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Intraovarian expression of GnRH-1 and gonadotropin mRNA and protein levels in Siberian hamsters during the estrus cycle and photoperiod induced regression/recrudescence

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Abstract

The hypothalamic-pituitary-gonadal (HPG) axis is the key reproductive regulator in vertebrates. While gonadotropin releasing hormone (GnRH), follicle stimulating (FSH), and luteinizing (LH) hormones are primarily produced in the hypothalamus and pituitary, they can be synthesized in the gonads, suggesting an intraovarian GnRH-gonadotropin axis. Because these hormones are critical for follicle maturation and steroidogenesis, we hypothesized that this intraovarian axis may be important in photoperiod-induced ovarian regression/recrudescence in seasonal breeders. Thus, we investigated GnRH-1 and gonadotropin mRNA and protein expression in Siberian hamster ovaries during (1) the estrous cycle; where ovaries from cycling long day hamsters (LD;16L:8D) were collected at proestrus, estrus, diestrus I, and diestrus II and (2) during photoperiod induced regression/ recrudescence; where ovaries were collected from hamsters exposed to 14wks of LD, short days (SD;8L:16D), or 8wks post-transfer to LD after 14wks SD (PT). GnRH-1, LH β , FSH β , and common α subunit mRNA expression was observed in cycling ovaries. GnRH-1 expression peaked at diestrus I compared to other stages ($p < 0.05$). FSH β and LH β mRNA levels peaked at proestrus and diestrus I ($p < 0.05$), with no change in the α subunit across the cycle ($p > 0.05$). SD exposure decreased ovarian mass and plasma estradiol concentrations ($p < 0.05$) and increased GnRH-1, LH β , FSH β , and α subunit mRNA expression as compared to LD and, except for LH, compared to PT ($p < 0.05$). GnRH and gonadotropin protein was also dynamically expressed across the estrous cycle and photoperiod exposure. The presence of cycling intraovarian GnRH-1 and gonadotropin mRNA suggests that these hormones may be locally involved in ovarian maintenance during SD regression and/or could potentially serve to prime ovaries for rapid recrudescence.

Keywords

Ovary; Seasonal breeding; Photoperiod; GnRH-1; FSH; LH

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1. Introduction

The hypothalamic-pituitary-gonadal (HPG) axis is the key regulator of reproduction in all vertebrates. Gonadotropin releasing hormone (GnRH) is released from the hypothalamus and promotes the synthesis and release of the gonadotropins, follicle stimulating hormone (FSH), and luteinizing hormone (LH), from the anterior pituitary gland. In females, FSH and LH bind to their gonadal receptors to initiate folliculogenesis, steroidogenesis, and ovulation.

GnRH is a decapeptide and has many isoforms across species in both vertebrates and invertebrates [reviews 14,24] including GnRH-I and GnRH-II [20]. In addition to its hypothalamic synthesis, GnRH-1 mRNA, protein and its receptors are also expressed in many non-hypothalamic tissues [for review, see 26] including the pituitary gland [28], bovine uterus and oviducts [36], and the gonads of humans [7,15,20,23], non-human primates [6], rodents [1,29,32,40], and non-mammalian vertebrates [37].

Follicle stimulating hormone and luteinizing hormone are synthesized and released into the blood by the anterior pituitary gland in response to pulsatile secretion of GnRH from the hypothalamus. Both FSH and LH are heterodimers, each containing a unique β subunit and share a common α subunit. Although the primary site of gonadotropin synthesis and release is the anterior pituitary gland, gonadotropin mRNA and protein have been observed in human [2,13] and rat testes [8,39,45,46], seabream gilthead ovaries [41], southern catfish ovaries [42], human breast tissue [10], and monkey epididymis [44]. Expression of FSH α and β subunit mRNA was also reported in mouse testes [17] and ovaries [18], with FSH α and β subunit proteins localized to interstitial cells and corpora lutea [18]. More recently, in rat ovaries the common α and the unique β subunit mRNA of both LH and FSH were detected in intact follicles, theca cells, corpus luteum, and oocytes, however; only LH β and the common α subunit mRNA were present in granulosa cells [29]. Finally, intraovarian LH content does not differ across intact and hypophysectomized rats, presenting strong evidence for extra-pituitary production of gonadotropins [29].

Intraovarian expression of GnRH, FSH, and LH mRNA and protein indicates potential presence of an “HPG-like” or GnRH-gonadotropin axis in the ovary. Although this possibility is very intriguing, specific function(s) of such an axis in the ovary are not yet fully clear. A wide range of functions for the intraovarian GnRH/GnRH-R system have been proposed including: autocrine/paracrine regulation, a role in gonadal steroidogenesis, antiproliferation, follicular atresia, and mediation of apoptosis [see reviews; 12,15,20,26]. Functions of intraovarian FSH and LH are yet to be defined, but a paracrine role in ovarian function [18], steroidogenesis, and ovum maturation [29] has been proposed. Regulation of the ovarian GnRH - gonadotropin axis is also not fully understood. Down regulation of GnRH mRNA levels in the ovary by estrogen, gonadotropins, and melatonin has been suggested (see reviews; 15,20,26), and, recently it has been shown that ovarian GnRH and pituitary or ovarian FSH, may regulate LH levels in rat ovaries [16].

In addition to the regulation of the cyclical ovarian cycle, the HPG axis also mediates seasonal changes in reproductive function [11]. In Siberian hamsters (*Phodopus sungorus*), for example, short (<12h of light per day) photoperiods shut down the HPG axis, which results in gonadal regression, effectively inhibiting reproduction. Exposure to stimulatory long days (>12 hours of light per day) restores HPG axis function and gonads resume reproductive activity. Previously we have reported that female Siberian hamsters exposed to 12-14 wks of short days (SD; 16D:8L) exhibit reduced ovarian and uterine mass and function; and their subsequent transfer to long days (LD; 16L:8D) for 4-8 wks restores

reproductive organ mass, steroidogenesis, and folliculogenesis to LD control levels [21,27,33,34].

Photoperiod-induced regression in Siberian hamsters is regulated by the HPG axis, with loss of estrus cyclicity following declines of pituitary and serum FSH [30]. Likewise, a decrease in serum gonadotropins occurs within one week of SD exposure in male Siberian hamsters [43], whereas transfer of SD exposed hamsters to LD restores testes mass and serum FSH concentrations [35]. Although few female Siberian hamster studies are available, photoperiod exposure alters hypothalamic GnRH mRNA in male Siberian [3,25] and Syrian [4,38] hamsters, and SD exposure decreases pituitary gonadotropin (FSH β and LH β) mRNA in male Siberian hamsters [19].

Based on studies showing the presence of GnRH and gonadotropins in the ovary, and the importance of these hormones to seasonal breeding, we hypothesized that intraovarian expression of GnRH and gonadotropins, if present in Siberian hamster ovaries, could be important in photoperiod-induced alterations in ovarian function. Our objectives for the present study were to investigate (1) whether GnRH-1 and gonadotropin mRNAs and protein are expressed in Siberian hamster ovaries; and (2) if their expression is modulated during the estrous cycle and photoperiod induced regression and recrudescence.

2. Methods and Materials

2.1 Animals

Female Siberian hamsters (*Phodopus sungorus*, > 60 days of age) were obtained from the breeding colony of Dr. Katherine Wynne-Edwards, Queen's University (Kingston, Ontario, Canada) and were housed individually in polypropylene cages (28 × 7.5 × 13 cm) at 20 ± 2°C, receiving food and water *ad libitum*. All experiments were conducted in our AAALAC-approved facilities, in accordance with California State University, Long Beach and NRC guidelines for use of laboratory animals and with specific project approval from our Animal Welfare Committee. After three weeks of acclimatization hamsters were subjected to the following treatments:

2.2 Estrous cycle experiment

To help stimulate the estrous cycle, four male hamsters were housed in the same room as the females. Synchronization of estrous cycles was aided by placing soiled male bedding into individual female cages prior to tissue collection [9,21]. Vaginal cytology was used to preliminarily stage ovaries (n=6/group) at proestrus (P), estrus (E), diestrus I (DI), and diestrus II (DII) as described previously [21]. Anesthetized (ketamine [200 mg/kg] plus xylazine [20 mg/kg]) hamsters (n=4-5/group) were subsequently euthanized via cervical dislocation, and ovaries cleaned of adipose tissue and removed. One ovary from each animal was snap frozen in liquid N₂. In addition, hypothalamic tissue including the arcuate nucleus was also removed and frozen in liquid N₂.

2.3 Regression/recrudescence experiment

Hamsters were divided into following groups; LD group (n=5) exposed to long days (16L:8D) for 14 weeks; SD group exposed to short days (8L:16D; n=10) for 14 weeks and PT group (n=10) exposed to SD for 14 weeks and then transferred to LD for eight weeks. Hamsters were euthanized in the proestrus phase following administration of ketamine (200 mg/kg) plus xylazine (20 mg/kg) and ovaries removed, with one ovary frozen in liquid N₂ for RNA extraction and real time PCR. Blood was collected retro-orbitally, centrifuged at 5000g for 5 min at 4°C and stored at -80°C until used to assay for sex steroids.

2.4 Total RNA extraction and cDNA synthesis

Total ovarian RNA was extracted using Trizol LS reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer directions. One μg of total RNA (260/280 ratio >1.6) was used for cDNA synthesis using ImProm Reverse Transcription System (Promega, Madison, WI) according to the manufacturers' directions. As a control, hypothalamic (primarily arcuate nucleus) total RNA was also extracted as described above.

2.5 Relative real time PCR

All primers were designed using hamster sequences and Primer 3 software or OligoPerfect Designer software (Invitrogen) matching the design criteria according to the MX3000 cyclor manual (Stratagene Corp., Cedar Creek, TX; Table 1). Because Siberian hamster sequences were not available for housekeeping genes GAPDH and HPRT1, we first used semi-quantitative RT PCR, then purified and sequenced the resulting bands (Macrogen Corp. Rockville, MD). These hamster sequences were then used to design primers (Table 1) for GAPDH and HPRT1 real time PCR. The relative real time PCR was done on Mx3000 thermocycler using Absolute QPCR SYBR green mix (ABgene, Surrey, UK). The PCR reaction mix contained 1 μl cDNA (1:5 dilution of cDNA transcribed using 1 μg total RNA) + 1 μl each of forward and reverse primers (80nM concentration) + 6 μl SYBRgreen mix + 3 μl water (Promega DNase, RNase, Protease free) to a total volume of 12 μl . PCR cycles consisted of 15 min hold at 95°C (1 cycle), then 40 amplification cycles at appropriate T_m (Table 1), extension (1 min at 72°C) followed by dissociation. Non-template negative controls and standards were included in each PCR analysis. In addition, PCR products were analyzed on agarose gels to confirm that the correct size (base pairs, Table 1) product was obtained and also to visualize potential secondary and nonspecific amplification. As an additional positive control we made cDNA from hypothalamic RNA (from the same hamsters used for ovaries) and tested our primers. For the standard curve, cDNAs from all samples were pooled and a 4-point curve was included with each run (for gene of interest and house keeping genes). The relative amounts of mRNA (arbitrary units) were calculated using standard curves of each gene of interest and house keeping genes GAPDH and HPRT1. Subsequently the ratios of gene of interest to both house keeping genes were calculated. No differences were observed between normalizations to both housekeeping genes; therefore, for simplicity, the data are presented as the mean $\pm$ SEM of the HPRT1-normalized ratios.

2.6 Plasma estradiol and progesterone

Estradiol and progesterone levels were determined by enzyme immunoassay using Progesterone EIA and Estradiol EIA kits (Cayman Chemical, MI) following manufacturers' instructions. All serum samples (50 μl) and standards curve were run in duplicates and the assay was conducted exactly as per kit instructions. Estradiol and progesterone concentrations were calculated using the manufacturer's template.

2.7 Immunohistochemical detection of GnRH-1 protein

Sectioned (6 μm) ovary tissue was deparaffinized in xylene, rehydrated through a graded series of ethanol, washed in phosphate buffer (PBS), and heated under pressure in 1mM EDTA pH 8.0 for 15 min, cooled, and washed in PBS. Sections were then placed in 0.3% hydrogen peroxide/methanol solution, blocked with species matched normal serum/Tween-20 (0.1%) for 40 min and after two washings incubated with either mouse anti-gonadotropin releasing hormone (GnRH-1) monoclonal antibody (1:500) (Millipore Corp., Temecula, CA), anti-FSH β mouse monoclonal antibody (1:20) or anti-LH goat polyclonal antibody (1:50) (Santa Cruz Biotechnology, CA) for one hr at room temperature in a humidified chamber followed by overnight incubation at 4°C. Sections were then processed using appropriate secondary

antibody using Vectastain Elite ABC kit and NovaRed Substrate (Vector Laboratories, Burlingame, CA) as per manufacturer's protocol. Sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls were processed without primary antibody, or by antigen preabsorption.

Both extent and intensity of immunostaining was recorded for GnRH and gonadotropin antibodies with sections assigned a numerical value ranging from 1-5. For intensity, a score of 1 indicated no staining; a score of 2 indicated faint staining in ovarian structures (follicles, corpora lutea, stroma), a score of 3 indicated medium-intense staining in most structures, a score of 4 indicated intense staining in most structures, and a score of 5 specified intense staining in all structures present in the ovary. For extent, a score of 1 specified no staining, a score of 2 specified staining across ~25% of the structures/stroma in the cross section, a score of 3 specified staining across ~50% of the structures/stroma of the cross section, a score of 4 specified staining across ~75% of the structures/stroma of the cross section, and a score of 5 specified intense staining ~100% of structures/stroma in the cross section. For all counts, scores for three sections (60-100 microns apart at minimum) per animal were averaged, and average extent was multiplied by average intensity for each animal. This index was included in the group mean (n=5-7 animals per group) used in the ANOVA analysis.

2.8 Statistical analysis

Results are presented as mean $\pm$ SEM and analyzed by one-way ANOVA with Newman Keuls post hoc tests using Prism 4 software (GraphPad Software, Inc, San Diego, CA). $p \leq 0.05$ was considered statistically significant. A logY transformation was used to determine statistical differences in gonadotropin estrous cycle immunostaining data to reduce variance.

3. Results

3.1 Real Time PCR of GnRH-1, FSH β and LH β and the common α subunit mRNA during the estrus cycle

In this study we measured the GnRH-1 isoform because it has been detected in both human and rat ovaries. GAPDH and HPRT-1 mRNA expression was used as a normalizing factor; since no differences were noted in relative expression results, ratios with HPRT-1 are presented for consistency. GnRH-1 mRNA levels were nearly 5-fold higher during diestrus I (DI) as compared to proestrus (P), estrus (E), or diestrus II (DII) ($p < 0.05$; Figure 1A). As a positive control we also measured GnRH-1 mRNA levels in the hypothalamus (arcuate nucleus) of the same hamsters and, as expected, GnRH-1 mRNA of the same product size was detected but in higher amounts than observed in the ovary (data not shown).

Relative levels of LH β mRNA increased approximately 2.5-4.3 fold ($p < 0.05$) in P and DI as compared to E and DII (Figure 1B). Similarly, relative levels of FSH β mRNA were higher during P (1.9 fold) and DI (2.2 fold) ($p < 0.05$) as compared to E and DII (Figure 1C). The common α subunit mRNA was expressed throughout the cycle but did not differ significantly between groups ($p > 0.11$, Figure 1D). LH β and FSH β mRNA were not detected in the arcuate nucleus as expected (data not shown). To further confirm the specificity of our primers, PCR products of GnRH-1, FSH β , LH β , common α subunit, and housekeeping genes GAPDH and HPRT1 were analyzed on 2% agarose gels and in each case a single band at the expected molecular sizes (Table 1) were observed (data not shown).

3.2 Photoperiod induced regression/recrudescence

3.2.1 Ovarian mass, steroidogenesis, and follicle and corpora lutea counts—

Exposure of hamsters to 14 weeks of SD significantly reduced ovarian mass (2.1 fold,

Figure 2A) and plasma estradiol concentrations (3.2 fold; Figure 2B) compared to LD controls, with both reproductive parameters returning to LD levels after transfer to LD for 8 weeks (PT) (Figures 2A and 2B). Body mass and plasma progesterone levels showed no differences between LD, SD, and PT groups ($p > 0.05$; data not shown). In addition, the number of antral follicles and corpora lutea per section was severely reduced in the regressed ovaries (SD) but returned to LD levels after transfer to LD for 8 weeks (PT) (Figures 2C and 2D).

3.2.1 QPCR analysis of intraovarian expression of GnRH-1, FSH β , and LH β and the common α subunit mRNA—Fourteen weeks of SD exposure increased relative ovarian GnRH-1 mRNA levels by 1.9 fold compared to LD control ovaries ($p < 0.05$; Figure 3A). Transfer of SD exposed hamsters to LD for eight weeks (PT) reduced GnRH-1 mRNA levels compared to both SD (8.5 fold) and LD control (4.5 fold) groups ($p < 0.05$; Figure 3A). Similar to GnRH-1, 14 wks of SD exposure increased ($p < 0.05$) relative mRNA levels of the specific LH β (4 fold, Figure 3B), FSH β (3 fold, Figure 3C), and the common α subunit (3 fold, Figure 3D) in the regressed ovaries (SD) as compared to LD control. However, unlike GnRH-1, FSH β and common α subunit mRNA expression returned to LD levels after transfer to LD for eight weeks (PT) ($p > 0.05$; Figure 3C, 3D).

Immunodetection of GnRH-1, FSH, and LH protein: Immunohistochemistry results showed presence of GnRH-1, FSH and LH protein across the estrous and photoperiod exposure groups (Figure 4). Immunostaining of GnRH was detected in the granulosa, especially in luteal granulosa cells, along the zona pellucida, in oocytes, and in endothelial cells surrounding blood vessels (representative GnRH-1 staining in LD Figure 4A). FSH immunostaining was localized predominantly to vascular endothelial cells, follicular fluid and scattered granulosa cells (Figure 4B, and inset). LH immunodetection was observed in granulosa cells, theca cells of antral follicles and oocytes (Figure 4C). No staining was noted in negative controls for all three antibodies (Figure 4 insets show representative negative controls).

Quantification of staining extent/intensity showed differences across the estrous cycle and with photoperiod exposure (Figures 5 and 6). GnRH immunostaining peaked in DI as compared to lower levels observed in both P and DII ($p < 0.05$; Figure 5A). FSH immunodetection was high throughout P, E, and DI, with a significant decline in DII ($p > 0.05$; Figure 5B). FSH immunostaining extent/intensity peaked during estrous with a decline in DI ($p < 0.05$; Figure 5C). Both GnRH and FSH immunodetection was observed in LD ovaries with a significant decline with SD exposure ($p < 0.05$); levels returned to values no different from LD in the PT group ($p > 0.05$; Figure 6A, B). LH immunostaining index showed a similar pattern, with high levels in both LD and PT groups as compared to lower SD detection ($p < 0.05$; Figure 6C).

4. Discussion

While the expression of GnRH and gonadotropin mRNA has been shown in human, rat, mouse, and fish ovaries among other extra-hypothalamic/pituitary tissues, their presence or potential involvement in seasonally breeding species during the photoperiodic response has not been explored. Results from the present study for the first time show differential expression of GnRH-1 and gonadotropin mRNA in Siberian hamster ovaries during the estrous cycle and during photoperiod induced regression and recrudescence, suggesting local production of these hormones. In addition, protein immunodetection for GnRH, FSH, and LH was quantified and also found to be dynamic across estrous and photoperiod cycles.

Intraovarian expression of GnRH-1 mRNA during the Siberian hamster estrous cycle peaked at diestrus I, but remained significantly lower in proestrus, estrus, and diestrus II (Figure 1). These data are consistent with the reported production of GnRH and GnRH-R mRNA by the granulosa luteal cells in rats [32], humans [15] and rhesus macaques [6], cells that are prominent in the ovary during diestrus I. In rat ovaries, GnRH mRNA expression levels peak during diestrus I and proestrus, specifically at 1000 h of diestrus I and 1400 h of proestrus [28]. Changes in GnRH-1 mRNA levels in cycling ovaries are dynamic, and thus it is likely that their detection may reflect the time of tissue sampling. In the present study, ovaries were harvested on the morning of proestrus, estrus, diestrus I, and diestrus II, between 0800-1100h and not at specific time intervals as in the above study [28], and it is possible that a proestrus peak may have been missed due to this more general sampling. Finally, ovarian GnRH mRNA may be elevated in DI in response to the low estradiol concentrations that characterize this stage. In human granulosa cells, both GnRH and its receptor are down-regulated by estrogen [22], and removing this inhibition may induce an increase in GnRH. The interplay of ovarian steroids with intraovarian GnRH has not been determined in Siberian hamsters, and these possible modes of regulation have yet to be elucidated.

The present study also shows intraovarian production of LH β , FSH β , and their common α subunit mRNA, along with changes in their relative levels during the estrus cycle (Figure 1). While common α subunit mRNA was present throughout the cycle, no significant changes in expression were observed, despite an apparent trend towards an increase in DI. In contrast, intraovarian LH β and FSH β mRNA levels peaked at proestrus and diestrus I, with significant declines at estrus and diestrus II (Figure 1). In PMSG-stimulated rats, intraovarian LH β mRNA levels peak in proestrus as compared to late estrus [29], similar to the pituitary release of LH itself in the classic pre-ovulatory spike [5,29]. Regulation of intraovarian gonadotropins is not fully understood; control may be local, by the hypothalamus, or the pituitary, or a combination of regulatory factors may act in concert. Indeed, ovarian GnRH, and ovarian or pituitary FSH can regulate ovarian LH β mRNA expression in isolated rat granulosa cells [16]. The additional intraovarian spike of both gonadotropins during diestrus (Figure 2B, 2C) mirrors the peak in GnRH (Figure 2A), a pattern that does not mimic pituitary mRNA, but does suggest differential regulation in the ovary and the pituitary.

While the focus of this paper was intra-ovarian GnRH-I and gonadotropin subunit mRNA, extent and intensity of protein immunostaining was also quantified. GnRH protein detection peaked DI, mimicking what was observed with mRNA expression (Figures 3 and 5). GnRH protein has been previously reported in rat ovarian tissue, albeit at reduced levels as compared to hypothalamic detection [28]. Cytoplasmic immunodetection in monkey luteal granulosa cells was strong throughout the CL lifespan [6], which matches the localization and DI peak observed in the current study (Figure 5). In mice, FSH protein has been predominantly immunodetected in interstitial cells, with select steroidogenic luteal cells also reacting [18]. This localization corresponds to the current study where endothelial cells in stromal blood vessels showed immunoreaction for FSH, in addition to some granulosa cells of follicles and CL (Figure 4). FSH protein was detected throughout P, E, and DI at high levels, similar to the peak in mRNA at P and DI. Pituitary peaks in FSH release can potentially explain the discrepancy of low FSH mRNA expression with high staining at the protein level at the estrus stage observed in the current study. The antibodies used react with the FSH peptide, but do not differentiate between ovarian or pituitary origin of the protein, whereas mRNA expression is specific to ovarian tissue. The same explanation is likely for the peak observed at E in LH protein immunostaining (Figure 5), with the possibility that, at least some of the protein detected in the ovary was released from the pituitary in the LH surge.

This is the first report to investigate potential alterations in intraovarian GnRH/gonadotropin production during photoperiod induced regression and recrudescence. Siberian hamsters are seasonal breeders and become reproductively quiescent following acute exposure to short photoperiods. Consistent with previous reports (e.g., 21, 27, 30), the present results show that 14 weeks of SD exposure reduced ovarian mass, plasma estradiol concentrations, and number of both antral follicles and corpora lutea. Most of these parameters returned to LD control levels following transfer to LD for 8 weeks (PT group), demonstrating photoperiod-induced regression and recrudescence of the ovary (Figure 2). In addition, our results show differential expression of GnRH-1, FSH β , LH β , and the common α subunit mRNA levels in functional, regressed, and recrudescing ovaries (Figure 3). GnRH-1 mRNA levels increased significantly in regressed ovaries (SD) compared to LD controls and decreased further in PT compared to both LD and SD groups (Figure 3). A similar hypothalamic increase following SD exposure is not reported; no changes were noted in GnRH mRNA content in forebrains of SD exposed male Syrian hamsters as compared to LD [4], despite SD declines in serum GnRH. In contrast to our ovarian data, transfer of photoinhibited Siberian hamster males to LD induces a transitory increase in the number of neural cells with detectable GnRH mRNA [25]; however exposure to both SD and LD photoperiod was truncated as compared to the current study. The photoperiod-induced changes in intraovarian GnRH observed in our study may be the result of interaction with ovarian steroids [see reviews; 15,20,26]. Because estrogen treatment decreases GnRH-1 mRNA levels in human luteal granulosa cells, a result reversed by estrogen antagonists [22], the observed SD increase in GnRH-1 mRNA in the present study (Figure 3) could be due to significantly lower estrogen levels, and lower GnRH-1 levels in the PT group may then be related to newly resumed estrogen production (Figures 2,3). This is further strengthened by immunolocalization of GnRH protein in granulosa, especially luteal granulosa cells in hamsters, primates, and rats (Figure 4) [6,16].

Intraovarian gonadotropin mRNA expression and protein levels in Siberian hamster ovaries also reacted to photoperiod changes. Plasma concentrations of gonadotropins are characteristically reduced with short photoperiod exposure (e.g., 30, 35). Indeed, in male Siberian hamsters, pituitary gonadotropin mRNA and serum FSH levels decrease during photoperiod-induced regression [3,19,43], and transfer of photoinhibited males to LD rapidly restores pituitary FSH β mRNA and serum FSH concentrations [3]. These patterns correspond with immunodetection data from the present study where intensity/extent of all three proteins decreased during short day inhibition (Figure 6). However, our results also show a SD increase in relative levels of ovarian FSH β , LH β , and common α subunit mRNA, with a return to LD control levels after transfer to LD for 8 weeks for FSH β and the common α subunit (Figure 3). Coupling the presence of immunostaining for these proteins in SD ovaries despite low to no pituitary contribution, along with high mRNA expression in SD, suggests that the gonadotropins in photoinhibited ovaries are locally produced.

Because folliculogenesis is arrested in regressed ovaries, the presence of these proteins and the increase in ovarian gonadotropin mRNA in SD conditions may have a separate paracrine role in (1) maintaining primary follicles when reproduction is inhibited and/or (2) keeping the primary follicles primed and thus responsive to hypothalamic or pituitary endocrine signals for rapid recrudescence of ovarian function upon LD stimulation or spontaneous recrudescence. Both ovarian mass and antral follicle number increase significantly within one week of transfer of photoinhibited Siberian hamsters to long days [27], with full recrudescence occurring within 8 weeks post transfer, indicating a rapid return to ovarian function. Maintaining low levels of gonadotropins to enable follicles to respond rapidly to pituitary gonadotropins has been suggested in the cycling ovary [31], and this is an intriguing possibility in the photoperiod-inhibited ovary. Additionally, studies in rat testes [46], and rat [29] and fish [41] ovaries have shown that the gonadal LH β subunit is longer than that observed in the pituitary, which may allow differential “organ-specific” function

between ovarian and pituitary gonadotropins. Intraovarian produced gonadotropins could potentially contribute towards maintenance or priming of primary follicles ovary to maximize the response of normal or photo-regressed ovaries to pituitary gonadotropin surges, allowing for rapid recrudescence, although this possibility has not yet been examined.

In summary, the present study for the first time clearly shows intraovarian expression of GnRH-1, FSH β , LH β and the common α subunit mRNA along with GnRH/gonadotropin protein localization in Siberian hamster ovaries. Importantly, mRNA expression suggests local production of these hormones in the ovary, and this expression is regulated differentially during the estrus cycle and during photoperiod induced regression/recrudescence. Taken together these findings suggest the possibility of a “GnRH-gonadotropin” axis in Siberian hamster ovaries; however, its functional presence, precise role, and regulation remain to be elucidated. Intraovarian GnRH and gonadotropins may play a potential supplementary local role in ovulation or follicular development, and may serve to maintain or prime the ovary during SD declines in the hypothalamic and pituitary support. While photoperiod induced changes in reproductive function have been well studied, little is known about how the gonads themselves may influence reproductive recrudescence. Further examination of the roles of this putative gonadal axis may be critical next steps to better understand the loss and return of ovarian function in seasonal breeders like Siberian hamsters.

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Figure 1. Mean $\pm$ SEM relative levels of intraovarian mRNA expression for GnRH and gonadotropins in LD exposed Siberian hamsters in the different stages of the estrous cycle (E= estrus, P= proestrus, DI= diestrus I, DII= diestrus II). (A) GnRH-1, (B) LH β , (C) FSH β , (D) the gonadotropin common α subunit. Results from real time PCR assays using HPRT as a control gene shown. Groups with different letters are significantly different ($p < 0.05$).

Figure 2.

(A) Ovarian mass (mg), (B) plasma estradiol concentrations (pg/ml), (C) average number of antral follicles per ovarian cross section, (D) average number of corpora lutea per ovarian cross section in Siberian hamsters exposed to: long photoperiods (LD; 16L:8D), 14 weeks of short inhibitory photoperiods (SD; 8L:16D), or 14 weeks of SD followed by 8 weeks of LD (PT; post transfer). Groups with different letters are significantly different ($p < 0.05$).

Figure 3.

Real time PCR mRNA expression for GnRH and gonadotropins. Mean $\pm$ SEM relative levels mRNA expression in Siberian hamsters exposed to long photoperiods (LD; 16L:8D), 14 weeks of short inhibitory photoperiods (SD; 8L:16D), or 14 weeks of SD followed by 8 weeks of LD (PT; post transfer). (A) GnRH-1, (B) LH β , (C) FSH β , (D) the gonadotropin common α subunit. Data normalized to HPRT control gene. Groups with different letters are significantly different (p<0.05).

Figure 4. Representative ovarian A) GnRH, B) LH and C) FSH protein immunodetection in long day exposed Siberian hamsters. Staining is red with purple/blue background. Insets show negative control immunostaining (no primary antibody present for GnRH, LH, antigen preabsorption for FSH); AF= antral follicle, At= atretic follicle; BV= medullary blood vessels, CL= corpus luteum, PA=preantral follicle.

Figure 5. Index of immunostaining (extent x intensity) for A) GnRH, B) LH, and C) FSH during the estrous cycle (E= estrus, P= proestrus, DI= diestrus I, DII= diestrus II). Bar graphs represent mean $\pm$ SEM immunostaining index levels. Groups with different letters are significantly different ($p < 0.05$).

Figure 6. Index of immunostaining (extent × intensity) for A) GnRH, B) LH, and C) FSH in Siberian hamsters exposed to long photoperiods (LD; 16L:8D), 14 weeks of short inhibitory photoperiods (SD; 8L:16D), or 14 weeks of SD followed by 8 weeks of LD (PT; post transfer). Bar graphs represent mean ± SEM immunostaining index levels. Groups with different letters are significantly different ($p < 0.05$).

Table 1

Real time PCR primer sequences, product sizes in base pairs, and primer melting temperature (T_m) for Siberian Hamsters (*Phodopus sungorus*).

Gene	Sequence	bp	T _m °C
GnRH-1	F- TCT GGT CAT GTT GTC CGT GT	170	61
	R- CTT GCT GGT GTG TGG TAT GC		
FSHβ (AF106914)	F-TGC ATC CTA TTC TGG TGC TG	196	60
	R- TTT CTG GGT ATT GGG TCT GG		
LHβ (AF106915)	F- CTG CTA TGG CTG TTG CTG AG	173	60
	R- AAC ACT CGG ACC ATG CTA GG		
GAPDH	F: GGA GAA AGC TGC CAA GTA	170	55
	R: TGT CAT TGA GAG CGA TGC		
HPRT1	F- TGA TCA GTC AAC AGG GGA CA	209	55
	R- CTG GCC GAT ATC CAA CAC TT		