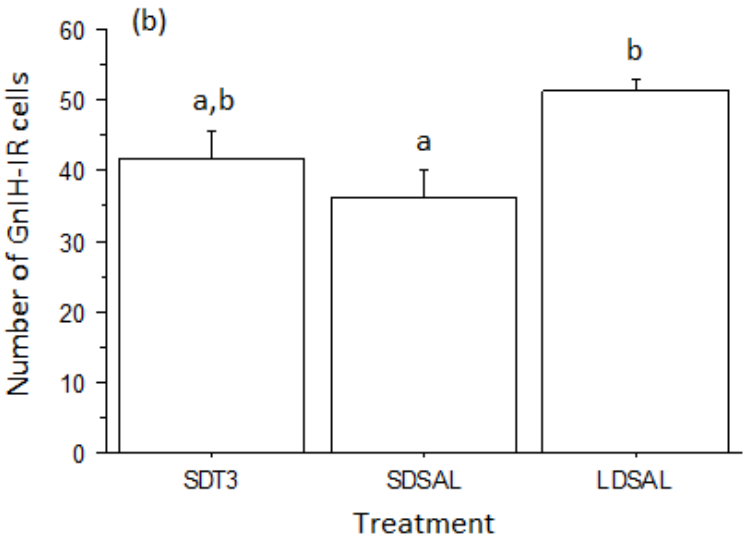


Figure 3. Number of RF-amide-ir cells; (A) Kiss1-ir (± SE) in the AVPV and (B) GnIH-ir (± SE) in the DMH. Bars with different letters differ significantly ($p < 0.04$ for each comparison). Groups are labeled as in Figure 1.

Discussion

The results indicate that exogenous T_3 mimics LD exposure with respect to both testicular growth and RFamide expression in SD-housed hamsters. Thus, T_3 administration resulted in recrudescence of the testes and increased the number of Kiss1 and GnIH expressing cells in the AVPV and DMH, respectively. Administration of T_3 did not, however, elicit the full LD phenotype. Instead, T_3 treatment resulted in intermediate testis size, similar to findings from a previous experiment that employed identical T_3 injections (Freeman et al., 2007). Our results support the hypothesis that T_3 stimulates gonadal growth and extend this finding by suggesting that T_3 impacts gonadal growth through alterations in RFamide expression. This result suggests that rather than

representing two separate neuroendocrine pathways, T_3 and the RFamides represent components of a single pathway through which environmental cues impact the HPG axis. While it is likely that Kiss1 and GnIH share reciprocal neural connections (Parhar et al., 2012), to our knowledge similar anatomical connections have not been documented between T_3-positive neurons involved in Mel-responsiveness and RFamide neurons.

Treatment with T_3 resulted in numbers of RFamide cells that were intermediate to the SD and LD control groups which were characterized by fewer and more RFamide positive neurons, respectively. This supports the hypothesis that T_3 stimulates the reproductive axis via up-regulation of Kiss1 (Oakley et al., 2009). Interestingly, the observed stimulatory effect of T_3 injections on the number of GnIH cells was the opposite of what was expected given that GnIH is thought to act as an inhibitor of the HPG axis (Bentley et al., 2010). Thus it was expected that T_3 administration would result in decreased numbers of GnIH cells, consistent with dis-inhibition of the HPG axis. This seemingly paradoxical pattern of GnIH expression has been noted in other experiments that documented increases in GnIH expression under conditions when the reproductive axis was stimulated. For example, Revel et al. (2009) and Ubuka et al. (2012) both noted elevated GnIH expression under LD exposure in Syrian and Siberian hamsters, respectively. It is possible that GnIH acts to counter balance the actions of Kiss1 on the HPG axis, thus it might be expected that photoperiod-dependent changes in GnIH expression may parallel changes in Kiss1 expression. For example, GnIH appears to regulate the acute inhibition of the reproductive axis in response to stress (Calisi et al., 2008; Kirby et al., 2009). Thus, it is possible that Kiss1 is responsible for the long term regulation of the reproductive axis whereas, GnIH acts as an acute brake. One recent

model of the interaction between Kiss1 and GnIH in the control of reproduction suggests that these peptides act through reciprocal connections to inhibit one another, thus it might be expected that their expression changes in parallel (Parhar et al., 2012).

As in a previous experiment (Freeman et al., 2007), T_3 administration did not elicit complete gonadal re-growth as compared to hamsters exposed to LD for an extended period. Instead, T_3 treatment resulted in an intermediate gonad size that differed significantly from both the SD and LD control groups. This partial recrudescence of the gonads could be due to a multiple factors. For example, the dose of T_3 administered may have been insufficient to elicit full LD gonadal stimulation, although some T_3 administered peripherally does enter the CNS (Palha et al., 2002), the amount of the T_3 that crossed the blood brain barrier may have been insufficient. Additionally, it is likely that multiple cues are necessary for the full transition from the SD- to the LD-phenotype. For example, the transfer of hamsters from a SD to a LD photoperiod results in the rapid truncation of the pineal Mel rhythm (Paul et al., 2008) that, in turn, actively stimulates gonadal growth (Carter and Goldman, 1983), likely by acting at the NRe (Teubner and Freeman, 2006; Teubner et al., 2008), whereas, the present treatment did not alter Mel duration. Lastly, it is possible that in order to attain summer-like testis size, prolactin secretion must also be stimulated. Inhibition of prolactin is necessary for complete testicular regression in short day lengths; thus, long-day like prolactin concentrations may be necessary to attain full testicular growth (Bartke et al., 1980; Amador et al.,1986).

Previous experiments across taxa indicate that the enzymes Dio2 and Dio3 are involved in T_3 production and inactivation, respectively, and that their expression is sensitive to photoperiod and Mel (Watanabe et al., 2004; Yasuo et al., 2005). In hamsters,

exposure to short day lengths results in a decrease in the expression of Dio2 and an increase in the expression of Dio3. This expression pattern likely results in lower T_3 availability in short day lengths. A hypothesis consistent with these findings is that elevated T_3 supports the long day phenotype, whereas the absence of T_3 elicits the short day phenotype. The present results support this hypothesis in Siberian hamsters; thus, T_3 is capable of mimicking long day lengths with regard to gonadal function and expression of the RFamides.

Conclusions

Our results indicate that exogenous T_3 mimics the effects of LD exposure on the reproductive axis of Siberian hamsters. Exposure to LD increases production of Dio 2 (Yoshimura et al., 2003) and decreases circulating levels of Dio 3 in the brain (Yasuo et al., 2005; Barrett et al., 2007). These alterations result in increased T_3 availability, which is thought to stimulate the HPG axis. Our results further suggest that the mechanism by which T_3 stimulates the HPG axis includes alterations in RFamide expression. Several questions remain unanswered, however. For example, the mechanism by which T_3 increases the number of RFamide expressing cells remains unknown. Future experiments could determine whether decreasing T_3 availability in LD hamsters elicits decreases in RFamide expression. Additionally, it remains to be determined whether the RFamides are capable of altering Dio2 and Dio3 expression, thereby regulating T_3 availability.

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CHAPTER 4: CONCLUSION

We determined from the results of Experiment 1, that while Mel cannulas elicited testicular regression when localized to either target tissue, they elicited site-specific effects on the expression of the two RF-amides. Mel administered to the SCN resulted in a reduction in the number of Kiss1 cells in the AVPV, whereas Mel localized to the NRe resulted in a reduction in the number of GnIH cells in the DMH. These results suggest that the changes in Kiss1 and GnIH typically observed after transfer to short photoperiods are not necessary for gonadal regression, but that decreases in either may be sufficient. We also determined in Experiment 2, that injections of T_3 that induced gonadal growth also elicited up-regulation of both RF-amides. These results indicate that T_3 acting via the RF-amides may serve as an intermediate step between the melatonin rhythm and the reproductive response. Taken together these experiments indicate that Mel regulates the RF-amides independently of one another by actions at separate target tissues. In addition, the results suggest that photoperiod-dependent alterations in T_3 may act through changes in the expression of the RF-amides to ultimately elicit changes in gonadal function.

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IACUC PROTOCOL ACTION FORM

To:	David Freeman
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	4-3-08

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. 0642 (Neuroendocrine Mechanisms Seasonal Reproduction)

☒ Your proposal is approved for the following period:

From: April 4, 2008 To: April 3, 2011

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: _____ To:

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Revision Memorandum dated _____ for the following period:

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☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Revision Memorandum dated _____

Prof. Guy Mittleman, Chair of the IACUC

Dr. Karyl Buddington, University Veterinarian
And Director of the Animal Care Facilities

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IACUC PROTOCOL ACTION FORM

To:	David Freeman
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	4-3-08

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. 0643 (rhythms in aggression by photic alterations in central estrogen)

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From: April 4, 2008 To: April 3, 2011

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

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