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Time-of-day-dependent Sensitivity of the Reproductive Axis to RFamide-related Peptide-3 Inhibition in Female Syrian Hamsters

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Abstract

In spontaneously ovulating rodent species, the timing of the luteinizing hormone (LH) surge is controlled by the master circadian pacemaker in the suprachiasmatic nucleus (SCN). The SCN initiates the LH surge through the coordinated control of two, opposing neuropeptidergic systems that lie upstream of the gonadotropin-releasing hormone (GnRH) neuronal system, the stimulatory peptide, kisspeptin, and the inhibitory peptide, RFamide-related peptide-3 (RFRP-3; the mammalian ortholog of avian gonadotropin-inhibitory hormone (GnIH)). We have previously shown that the GnRH system exhibits time-dependent sensitivity to kisspeptin stimulation, further contributing to the precise timing of the LH surge. To examine whether this time-dependent sensitivity of the GnRH system is unique to kisspeptin, or a more common mechanism of regulatory control, we explored daily changes in the response of the GnRH system to RFRP-3 inhibition. Female hamsters were ovariectomized to eliminate estradiol (E2) negative feedback and RFRP-3 or saline were centrally administered in the morning or late afternoon. LH concentrations and *Lhb* mRNA expression did not differ between morning RFRP-3 and saline-treated groups but were markedly suppressed by RFRP-3 administration in the afternoon. However, RFRP-3 inhibition of circulating LH at the time of the surge does not appear to act via the GnRH system as no differences in mPOA *Gnrh* or RFRP-3 receptor *Gpr147* mRNA expression were observed. Rather, RFRP-3 suppressed arcuate nucleus *Kiss1* mRNA expression and potentially impacted pituitary gonadotropes directly. Together, these findings reveal time-dependent responsiveness of the reproductive axis to RFRP-3 inhibition, potentially via variation in the sensitivity of arcuate nucleus kisspeptin neurons to this neuropeptide.

Keywords

Reproduction; Kiss1; HPG; circadian rhythms

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Introduction

Circadian timing is critical for female reproduction with disruptions to circadian timing leading to pronounced deficits in female reproductive health. For example, women with irregular sleep or work cycles have decreased fertility and increased rates of miscarriages (1–4). In spontaneously ovulating species, the timing of the luteinizing hormone (LH) surge required for ovulation is under strict circadian regulation by the suprachiasmatic nucleus (SCN) of the hypothalamus, the master mammalian brain clock (4–7). The dependence of ovulation on circadian timing coordinates a limited time window of fertility with sexual motivation and activity to maximize reproductive success, with the preovulatory LH surge occurring during early mornings in women and diurnal rodents (8–10) and in late afternoon in nocturnal rodents (11–13). To ensure appropriate oocyte maturation at the time of ovulation, the neuroendocrine circuit initiating ovulation has an additional reliance on estradiol (E2) signaling from developing follicles. As maturing follicles develop during the follicular phase of the ovulatory cycle, increasing concentrations of E2 are secreted to maintain LH at low concentrations through negative feedback. However, just prior to ovulation, peak E2 concentrations act through positive feedback to initiate the LH surge that triggers ovulation (3, 6, 7, 14–17). Previous findings by our group and others suggest that the temporary shift from negative to positive feedback is coordinated by the SCN (11, 17–20).

The SCN modulates reproductive axis function via direct and indirect communication to the hypothalamic-pituitary-gonadal (HPG) axis. At the time of the LH surge, monosynaptic vasoactive intestinal peptide (VIP) projections from the SCN directly stimulate GnRH neurons, with FOS expression increased in GnRH neurons receiving VIP input around the time of the LH surge (21, 22). GnRH neurons do not express estrogen receptor α (ER α), the receptor subtype that mediates E2 positive and negative feedback. To modulate the balance of negative and positive E2 feedback, the SCN coordinates the activity of two opposing, ER α -expressing neuropeptidergic systems that lie upstream of the GnRH system, the stimulatory neuropeptide, kisspeptin, and the inhibitory neuropeptide, RFRP-3 (RFRP-3; the mammalian ortholog of avian gonadotropin-inhibitory hormone (GnIH)) (4, 11, 18, 23–30). The SCN coordinates cellular activity of RFRP-3 neurons to suppress the reproductive axis outside the time window of the LH surge and allow for the transient suppression of E2 negative feedback around the time of the surge (11, 18, 23). RFRP-3 neurons are concentrated in the dorsomedial hypothalamus (DMH) and project broadly to hypothalamic loci that contain GnRH neurons and fibers (i.e., medial septum, diagonal band of Broca, preoptic area, anterior hypothalamus, and arcuate nucleus) in addition to the ventromedial nucleus of the hypothalamus and brainstem (24). RFRP-3 cell projections form direct contacts with GnRH neurons expressing the RFRP-3 receptor, GPR147 (24, 26, 31). Furthermore, RFRP-3 directly suppresses GnRH neuron activity and consequent LH release (18, 24, 29, 32–35). In some species, RFRP-3 neurons may also act on the anterior pituitary to mediate LH release as RFRP-3 neurons directly project to the median eminence and GPR147 is expressed in the pituitary (11, 34, 36–38). Finally, RFRP-3 may modulate the HPG axis via a subpopulation of arcuate nucleus (ARC) kisspeptin neurons that express GPR147 (39).

Concomitant with RFRP-3 suppression at the time of the LH surge, the SCN stimulates kisspeptin neurons located in anteroventral periventricular nucleus (AVPV) that, in turn, stimulate the GnRH system and the LH surge (22, 29, 40, 41). Whereas we have previously shown that kisspeptin neurons are indiscriminately sensitive to SCN signaling across the day in Syrian hamsters, GnRH neurons exhibit time-dependent sensitivity to kisspeptin stimulation, responding more robustly in the afternoon than in the morning (29). This additional mechanism of temporal control likely further ensures precision in the timing of the LH surge and ovulation. The present study examined whether this time-dependent sensitivity of the GnRH system is unique to kisspeptin or if daily changes in reproductive system sensitivity also occur in response to RFRP-3 inhibition to further precise timing of the LH surge.

Because reproductive axis inhibition is essential prior to ovulation, we hypothesized that the GnRH system is maximally responsive to RFRP-3 in the morning, prior to the LH surge. If true, then LH concentrations should be inhibited by RFRP-3 in the morning but not (or to a greater degree than) in the afternoon. However, it is also possible that the GnRH system is maximally responsive to RFRP-3 in the afternoon, because this is a time during which RFRP-3 neurons are typically transiently inactive (11, 18, 42). If it is the case that maximal responsiveness of the GnRH system occurs in the afternoon, then LH concentrations should be inhibited by RFRP-3 in the afternoon but not (or to a greater degree than) in the morning. RFRP-3 inhibition of LH occurs through changes in LH peptide secretion, which may or may not reflect changes in mRNA expression. Likewise, inhibition of LH is possibly accompanied by changes in GnRH peptide release which may or may not be reflected in changes in *Gnrh* mRNA expression. Finally, RFRP-3 may modify LH production and/or release via direct impact on pituitary gonadotropes, or indirectly via kisspeptin neurons in the ARC that control GnRH pulsatility (43–45). To select among these possibilities, we examined daily changes in HPG axis sensitivity to RFRP-3 inhibition in ovariectomized (OVX) female hamsters administered RFRP-3 or saline in the morning (prior to the LH surge) or late afternoon (around the time of the LH surge).

Material and Methods

Animals

Thirty-four >8-week-old female Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River (Wilmington, MA) and maintained on a 14:10 light:dark cycle (lights on at 06:00, lights off at 20:00) at $23 \pm 1^\circ\text{C}$ with food and water available *ad libitum*. A 14:10 light:dark cycle was employed to create a “long day” light regimen, as Syrian hamsters are seasonal breeders that breed under long day conditions. All procedures were approved by the Animal Care and Use Committee at the University of California, Berkeley and conformed to principles enumerated in the NIH guide for the use and care of laboratory animals.

Experimental Procedure

Surgical Procedures

After a 2 wk acclimation period, all hamsters were ovariectomized (OVX) to eliminate E2 negative feedback. Surgeries were conducted under isoflurane anesthesia with buprenorphine (s.c., 0.1 mg/kg) provided for analgesia. After a 2 wk recovery, a guide cannula (22GA, 6 mm; PlasticsOne, San Diego, CA, USA) was stereotaxically implanted under deep anesthesia (ketamine-xylazine cocktail (i.p., 60/5 mg/kg) directed at the lateral ventricle. For cannular implantation, the head was shaved, prepared for surgery, and animals were placed in a stereotaxic apparatus (Kopf, Tujunga, CA). Guide cannulae were placed at the following coordinates relative to bregma: 1.3 mm mediolateral, 1.1 mm posterior, and 3 mm ventral from the surface of the dura mater. Following surgery, a dummy cannula (6.5 mm; PlasticsOne, San Diego, CA, USA) was inserted into each guide cannula to prevent obstruction. Buprenorphine was administered before and after the surgeries for analgesia (s.c., 0.1 mg/kg). Following the procedure, hamsters were singly housed for the remainder of the study. Animals were given 1 wk to recover before assessing cannula placement via injections of angiotensin-II (5 ng angiotensin-II in 2 μ L sterile 0.9% saline) and examination of subsequent drinking behavior. Immediate drinking exhibited by hamsters confirmed the location of the cannula in the lateral ventricle.

Pharmacological manipulations and sample collection

Five μ L of saline or RFRP-3 (100 or 500 ng in saline) (Syrian hamster RFRP: ILSRVPSLPQRF-NH₂, purchased from Phoenix Pharmaceuticals, CA, USA) were injected (i.c.v.) in the morning (3 h after lights on, n=6/group) or in the afternoon (3h before lights off, n=6–7/group), at a rate of 0.5 μ L per 30s, while the animals were freely moving about their home cage. Blood samples were collected from the retro-orbital sinus 20 mins following injection and centrifuged at 1400 RCF for 15 mins. Serum was collected and stored at –20°C until assayed. 2 weeks later, animals were injected again with RFRP-3 (100 ng/5 μ L saline) or saline (5 μ L) in the morning or afternoon (n=6–9/group) and animals were sacrificed 2h later. Brains and pituitaries were flash frozen and brains were sectioned at 300 μ m and transferred to RNAlater (AMBION, AM7021, Grand Island, NY, USA) for one night at 4°C and –20°C thereafter until further processed. A 3 mm biopsy punch was used to microdissect the DMH and ARC in a single punch, and a 2 mm biopsy punch was used to microdissect the mPOA and AVPV bilaterally (Figure 1). RNA was extracted using ISOLATEII RNA mini kit (Bioline, BIO-52073, Memphis, TN, USA) and reversed transcribed for RT-PCR (iScript RT supermix, BIO-RAD, 170–8841, Hercules, CA, USA). A random, representative sample of RNA (for each tissue n=8) was assessed for RNA quality on an Agilent Technologies Bioanalyzer and yielded an average RNA integrity number (RIN) of 7.3 or higher. To confirm LH suppression and further validate the detection levels of the LH ELISA at low concentrations, 5 hamsters were injected with estradiol benzoate (EB, 100 μ g in 200 μ L sesame oil) and retro-orbital blood samples were collected 90 min later as described above.

qRT-PCR—Analysis of relative gene expression via qRT-PCR was performed using SSOAdvanced SYBR Green supermix (BIO-RAD, 1725272, Hercules, CA, USA). Samples

were run on a BIO-RAD CFX384 machine with 10 μ l reaction volumes with a 2-step amplification for 40 cycles followed by a melt curve. Primers were designed from published sequences for Syrian hamsters using NCBI Primer BLAST software (Table 1). Primer sets were validated for specificity using positive, negative, no reverse transcriptase, and no template controls, and confirmed with a single-peak melt curve and correct product length. Efficiency of each primer set was determined by standard curve; primers were 94.7–105.4% efficient with R^2 values above 0.99. All samples were run in triplicate. Replicate sets in which Cq values varied beyond 0.5 cycles were excluded from analysis and resulting data were analyzed in Microsoft Excel following the delta delta Cq method (46). The geometric mean of 2 housekeeping genes' expression was used for reference. Because the expression of housekeeping genes was found to vary with time of day or treatment between brain regions, samples from different brain regions were analyzed with different reference genes. *Gapdh* and *Actb* were used as reference genes for the pituitary (Cq ranges were 20.3–24.26 and 20.6–24.64, respectively), whereas *Hmbs* and *Tbcc* were used as reference genes for the DMH and ARC (Cq ranges were 23.9–26.8 and 21.35–24.24, respectively), and *B2m* and *Rplp16* were used as their reference genes for AVPV and POA samples (Cq ranges were 20.78–23.88 and 18.77–21.53, respectively). Housekeeping genes were not significantly different between all groups, and in all gene replicate groups Cq Standard deviation was smaller than 0.2. Whereas *Kiss1* mRNA expression was measured in the DMH and ARC, it was not assessed in the AVPV and POA due to late and unstable amplification, indicating low mRNA expression, possibly as a result of the OVX. All data are expressed as a fold-change over morning, saline hamsters. Some samples did not have sufficient cDNA to quantify the expression of all genes, thus sample sizes vary for different genes measured.

Assessment of LH levels—LH concentrations were quantified with an ELISA, using a modified protocol that was kindly provided by Jens D. Mikkelsen (Copenhagen University Hospital, Denmark) (47), and all samples were run in duplicate. Briefly, 96-well microtiter plates were coated with 50 μ l of bovine LH β 518B7 monoclonal antibody (kindly provided by Lillian E Sibley, UC Davis, CA, USA) and incubated overnight at 4°C. Excess antibody was removed, and plates were washed 3 times with 200 μ l of 10mM PBS with 0.05% Tween 20 (PBS-T). Plates were blocked for 1h at room temperature using 5% skim milk powder in PBS-T. Following washes, 50 μ l of each sample and standards (mouse RIA kit, AF Parlow, National Hormone and Pituitary Program, University of California, Harbor Medical Center, Los Angeles, CA, USA) diluted in assay buffer was added to each well and incubated for 2 h at room temperature. Plates were then washed and 50 μ l of rabbit polyclonal LH antibody (AFP240580Rb, AF Parlow, National Hormone and Pituitary Program, University of California, Harbor Medical Center, Los Angeles, CA, USA) were added into each well and incubated for 90 mins at room temperature. After washing, a 1:2000 dilution of polyclonal goat anti-rabbit IgG conjugated to horseradish peroxidase (DAKO Cytomation, catalog # P0448, Santa Clara, CA, USA) was added to each well and incubated for 1 h at room temperature. After washing, o-Phenylenediamine (OPD, Invitrogen, catalog # 00–2003, Camarillo, CA, USA) in citrate buffer was added to each well and the reaction was allowed to proceed for 30 mins at room temperature in darkness before being stopped by the addition of 3M HCl to each well. Light absorbance was immediately read at 490 nm with a reference of 655 nm. Representative random serum samples were assessed by the Center for Research

in Reproduction at the University of Virginia (UVA), and Pearson's $r=0.97$ correlation was found between the LH values obtained at UVA and the values generated by the 'in-house' LH ELISA. The assay was also validated by assessing parallelism with the standard curve as well as blood samples collected 90 min following E2 benzoate (EB) administration used to suppress LH concentrations. Assay sensitivity was 0.002 ng/ml and intra- and inter-assay variability were 1.1% and 3.4%, respectively.

Statistical analysis—Group comparisons were examined using a two-way analysis of variance (ANOVA). In instances which assumptions of normality and/or equal group variance were violated, data was analyzed by planned contrasts for comparisons between specific groups was made on the basis on *a priori* hypotheses and corrected for multiple comparison with Bonferroni's inequality test. Statistical analyses were performed in SPSS (Armonk, New York, USA) and Prism (San Diego, CA, USA). All data are reported as mean \pm standard error of the mean (SEM) with $p < 0.05$ considered statistically significant. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Circulating LH

LH concentrations were measured to examine whether the GnRH system exhibits daily changes in sensitivity to RFRP-3 inhibition. Consistent with the timing of the LH surge to the afternoon, baseline LH concentrations (saline groups) were significantly different across the day, increasing from 18.65 ng/ml \pm 3.8 in the morning to 29.6 ng/ml \pm 4.3 in the afternoon ($p < 0.044$, 95% confidence interval: -1.998 to 23.87) (Figure 2, $n=6-7$ /group). 100 ng RFRP-3 significantly decreased circulating LH concentrations in the afternoon 20 mins after administration (from 29.6 ng/ml \pm 4.3 to 14.7 ng/ml \pm 2.8 ($p < 0.006$, 95% confidence interval: -25.89 to -3.883). At this same dose, no differences were found between saline and RFRP-3 administration in the morning ($p > 0.05$). No effects were observed with the 500 ng dose of RFRP-3 ($p > 0.05$ in all cases; data not shown). Finally, EB markedly suppressed LH concentrations 90 mins post administration (decreasing to 5.47 ng/ml \pm 0.9, $t=2.808$, $p < 0.01$, data not shown), further validating the LH assay.

Pituitary gene expression

Pituitary gene expression was measured to examine whether changes in LH concentrations are accompanied by changes at the mRNA level and whether pituitary cells exhibit the potential for direct inhibition by RFRP-3 (i.e., changes at the level of the pituitary independent of changes in the GnRH or kisspeptin systems) (Figure 3, $n=6-9$ /group). Within each treatment (saline or RFRP-3), no differences in *Lh β* mRNA expression were found across time of day. However, RFRP-3 significantly decreased pituitary *Lh β* subunit mRNA expression in the afternoon ($p < 0.04$, 95% confidence interval: -1.092 to 0.07406) but not in the morning ($p > 0.05$), compared to saline, consistent with the impact of this peptide on circulating LH. In contrast, pituitary *Gnrh-r* mRNA expression did not differ at any time point regardless of treatment. However, a significant time X treatment interaction was found for pituitary *Gpr147* mRNA expression ($F(1,24)=2.427$, $p < 0.019$), with RFRP-3

significantly decreasing pituitary *Gpr147* mRNA expression in the afternoon ($p < 0.016$, 95% confidence interval: -1.702 to -0.08974) but not in the morning ($p > 0.05$).

mPOA gene expression

To examine whether changes in LH concentrations are mediated via the classic GnRH-LH pathway, mPOA *Gnrh* and *Gpr147* mRNA expression were assessed (Figure 4, $n = 5-9$ /group). Baseline mPOA *Gnrh* expression (i.e., saline groups) was significantly reduced in the afternoon relative to morning ($p < 0.01$, 95% confidence interval: -0.5464 to -0.05362). Additionally, RFRP-3 significantly decreased mPOA *Gnrh* mRNA expression in the morning ($p < 0.024$, 95% confidence interval: -0.5581 to -0.002333) but not in the afternoon ($p > 0.05$). mPOA *Gpr147* mRNA expression did not differ at any time point for either treatment ($p > 0.05$ in all cases).

ARC gene expression

RFRP-3 may modify LH production and/or release indirectly via kisspeptin neurons in the ARC that control GnRH pulsatility (48). Thus, we examined the expression of *Kiss1* and *Gpr147* mRNA in the ARC under RFRP-3 and saline treatments (Figure 5, $n = 7-9$ /group). Within each treatment (saline or RFRP-3), no difference in mRNA expression was found across time of day. However, RFRP-3 significantly decreased ARC *Kiss1* mRNA expression in the afternoon ($p < 0.022$, 95% confidence interval: -1.229 to -0.01701) but not in the morning ($p > 0.05$), compared to saline controls. In the ARC, *Gpr147* mRNA baseline expression (saline groups) exhibited a non-significant trend in which afternoon levels were reduced compared to morning ($p < 0.054$, 95% confidence interval: -0.2739 to 2.418). No effect of RFRP-3 was observed for ARC *Gpr147* mRNA expression at either time point ($p > 0.05$ in each case).

Discussion

The present findings indicate that the reproductive axis responds to RFRP-3 in a time-dependent manner, with central RFRP-3 administration in the afternoon, but not the morning, reducing circulating LH and downregulating pituitary *Lhb* subunit mRNA expression. These findings support the notion that the reproductive axis is most sensitive to RFRP-3 inhibition around the time of the LH surge (i.e., late afternoon). As previous studies have established that administration of RFRP-3 around the time of ovulation suppresses the GnRH/LH surge (49) and sexual motivation (50), this finding further underscores the importance of RFRP-3 cellular inhibition at this time as we and others have previously shown (11, 18, 23). Additionally, consistent with previous findings in this species (29), the present findings further establish daily changes in the reproductive axis that are coordinated with the timing of the LH surge, even in the absence of estrogen. Together, these outcomes underscore the importance of circadian-controlled RFRP-3 system inhibition to permit the LH surge and coordinate maximal fertility with sexual motivation.

To explore where daily changes in sensitivity to RFRP-3 are mediated, we examined the expression of *Gpr147*, the cognate receptor for RFRP-3. In the brain, *Gpr147* is expressed in GnRH cells (26, 32, 33, 51), in the pituitary (11, 34, 36, 38), and in kisspeptin neurons (26,

39), providing three potential loci at which such changes may occur. Specifically for kisspeptin neurons, 12–15% of AVPV kisspeptin cells express *Gpr147* and 25% of KNDy neurons express *Gpr147* in both male and female mice (26, 39). Likewise, ~35% of ARC kisspeptin neurons receive RFRP-3 immunoreactive fiber contacts (39). In the present study, RFRP-3 had no effect on the expression of *Gpr147* in the mPOA and AVPV, suggesting that enhanced RFRP-3 signaling via *Gpr147* in these regions is not responsible for increased responsiveness to RFRP-3 inhibition in the afternoon. Likewise, hypothalamic *Gnrh* mRNA levels were not reduced in the afternoon by infusion of RFRP-3. Furthermore, RFRP-3 did not influence *Gnrh-r* mRNA expression in the pituitary. These findings suggest that changes in the sensitivity of hypothalamic GnRH neurons, or reduced pituitary sensitivity to GnRH, do not underlie the enhanced suppression of LH by RFRP-3 in the afternoon. Whether or not the enhanced suppression of LH in the afternoon by RFRP-3 is a result of inhibition of GnRH peptide release, post transcriptional/translational events regarding GPR147 (e.g., more GRP147 receptors are available/translated in the afternoon), or the specific time intervals between RFRP-3 administration and sampling represents an important area for future inquiry.

Although the present findings do not support a role for altered GnRH cell sensitivity to RFRP-3 signaling or changes in pituitary sensitivity to GnRH across the day, the findings suggest that daily changes in the suppressive actions of RFRP-3 might occur at the level of ARC kisspeptin cells. Specifically, we observed a substantial reduction in *Kiss1* mRNA expression in the ARC following afternoon, but not morning, RFRP-3 administration. These findings point to the possibility that ARC kisspeptin cells may act on GnRH terminals to modulate their output across the day in response to upstream mediators. GnRH neurons possess unique axonal projections to the median eminence that also exhibit dendritic functions (43, 52, 53). These so-called ‘dendrons’ allow for synaptic input and the integration of information to control the release of GnRH. In several species, ARC kisspeptin neurons exhibit axo-axonal contacts with GnRH neurons (54, 55) as well as projections to the internal and external layer of the median eminence (43). Our results show that the expression of ARC *Kiss1* mRNA co-varies with circulating LH levels, with RFRP-3 acting to reduce both *Kiss1* mRNA expression and circulating concentrations of LH in the afternoon but not in the morning, consistent with this pathway of control. In support of this possibility, ablation of ARC KNDy neurons leads to atypical LH surge amplitude (56, 57). The present findings are also in agreement with a recent study demonstrating RFRP-3 suppression of ARC kisspeptin expression in free cycling Syrian hamsters maintained in long photoperiods (23). This same study found that hamsters injected with RFRP-3 in the afternoon, but not in the morning, exhibit suppression of LH concentrations when in proestrus. Furthermore, ARC kisspeptin neurons receive monosynaptic input from RFRP-3 neurons and express the RFRP-3 receptors (39). Together, the present and previous findings support the notion that RFRP-3 cells are in a position to modify LH secretion through actions on ARC kisspeptin cells and these cells differ in their response to RFRP-3 across the day.

In addition to actions on the ARC kisspeptin cell population, daily changes in RFRP-3 sensitivity may also be mediated at the level of the pituitary, as pituitary *Gpr147* and *Lhb* mRNA expression are reduced following RFRP-3 treatment in the afternoon but not in the

morning. Future studies in which RFRP-3 are administered peripherally in the morning and afternoon are necessary to examine this possibility as it is unclear whether or not injections of RFRP-3 in the present study enter the hypophyseal portal system. Across species (e.g., sheep, mice, hamsters, macaques, and humans), RFRP-3 projections to the median eminence and RFRP-3 receptor expression in the pituitary have been reported (11, 34, 36, 58, 59). In contrast, neither RFRP-3 projections to the median eminence nor its receptor are found in some species (60–63). In cultured pituitaries across species, RFRP-3 administration inhibits gonadotropin production and release (64–66), suggesting the potential for inhibition *in vivo*. Although our study did not assess this pathway directly, the expression of pituitary *Gpr147* mRNA exhibited a similar pattern to that of LH and *Lhb*, with RFRP-3 inhibition of *Gpr147* mRNA expression in the afternoon but not in the morning. These findings suggest potential actions of RFRP-3 that ultimately affect pituitary level responsiveness to this neuropeptide.

In the current study, hamsters were ovariectomized to eliminate E2 negative feedback. In the absence of E2, the pattern of LH in the saline (control) groups resembled the expected pattern, with LH concentrations being higher in the afternoon than in the morning (11). However, this daily change is not reflected in *Gnrh* mRNA expression. Also contrary to expectation, RFRP-3 suppressed *Gnrh* expression in the mPOA in the morning but not in the afternoon, contrasting with patterns of circulating LH that are not inhibited by morning RFRP-3 treatment. These unexpected relationships between the pattern of *Gnrh* mRNA and daily change in LH is possibly due to the disparity between the time at which blood and brain samples were collected (i.e., blood samples were collected 20 min post treatment, whereas brains were collected 2 h post treatment). It is also possible, that post transcription/translation modifications lead to differential GnRH peptide release (67). Finally, we cannot exclude the possibility that the removal of ovarian hormones alters the typical hypothalamic response to RFRP-3 communication. Future studies examining the time course of gene transcription/translation and the association with peptide release will help to select among these possibilities.

The present findings suggest that the mechanisms driving LH secretion differ depending on the time of day and neurochemical environment. Specifically, in the absence of RFRP-3 administration (i.e., saline conditions), or during times that RFRP-3 is administered when it is typically released (i.e., the morning), the mechanism driving LH secretion converge at the level of GnRH neurons. However, when RFRP-3 is administered in the afternoon, a time during which it is not typically released, it appears to bypass direct communication with the GnRH system, instead acting through ARC kisspeptin cells and/or directly on the pituitary. This latter circumstance might result in the case of circadian disrupted individuals, including women who are jetlagged, have irregular shift work hours, or are exposed to light at nighttime (e.g., from electronic devices), conditions associated with marked deficits in ovulatory cycling (1, 2).

In conclusion, the present findings indicate that time-dependent sensitivity to regulators of the HPG axis is not unique to kisspeptin stimulation of the GnRH system, at least in Syrian hamsters. The reproductive axis is maximally responsive to RFRP-3 administration in the afternoon, with no effect in the morning, even in the absence of estrogen. During the afternoon, RFRP-3 appears to inhibit LH secretion through actions on ARC kisspeptin cells

and the pituitary rather than the GnRH system. These findings further highlight the importance of timed suppression of the RFRP-3 system at the appropriate time of day to allow for the LH surge and ovulation. These findings raise the possibility that, in cases of circadian disruption (e.g., irregular sleep patterns, nighttime exposure to light-emitting devices, shift work), mistimed RFRP-3 release may be responsible for compromised fertility seen across species, including humans.

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Circulating LH

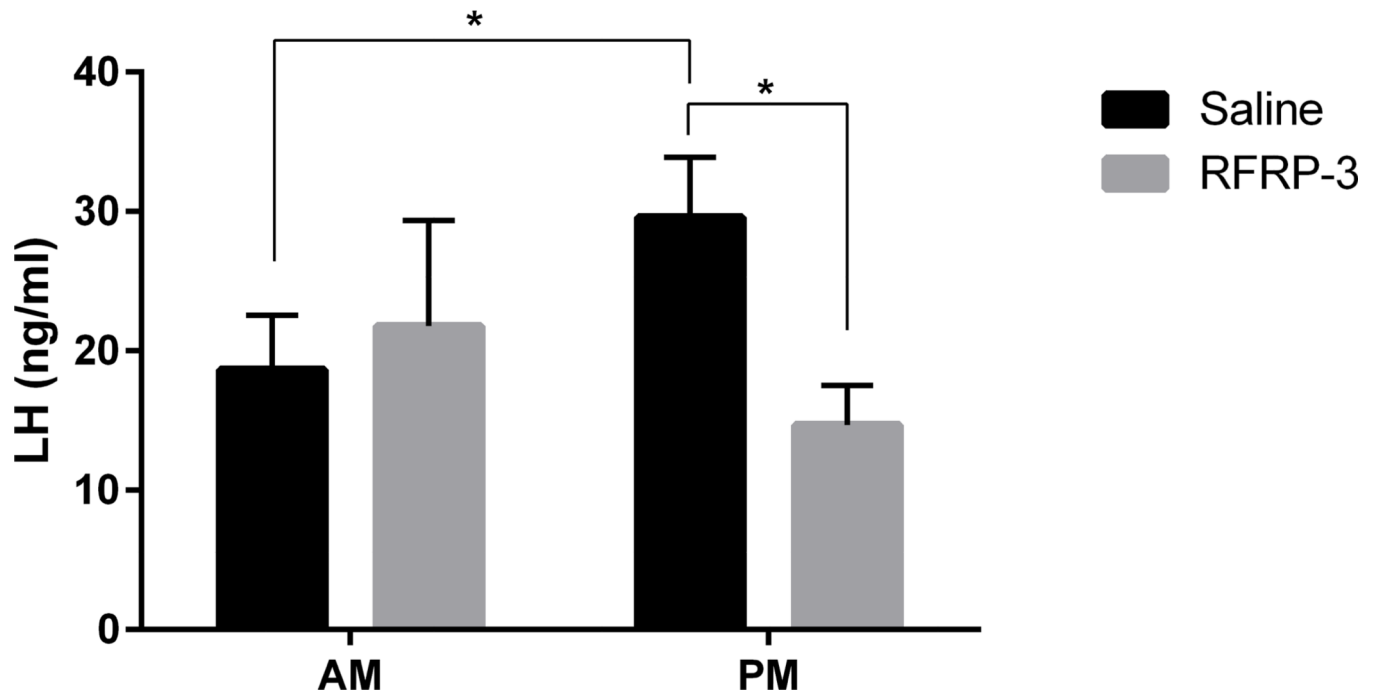


Figure 2. Central administration of RFRP-3 (100 ng) inhibits circulating LH in the afternoon 20 min post administration. $n = 6, 6, 6, 7$, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively. Data are presented as mean \pm standard error of the mean. * $p < 0.05$.

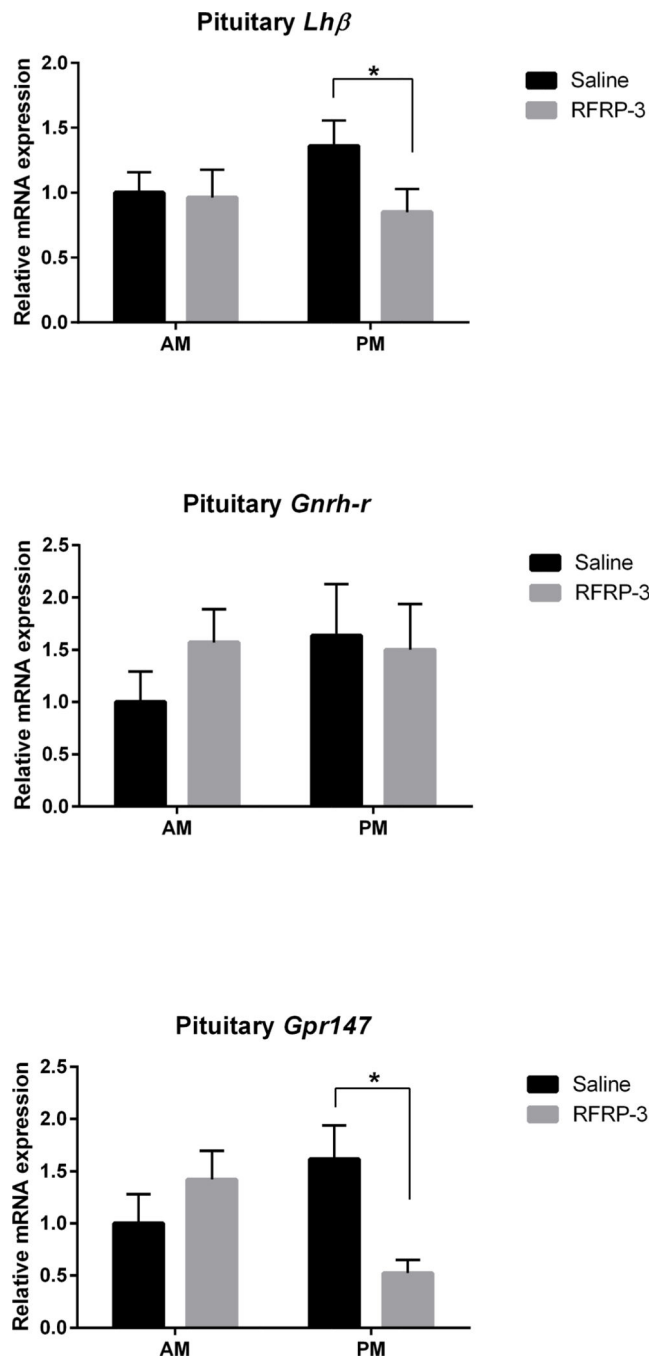


Figure 3. Central administration of RFRP-3 suppresses pituitary *Lhb* subunit mRNA expression in the afternoon but not the morning (top; n = 6, 6, 6, 7, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). Pituitary *Gnrh-r* mRNA expression is not affected by either RFRP-3 or time of day (middle; n = 6, 8, 7, 7, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). RFRP-3 suppresses pituitary *Gpr147* mRNA expression in the afternoon but not the morning (bottom; n = 6, 8, 9, 5, for AM saline, AM RFRP-3, PM

saline, PM RFRP-3, respectively). Data are presented as mean \pm standard error of the mean.
* $p < 0.05$.

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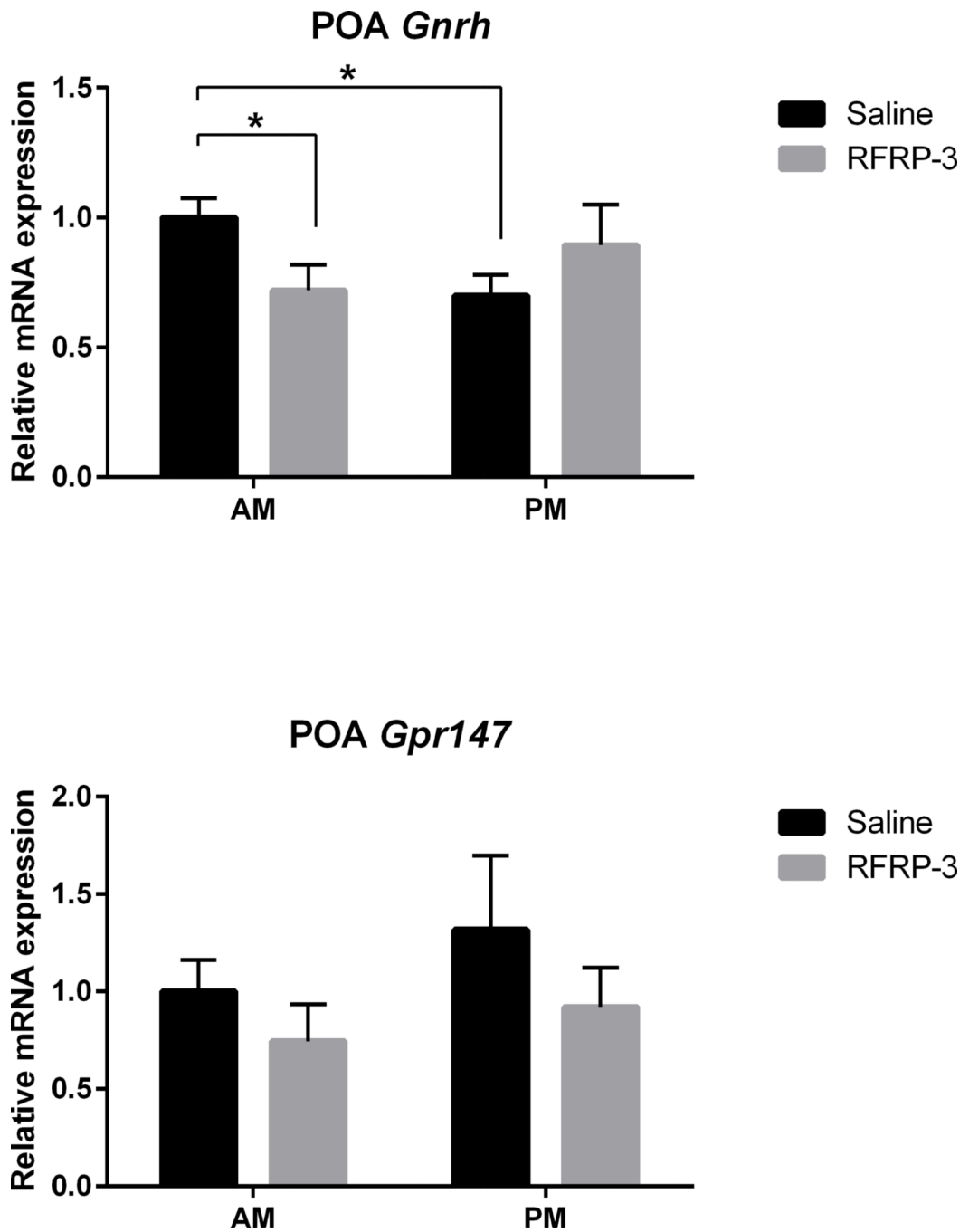


Figure 4. mPOA *Gnrh* mRNA expression is suppressed by central administration of RFRP-3 in the morning but not in the afternoon (top; n = 6, 6, 7, 9, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). RFRP-3 does not alter mPOA *Gpr147* mRNA expression (bottom; n = 7, 5, 7, 8, for AM saline, AM RFRP-3, PM saline, PM RFRP-r, respectively). Data are presented as mean ± standard error of the mean. * $p < 0.05$.

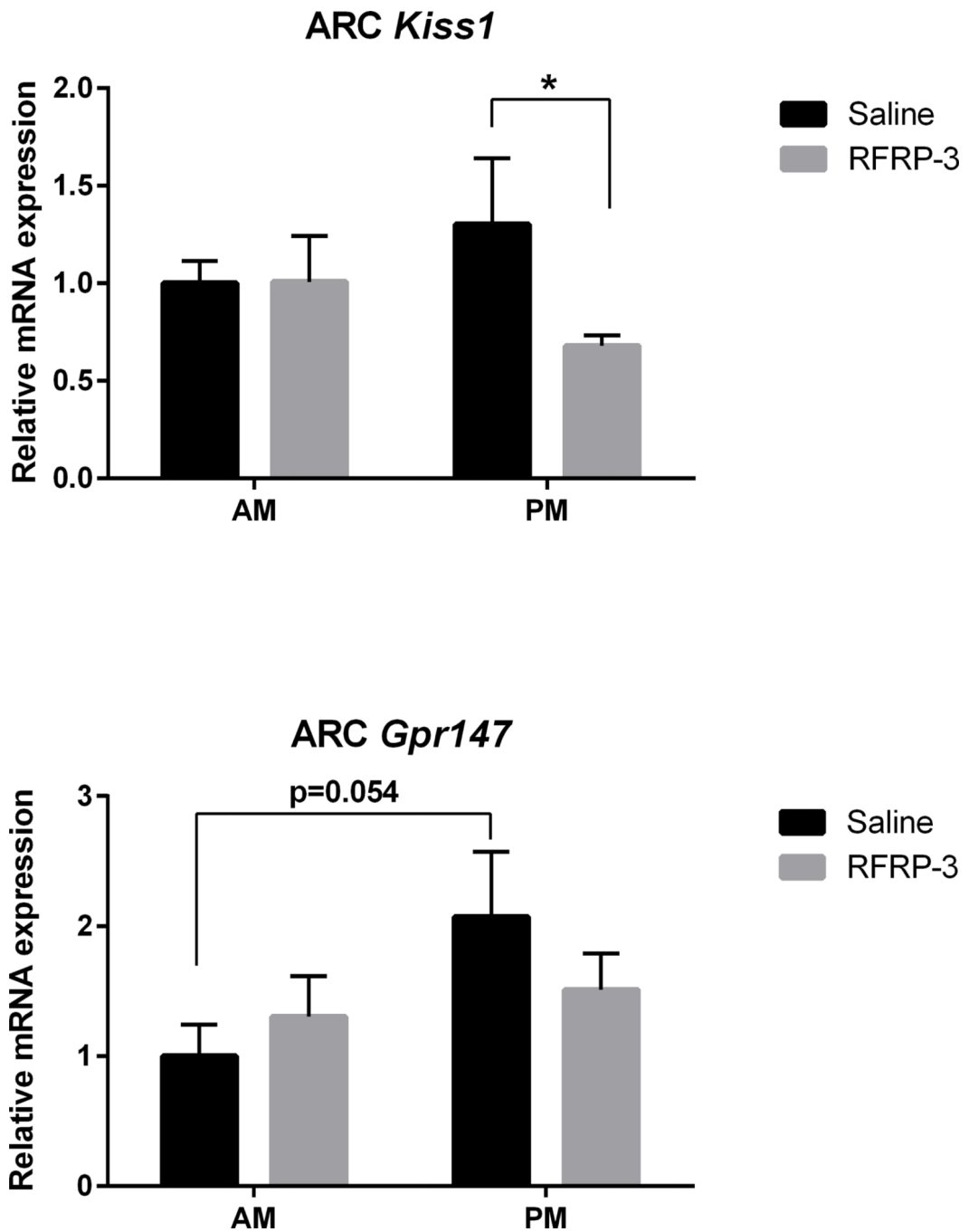


Figure 5. Central administration of RFRP-3 suppresses ARC *Kiss1* mRNA expression in the afternoon but not in the morning (top; n = 7, 8, 7, 9, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). No effect of RFRP-3 on ARC *Gpr147* mRNA expression was observed (bottom; n = 6, 8, 7, 9, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). Data are presented as mean \pm standard error of the mean. * $p < 0.05$.

Table 1.

Primers used for qRT-PCR

Primer	Forward	Reverse	Product Size
<i>Lhβ</i>	CGGCTACTGTCCTAGCATGG	AGGCGGACAGATGTGAAGTG	102
<i>Gnrh-r</i>	TCATCTTCACCTCACACG	GTGGCAAATGCGACTGTCAT	121
<i>Gnrh</i>	AGGGACCTTCGAGGAGTTCT	TGTGGATCCTTTGGTGCTGAT	88
<i>Kiss1</i>	TGGTTATCTTTGACCTCCGGC	TGCCAAGAAGCCAATGTGGT	105
<i>Gpr147</i>	CCGGTTGGCCTTTTGACAAT	CAGCTTCTCACGAAAGGGT	140
<i>Gapdh</i>	ACAGTCAAGGCTGAGAACGG	TCCACAACATACTCGGCACC	116
<i>Actb</i>	GACCCAGATCATGTTGAGACCT	TCCGGAGTCCATCACAATGC	112
<i>B2m</i>	TGGCCGTGGTCTTTCTGATG	TGGAAGTGCACACATAGCA	139
<i>Rplp16</i>	ATCTACTCCGCCCTCATCCT	GCAGATGAGGCTTCCAATGT	159
<i>Hmbs</i>	TATCCTGGATGTTGCACGGC	TCTCAACACCCAGTGGTTCA	165
<i>Tbcc</i>	CAGTGGGACTGAGCACTAGC	TAGCAAAAGCCCCGGGTTAG	156

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