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Photic and non-photic seasonal cues differentially engage hypothalamic kisspeptin and RFamide-related peptide mRNA expression in Siberian hamsters

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Abstract

Seasonally breeding animals use a combination of photic (i.e., day length) and non-photic (e.g., food availability, temperature) cues to regulate their reproduction. How these environmental cues are integrated is not understood. To assess the potential role of two candidate neuropeptides, kisspeptin and RFamide-related peptide-3 (RFRP), we monitored regional changes in their gene expression in a seasonally breeding mammal exposed to moderate changes in photoperiod and food availability. Adult male Siberian hamsters (*Phodopus sungorus*) were housed in a long (16 h light/day; 16L) or intermediate (13.5L) photoperiod and fed *ad libitum* or a progressive food restriction schedule (FR; reduced to 80% of *ad libitum*) for 11 weeks. Gonadal regression occurred only in FR hamsters housed in 13.5L. Immunohistochemistry was used to identify diencephalic populations of kisspeptin- and RFRP-immunoreactive cells, and quantitative PCR was used to measure gene expression in adjacent coronal brain sections. Photoperiod but not food availability altered *RFRP* mRNA expression in the dorsomedial sections, whereas food availability but not photoperiod altered *Kiss1* expression in the arcuate sections; intermediate photoperiods elevated *RFRP* expression, and food restriction suppressed *Kiss1* expression. Regional- and neuropeptide-specific activity of RFamides may provide a mechanism for integration of multi-modal environmental information in the seasonal control of reproduction.

Keywords

RFamide; seasonality; energetics; reproduction; neuropeptides

INTRODUCTION

In environments where robust seasonal cycles of temperature and food availability prevail, seasonal cues regulate the phase of the geophysical cycle during which critical events in mammalian reproduction occur (puberty, ovulation, breeding, parturition, weaning) (Paul *et al*., 2008, Prendergast *et al*., 2009). In Siberian hamsters (*Phodopus sungorus*), as in other small photoperiodic rodents, summer photoperiods ≥14 h light/day (14L) stimulate, and winter photoperiods <12L inhibit, testicular development, gametogenesis, ovarian function, and reproductive behavior (Hoffmann, 1982, Park *et al*., 2004, Schlatt *et al*., 1995). The

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light-dark cycle entrains a circadian rhythm of nocturnal pineal melatonin secretion (Illnerová, 1991), the duration of which acts on pituitary, thalamic, and hypothalamic melatonin-sensitive target tissues to convey photoperiodic information to the reproductive axis (Badura & Goldman, 1992, Bartness *et al*., 1993, Carter & Goldman, 1983a, b, Glass & Lynch, 1982, Morgan & Hazlerigg, 2008).

Recent work has suggested that photoperiodic regulation of two hypothalamic RFamides (Arg-Phe-NH_2), kisspeptin and RFamide-related peptide-3 (RFRP), may figure prominently in the transduction of melatonin signals to the HPG axis. Kisspeptin stimulates (Gottsch *et al*., 2004, Greives *et al*., 2007, Irwig *et al*., 2004, Messager *et al*., 2005), and RFRP inhibits (Kriegsfeld *et al*., 2006) LH secretion in mammals. In photoperiodic rodents, short photoperiods that trigger gonadal regression yield decreases in *Kiss1* and *RFRP* mRNA and their respective protein expression in the anteroventral periventricular nucleus (AVPV; for kisspeptin) and mediobasal hypothalamus including the dorsomedial hypothalamus (DMH; for RFRP) (Greives *et al*., 2007, Mason *et al*., 2007, Revel *et al*., 2008, Revel *et al*., 2006). In Siberian hamsters, kisspeptin peptide and mRNA expression increase in the arcuate nucleus (ARC) after transfer to short photoperiods (Greives *et al*., 2007, Mason *et al*., 2007, Simonneaux *et al*., 2009); in Syrian hamsters, however, transfer to short photoperiods decreases kisspeptin expression in this nucleus (Revel *et al*., 2006). Kisspeptin and RFRP may act in concert to stimulate or inhibit the HPG axis during photoperiod-induced reproductive transitions (Kauffman *et al*., 2007, Smith *et al*., 2008).

Non-photic environmental cues, such as food availability, ambient temperature, and social interactions, also contribute to seasonal timekeeping, but their underlying neurobiology is not understood (reviewed in Ball, 1993, Paul *et al*., 2008). Recently, a model was developed whereby non-photic seasonal regulation can be studied under static photoperiods. Siberian hamsters housed in an intermediate day length (13.5 h light/day; 13.5L) from birth undergo gonadal growth. After this gonadal development occurs, mild food restriction or increased population density can trigger substantial reproductive inhibition in hamsters housed in 13.5L, but these non-photic cues are without effect on hamsters housed in a long photoperiod (16L; Paul *et al*., 2009). This model permits investigation into candidate neural substrates responsible for the integration of photoperiodic and non-photic cues for the reproductive axis (e.g., kisspeptin and RFRP). For example, if kisspeptin is one such site of environmental cue integration, then intermediate photoperiods and mild food restriction, neither of which elicits gonadal responses alone, should each induce partial changes in *Kiss1* mRNA (e.g., moderate decreases in *Kiss1* mRNA in the AVPV); when 13.5L and mild food restriction are presented in combination— and reproductive responses occur— larger *Kiss1* mRNA responses would be expected. Alternatively, photoperiod and food restriction may be integrated via distinct kisspeptin neuronal populations: transfer to intermediate photoperiods may decrease *Kiss1* mRNA in the AVPV and mild food restriction may decrease *Kiss1* mRNA in the ARC. Similar predictions could be made for RFRP or for coordinated changes in both kisspeptin and RFRP signals among the AVPV, ARC, and DMH. On the other hand, if the integration of photoperiod and food restriction occurs upstream from the RFamides, then mRNA patterns in each nucleus should simply mirror gonadal responses: altered RFamide mRNA expression should only occur in hamsters challenged with both intermediate photoperiods and food restriction.

Here we describe an experiment that examined changes in *Kiss1* and *RFRP* gene expression in response to modest changes in photoperiod and food availability, which combined, were sufficient to induce gonadal regression. The results suggest that these two RFamides respond to photic and non-photic cues in a peptide- and regionally-specific manner and may work in concert to integrate seasonal cues via distinct neuronal pathways.

MATERIALS and METHODS

Animals and housing conditions

Adult (>4 months) male Siberian hamsters (*Phodopus sungorus*) were obtained from 13.5:10.5 h light:dark cycle (13.5L) or 16L (lights off at 1230 h CST for both photoperiods) breeding colonies maintained at the University of Chicago and housed singly from weaning in polypropylene cages (28 × 17 × 12 cm) with wood shavings (Harlan Sani-Chips, Harlan Inc., Indianapolis, IN, USA) under their same natal photoperiods. Ambient temperature of the experimental rooms was 20 ± 0.5°C and relative humidity was maintained at 53 ± 2%. Food (Teklad Rodent Diet 8604, Harlan Inc.) and filtered tap water were provided *ad libitum*, except where otherwise indicated. The data reported here are derived from a subset of 23 animals in a recent study that described the efficacy of intermediate day lengths in unmasking morphological reproductive and hormonal responses to food restriction (Paul *et al*., 2009). All procedures conformed to the USDA Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Chicago.

Reproductive and somatic measures

The length and width of the left testis were measured (±0.1 mm) through the abdominal skin using calipers while hamsters were under light isoflurane anesthesia. The size of the testis was estimated as the product of the length × width^2 (estimated testis volume; ETV), which is correlated with testis weight, circulating testosterone, and spermatogenesis (Gorman, 1995, Schlatt *et al*., 1995). Body masses (±0.1 g) were also recorded at the time of reproductive measurements.

Food restriction

Food restriction (FR) was accomplished by progressive reductions from mass-specific *ad libitum* intake (0.13 g food / g body mass; determined in a pilot study; data not shown) administered to each hamster in a single daily ration shortly after the onset of darkness (16L-FR: n=6; 13.5L-FR: n=6). From weeks 0 through 6, FR hamsters were initially provided with 90% of ad libitum daily intake (0.117 g/g), and thereafter (weeks 6 though 11) received 80% of ad libitum intake (0.104 g/g). This pattern of food restriction was designed to more closely simulate the progressive late-summer decrease in food availability that occurs in this species' natural environment (Weiner, 1987). Control hamsters had free access to food throughout the study (AdLib; 16L-AdLib: n=5; 13.5L-AdLib: n=6).

Selection of regionally specific tissue samples for mRNA quantification

Given the opposite photoperiodic responses of kisspeptin peptide and mRNA expression in the AVPV and ARC of Siberian hamsters (Greives *et al*., 2007, Mason *et al*., 2007, Simonneaux *et al*., 2009), measures of whole hypothalamic *Kiss1* expression is problematic. To achieve both quantitative and regionally specific RFamide data, a method combining immunohistochemistry (IHC) and quantitative PCR (qPCR) was used. One of two alternating sets of 30 μm coronal sections, rostral to caudal, was processed using IHC for RFamide immunoreactivity (RFamide-ir) in the anteroventral periventricular nucleus (AVPV), the arcuate nucleus (ARC), and the dorsomedial nucleus of the hypothalamus (DMH). The remaining set of sections was reserved for qPCR. Total RNA was extracted from the fixed sections adjacent to those that exhibited immunoreactivity in the AVPV, ARC, and DMH (3 sections per brain region). Sections were pooled within each brain region to generate 3 tissue samples for each hamster, 1 containing the AVPV, 1 containing the ARC, and 1 containing the DMH. Immunopositive perikarya were restricted to the AVPV, ARC, and DMH nuclei (Fig. 1A-C; cf. Gottsch *et al*., 2004). We further quantified *Kiss1*

and *RFRP* mRNA levels within the hypothalamus, thalamus, and cortex of a separate set of hamsters to validate claims that the changes in gene expression were in fact of hypothalamic origin.

Immunohistochemical localization of RFamide-ir cells

FR and AdLib treatments continued through the day of sacrifice. At the midpoint of the light phase, hamsters were deeply anesthetized with an overdose of sodium pentobarbital (15 mg/animal, i.p.) and were perfused transcardially with 40 ml of 0.9% saline, followed by 40 ml of 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Brains were postfixed for 2 days in fixative before cryoprotection with a mixture of buffered fixative and 30% sucrose for 2–3 days, and freezing at −80°C until sectioned.

Brains were sliced coronally at 30 μm on a freezing sliding microtome and free-floating sections were stored in cryoprotectant (Watson *et al*., 1986) at −20°C until processed for RFamide-ir. For each animal, alternating sections, rostral to caudal, were stained for using standard ABC immunocytochemistry, following the methods of Kramer et al. (2006). RFamide-ir cells were labeled using a rabbit anti-human kisspeptin antiserum diluted at 1:5000 (T-4771; Peninsula Laboratories Inc, Bachem, San Carlos, CA) raised against the following amino acids Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2, corresponding to amino acids 4-13. Sections were examined under bright field illumination on a Nikon Eclipse 80i microscope.

In the Siberian hamster hypothalamus, the T-4771 antibody cross-reacts with kisspeptin and RFRP, detecting cells in the AVPV, the ARC, and the DMH. Pre-adsorption procedures have indicated that the immunoreactive cells in the AVPV and ARC express kisspeptin, whereas those in the DMH express RFRP (Greives *et al*., 2007). Nonetheless, concerns remain regarding the specificity of this antibody (Goodman *et al*., 2007, Mikkelsen & Simonneaux, 2009), and recent studies have indicated that a diffuse population of kisspeptin cells may also exist in the DMH, at least in the laboratory mouse (Clarkson *et al*., 2009). Given these concerns and the fact that IHC is only semi-quantitative by nature, we restricted our use of this staining for localization of RFamide expression within the target nuclei, allowing selection of sections containing the AVPV, ARC, or DMH for qPCR.

Quantification of Kiss1 and RFRP expression via qPCR

Extractions were performed according the manufacturer's protocol (RNeasy FFPE kit, Qiagen, Valencia, CA, USA) with one exception: paraffin removal steps were skipped. Extracted RNA was suspended in 30μl RNase-free water and RNA concentration and quality were determined by spectrophotometer. All RNA samples were stored at −70° C until further analysis. cDNA was created via reverse transcription of 2μg of RNA from each sample with MMLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

To design primers and a probe for quantitative PCR with high specificity for this species, a portion of each gene of interest was sequenced. To sequence portions of these genes, semi-quantitative PCR was conducted on 1μl of pooled Siberian hamster hypothalamic cDNA with Taq DNA Polymerase enzyme (Invitrogen) according to the manufacturer's protocol in a thermocycler for 40 cycles (Bio-Rad). Degenerate primers were designed based on conserved regions among multiple species with known gene sequences (GenBank) using PrimerExpress software (Applied Biosystems, Foster City, CA, USA). PCR gene product amplification was visualized on 2% TAE-agarose gels containing ethidium bromide using a CCD camera. To verify amplification of the correct gene, PCR products were purified (Centricon-100, Millipore, Billerica, MA, USA) and directly sequenced at the University of

Chicago Cancer Research Center DNA Sequencing Facility. The resulting amplicon sequences for Siberian hamster *Kiss1* and *RFRP* were >90% homologous to published sequences for *Mus musculus Kiss1* and *RFRP*. Sequencing information was entered in the GENBANK database: *RFRP* (Accession # EU365871) and *Kiss1* (Accession # EU365872).

After confirmation of gene products, primers and probes for quantitative PCR were designed using PrimerExpress. Primers and probes were synthesized as follows, with probes labeled with 6-FAM and MGB (non-fluorescent quencher) at the 5′ and 3′ ends, respectively: *RFRP* forward 5′-GCCCCTGCCAACAAAGTG-3′, *RFRP* reverse 5′-CAGGGTCCTCCCAAATCTCA-3′, *RFRP* probe 5′-CCCACTCAGCAGCCA-3′; *Kiss1* forward 5′-AACTCATCAATGCCTGGGAAA-3′, *Kiss1* reverse 5′-GCTCGCAGTCCTCCAGGTT-3′, *Kiss1* probe 5′-CGGTGCGCAGAGAG-3′. A TaqMan 18S Ribosomal RNA primer and probe set (labeled with VIC; Applied Biosystems) was used as the control gene for relative quantification. Amplification was performed on an ABI 7900HT Sequencing Detection System by using Taqman® Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were: 50° C for 2 min, 95° C for 10 min, followed by 40 cycles of 95° C for 15 sec and 60° C for 1 min. Relative gene expression of individual samples run in duplicate was calculated by comparison to relative standard curve consisting of serial dilutions of pooled *P. sungorus* hypothalamic cDNA (1:1, 1:10, 1:100, 1:1000, 1:10,000) followed by normalization to *18S rRNA* gene expression. RNA quality for each sample was assessed via 260/280 ratio using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) before reverse transcription. All samples had acceptable ratios between 1.8 and 2.0 (mean: 1.98; range: 1.81-2.0).

Cerebral localization of mRNA signals—Hamsters (n=3) fed ad libitum were perfused in a manner identical to that described above for experimental animals. Brains were removed, post-fixed, frozen, and sectioned. Free-floating sections from each of three rostro-caudal brain regions (AVPV: bregma −0.3 mm; DMH: bregma −2.5 mm; ARC: bregma −3.3 mm) (Paxinos & Watson, 1998) were then dissected into cortex (including limbic structures), hypothalamus, and thalamus under a 4X dissecting microscope at 4°C (Fig. 1D-F). Between 10-12 microdissections from each brain region were pooled and expression levels of *Kiss1* and *RFRP* were determined using qPCR as described above.

Statistics

Data are expressed as means ± SEM for each group. Repeated measures and factorial ANOVAs were used to measure variation within and between experimental groups, and differences between means were assessed by least significant difference tests and t-tests, where warranted by a significant *F* statistic. Significance level was set at $P \leq 0.05$ for all tests. Analyses were performed using Statview 5.0.1 for the PC (SAS Institute Inc., Cary, NC, USA).

RESULTS

Food restriction induces gonadal regression only in intermediate day lengths

FR treatments inhibited body mass gains in both photoperiods ($F=9.9$; $P=0.005$; Fig. 2A); the magnitude of body mass inhibition tended to be greater in FR hamsters housed in 13.5L relative to those in 16L but fell short of significance ($P<0.10$). FR treatments and photoperiod interacted to affect testis size ($F=24.4$; $P<0.0001$); as in the parent population (Paul *et al*., 2009), significant gonadal regression only occurred in 13.5L hamsters ($P<0.0001$; Fig. 2B,C).

Kiss1 and RFRP mRNA expression

qPCR analysis of microdissections permitted evaluation of cortical, thalamic, and hypothalamic contributions to *Kiss1* and *RFRP* gene expression obtained from whole coronal sections at each of the 3 rostro-caudal levels investigated (*Kiss1*: AVPV and ARC; *RFRP*: DMH; Fig. 3). At the level of AVPV, most of the *Kiss1* signal (83%) was derived from hypothalamic cells; the remainder originated from cortex and thalamus. At the level of the ARC, the hypothalamus was the largest source of *Kiss1* gene expression (45%), but a substantial *Kiss1* signal (37%) was detected in cortex. At the level of the DMH, over 99% of the *RFRP* signal was hypothalamic in origin (Fig. 3).

In sections containing the ARC, *Kiss1* gene expression was significantly decreased in FR relative to AdLib hamsters ($F=7.9$; $P=0.01$; Fig. 4A) but was not affected by photoperiod ($F=1.1$; $P>0.3$); the interaction between these factors was not significant ($F=0.2$; $P>0.8$). In 16L hamsters, FR significantly inhibited *Kiss1* expression at the level of the ARC ($P<0.05$); decreases in 13.5L FR hamsters fell just short of significance ($P<0.06$). At the level of the AVPV, neither photoperiod ($F=0.2$; $P>0.6$), nor FR ($F=0.9$; $P>0.3$) significantly affected *Kiss1* gene expression (Fig. 4B); the interaction was also not significant ($F=0.2$; $P>0.6$).

In sections containing the DMH, *RFRP* expression was significantly greater in 13.5L relative to 16L hamsters ($F=9.1$; $P<0.01$), independent of food manipulations ($F<0.1$; $P>0.9$; Fig. 4C); again, there was no significant interaction between photoperiod and feeding ($F<0.1$; $P>0.9$). In both AdLib and FR hamsters, *RFRP* expression was ~40-fold higher in 13.5L relative to 16L hamsters (Fisher's PLSD; $P<0.05$, both comparisons).

DISCUSSION

Intermediate day lengths render the reproductive axis of Siberian hamsters more responsive to non-photic, exteroceptive cues such that testicular regression occurs only in hamsters exposed to both food restriction and intermediate day lengths (Paul *et al*., 2009). The present work extended this finding to investigate neuroendocrine mechanisms of photic and non-photic cue integration.

At the level of the ARC, food restriction induced a ~5-fold decrease in *Kiss1* expression but no main effect of photoperiod was detected. Thus, modest reductions in food availability decrease *Kiss1* mRNA at the level of the ARC, either by downregulating *Kiss1* transcription or by increasing post-transcriptional processing (e.g., translation and degradation). These data are consistent with reports in other non-photoperiodic species (Castellano *et al*., 2005, Luque *et al*., 2007, Smith *et al*., 2006). Given the well-established role of the ARC in regulating energy balance (Hill *et al*., 2008, Morgan *et al*., 2006), ARC kisspeptin neurons are ideally situated to monitor the metabolic state of the organism. In sections containing the AVPV, neither photoperiod nor food restriction affected *Kiss1* mRNA expression. At the level of the DMH, food restriction did not alter *RFRP* gene expression; however, exposure to intermediate photoperiods increased *RFRP* mRNA in the DMH ~40-fold.

The current data provide novel insights into environmental influences on the RFamide system. Non-photic (reduced food availability) and photic (intermediate day lengths) stimuli inadequate to impact reproductive physiology, nonetheless alter *Kiss1* and *RFRP* gene expression, respectively. Remarkably, in neither the ARC nor the DMH were any additive effects of food restriction or photoperiod evident. Gonadal responses were only associated with changes in both ARC *Kiss1* and DMH *RFRP*. Such a pattern of responses is consistent with a role for the RFamide system as an integrator of multimodal seasonal cues (Kriegsfeld, 2006). Cue integration is a complex process and undoubtedly involves other neuropeptides

as well. Nonetheless, a simple model in which environmental cues act on distinct GnRH input pathways, including kisspeptin and RFRP, is compatible with the present data.

Microdissections (cortex, thalamus, hypothalamus) of coronal brain sections indicated that at the level of the AVPV and the DMH the overwhelming majority of the *Kiss1* (>80%) and *RFRP* (>99%) mRNA, respectively, originated in the hypothalamus. Given the highly localized patterns of kisspeptin-ir and RFRP-ir in adjacent sections, we are confident that the vast majority of the *Kiss1* and *RFRP* mRNA obtained from whole coronal sections originated in the AVPV and DMH, and that the differences in mRNA expression between treatment groups reflect differences within these nuclei. Some caution is warranted, however, in interpreting data from the coronal sections at the rostro-caudal level of the ARC: although the strongest *Kiss1* signal originated in the hypothalamus (45%), a substantial amount of *Kiss1* mRNA was also expressed in cortex. Limbic structures (medial amygdala and hippocampus) are the likely source of this cortical *Kiss1* (37% of total; cf. Arai, 2009, Gottsch *et al*., 2004). Although the efferent targets of these populations of kisspeptin neurons have not been identified, numerous amygdalo-hypothalamic projections exist that govern reproductive responses (e.g., pheromone-processing circuits; Baum, 2009). Amygdala *Kiss1* may also participate in the regulation of gonadotrophin production by photoperiod or energetic cues. Future studies may attempt more precise neuroanatomical localization via *in situ* hybridization; however, in common with immunocytochemistry, such an approach is only semiquantitative. The approach we report here describes novel quantitative measurement of hypothalamic gene expression, in conjunction with neuroanatomical localization on adjacent sections, which may prove useful in future studies of brain gene expression.

A recent report indicated decreased *RFRP* mRNA in the mediobasal hypothalamus including the DMH following 10 weeks of exposure to a short photoperiod (Revel *et al*., 2008), but several methodological differences, most notably the use of intermediate photoperiods in the present report (cf. categorically short photoperiods in Revel *et al*., 2008), preclude direct reconciliation of these two studies. However, dynamic changes in the magnitude of RFRP restraint on GnRH neurons (Gibson *et al*., 2008, Kriegsfeld *et al*., 2006) may occur over time during photoperiod-induced gonadal regression. For example, an increase in *RFRP* expression may be required to initiate gonadal regression, but less critical for the maintenance of gonadal involution once regression is completed. Such a mechanism would be analogous to the dynamic stimulatory neuroendocrine events that evolve during photoperiod-induced gonadal growth in mammals and birds: robust, transient increases in *GnRH* expression and gonadotropin secretion initiate testicular development, but decline markedly thereafter, and are less critical to the maintenance of the long-day reproductive phenotype, once achieved (Bernard *et al*., 1999, Yellon & Goldman, 1984). Measurement of *RFRP* expression at several time points during the course of seasonal gonadal growth and regression should permit evaluation of this conjecture.

Gonadal steroids provide feedback regulation of kisspeptin and RFRP (Greives *et al*., 2008, Kriegsfeld *et al*., 2006, Smith *et al*., 2005a, Smith *et al*., 2005b). Because gonad-intact hamsters were used in the present study, gonadal steroid-dependent effects on *Kiss1* and *RFRP* in the present report cannot be definitively excluded. However, it is unlikely that circulating steroid concentrations account for the broad patterns of altered gene expression for the following reasons. Testosterone concentrations would be expected to be lowest in the group that underwent gonadal regression. However, FR caused decreases in *Kiss1* mRNA at the level of the ARC in hamsters maintained in both 16L and 13.5L, whereas gonadal regression occurred only in FR hamsters maintained in 13.5L. Similarly, exposure to 13.5L caused increases in DMH *RFRP* mRNA in both *ad libitum* and FR hamsters, despite gonadal regression only occurring in the latter group. In addition, circulating testosterone

concentrations of *ad libitum* fed Siberian hamsters maintained in 13.5L or transferred to 16L do not differ (Paul *et al*., 2009), yet in the present investigation, *RFRP* mRNA was elevated in 16L hamsters compared to 13.5L hamsters. Lastly, the direction of these changes in *Kiss1* and *RFRP* expression are opposite of what would be predicted by decreased gonadal steroids (Kriegsfeld *et al*., 2006, Smith *et al*., 2005b). Collectively, these arguments suggest that gene expression responses to photoperiod and food reported here are unlikely to be driven by altered gonadal steroid production. This conclusion is consistent with by several other studies which have demonstrated gonadal steroid-independent modulation of RFamide mRNA levels (Greives *et al*., 2008, Revel *et al*., 2008, Revel *et al*., 2006).

In nature, seasonal adaptations are regulated by both day length and non-photic cues. Early-spring and late-summer intermediate-duration day lengths usher an interval of heightened reproductive responsiveness to non-photic cues that ultimately govern the precise timing of seasonal reproductive transitions (Paul *et al*., 2009). The present results point to two hypothalamic RFamide peptides, kisspeptin and RFRP, in the mediation of photic and non-photic cues on the reproductive system, and suggest that they serve different roles. Intermediate day lengths increase DMH *RFRP* gene expression, and decreased food availability suppresses *Kiss1* mRNA at the level of the ARC. Taken together, the data suggest regional- and neuropeptide-specific recruitment of the RFamide system in the integration of photic and non-photic cues for the control of reproduction.

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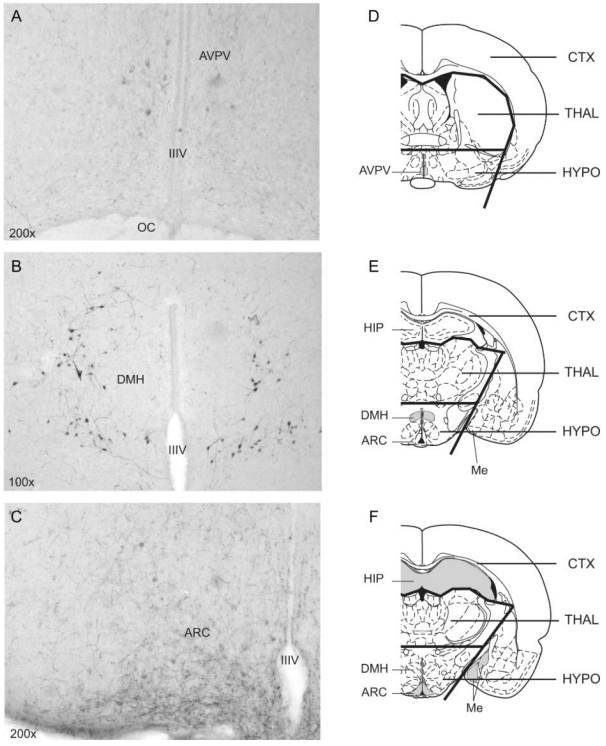

Figure 1.

Representative photomicrographs of T-4771-ir cells in the (A) anteroventral periventriculr nucleus (AVPV), (B) dorsomedial hypothalamus (DMH), and (C) arcuate nucleus (ARC) of male Siberian hamsters. (D-F) Illustrations of cortical (CTX), thalamic (THAL), and hypothalamic (HYPO) microdissections of coronal sections for qPCR analysis at the level of the (D) AVPV, (E) DMH, and (F) ARC. IIIV = third ventricle; OC = optic chiasm; HIP = hippocampus; Me = medial amygdala. Shaded areas represent putative areas containing *Kiss1* (in D and F) or *RFRP* mRNA (in E; see Discussion).

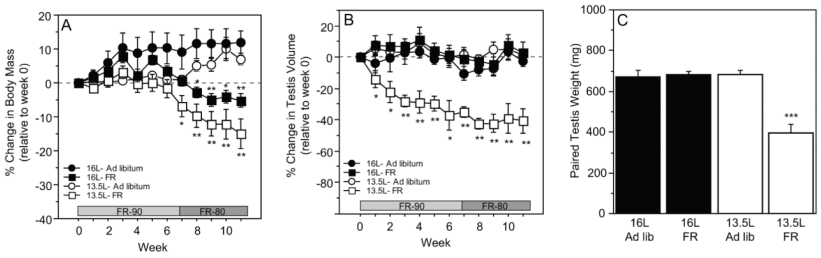

Figure 2.

Mean (±SEM) change in (A) body mass, (B) estimated testis volume, and (C) mean (+SEM) paired testis weights of hamsters singly housed in 16L or 13.5L and subsequently subjected to progressive food restriction (FR) or *ad libitum* feeding (AdLib) for 11 weeks, beginning on week 0. These data were derived from a subset of animals from a recent study in our laboratory (Paul *et al*., 2009). * P<0.05, ** P<0.005, ***P<0.0001 vs. AdLib-fed hamsters within photoperiod.

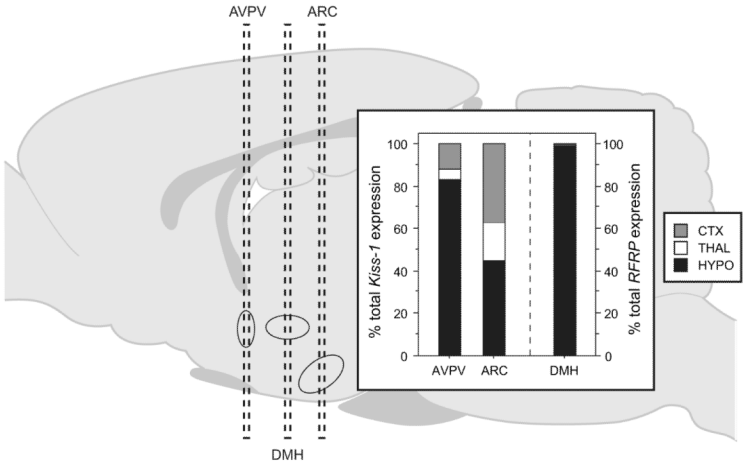

Figure 3.

Relative contributions of the hypothalamus (HYPO), thalamus (THAL), and cortex (CTX) to total brain *Kiss1* (left) and *RFRP* (right) mRNA expression in coronal sections at each of the three rostro-caudal levels analyzed (AVPV, ARC, and DMH).

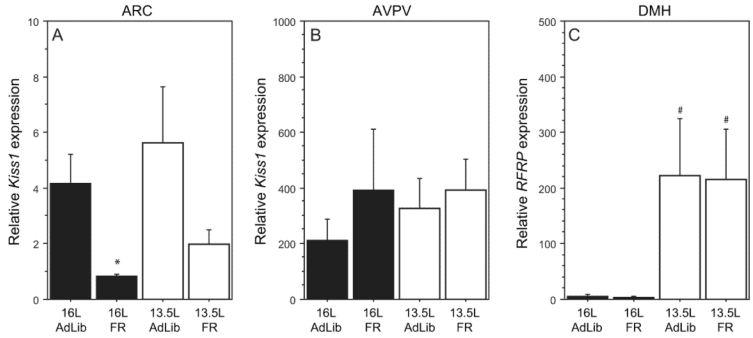

Figure 4.

Mean (±SEM) relative expression of *Kiss1* mRNA in coronal brain sections at the level of the (A) AVPV and (B) ARC of male Siberian hamsters; (C) mean (±SEM) relative expression of *RFRP* mRNA in sections at the level of the DMH. *Kiss1* and *RFRP* mRNA levels were normalized to *18S* rRNA gene expression. Photoperiod and feeding conditions as in Figure 2. The sample sizes for each group were 16L-AdLib: n=5; 16L-FR: n=6; 13.5L-AdLib: n=6; 13.5L-FR: n=6. * $P<0.05$ vs. AdLib hamsters within photoperiod; # $P<0.05$ 13.5L vs. 16L within feeding condition.