Sex Differences in Steroid Receptor Coexpression and Circadian-Timed Activation of Kisspeptin and RFRP-3 Neurons May Contribute to the Sexually Dimorphic Basis of the LH Surge

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In rodents, the ovulation-inducing luteinizing hormone (LH) surge is sexually dimorphic, occurring only in females, but the reasons for this sex difference are unclear. Two neuropeptides, kisspeptin and RFamide-related peptide 3 (RFRP-3), are hypothesized to regulate the gonadotropin-releasing hormone (GnRH)/LH surge. In females, both of these systems show circadian changes coincident with the LH surge, but whether males show similar temporal changes under comparable hormonal conditions is unknown. Here, we evaluated circadian time (CT)–dependent changes in gene expression and neuronal activation of *Kiss1* and *Rfrp* neurons of female and male mice given identical LH surge-inducing estrogen regimens. As expected, females, but not males, displayed a late afternoon LH surge and GnRH neuronal activation. *Kiss1* expression in the anteroventral periventricular nucleus (AVPV) was temporally increased in females in the late afternoon, whereas males demonstrated no temporal changes in AVPV *Kiss1* expression. Likewise, neuronal activation of AVPV *Kiss1* neurons was dramatically elevated in the late afternoon in females but was low at all circadian times in males. Estrogen receptor α levels in AVPV *Kiss1* neurons were sexually dimorphic, being higher in females than males. AVPV progesterone receptor levels were also higher in females than males. Hypothalamic *Rfrp* messenger RNA levels showed no CT-dependent changes in either sex. However, *Rfrp* neuronal activation was temporally diminished in the afternoon/evening in females but not males. Collectively, the identified sex differences in absolute and CT-dependent AVPV *Kiss1* levels, AVPV sex steroid receptor levels, and circadian-timed changes in neuronal activation of both *Kiss1* and *Rfrp* neurons suggest that multiple sexually dimorphic processes in the brain may underlie proper LH surge generation. **(*Endocrinology* 158: 3565–3578, 2017)**

Ovulation in female mammals is gated at the neuroendocrine level by an estrogen-mediated positive feedback induction of luteinizing hormone (LH) secretion. This "surge" release of LH is controlled by a preceding surge in gonadotropin-releasing hormone (GnRH) secretion from the brain. The secretion of GnRH is itself tightly regulated by a collection of upstream neurotransmitters and neuropeptides (1, 2). In rodents, the estrogen-induced GnRH/LH surge is circadian timed, occurring only in the late afternoon/early evening of proestrus, but not at other times of the day (3). The neural and molecular mechanisms that underlie the circadian and estrogen-dependent nature of the LH surge still remain incompletely characterized (4, 5), but are thought to include temporal and hormonal gating controlled by two hypothalamic neuropeptide systems, kisspeptin (encoded by *Kiss1*) and RFamide-related peptide 3 (RFRP-3, encoded by *Rfrp*).

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA
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Received 24 April 2017. Accepted 4 August 2017.
First Published Online 9 August 2017

Abbreviations: ANOVA, analysis of variance; AVP, arginine-vasopressin; AVPV, anteroventral periventricular nucleus; CT, circadian time; DIG, digoxigenin; DMN, dorsalmedial nucleus of the hypothalamus; E_2, 17-β estradiol; ERα, estrogen receptor α; GnRH, gonadotropin-releasing hormone; ISH, *in situ* hybridization; LH, luteinizing hormone; mRNA, messenger RNA; PeN, rostral periventricular nucleus; PR, progesterone receptor; RFRP-3, RFamide-related peptide 3; SCN, suprachiasmatic nucleus; SSC, sodium citrate, sodium chloride.

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Kisspeptin, acting on its receptor, Kiss1r, is an essential regulator of the reproductive axis. Humans or rodents with mutations in the *Kiss1* or *Kiss1r* genes suffer from hypogonadotropic hypogonadism, impaired sexual development, and infertility (6–9). Kisspeptin directly acts on GnRH neurons (10–12), evidenced by the fact that kisspeptin stimulates both c-fos induction (a marker of neuronal activation) and electrical firing of GnRH neurons in brain explants (10, 13–15) and, subsequently, a robust secretion of gonadotropins (9, 15–19). Within the rodent brain, *Kiss1* is expressed in two primary neuronal populations: the hypothalamic continuum of the anteroventral periventricular nucleus (AVPV) and neighboring rostral periventricular nucleus (PeN), and more caudally, the arcuate nucleus (20, 21). AVPV/PeN kisspeptin neurons in female rodents have been shown to be circadian regulated when estrogen is present, with *Kiss1* messenger RNA (mRNA) and *Kiss1* neuronal activation being significantly elevated just prior to and during the beginning of the dark phase, aligning with the occurrence of the LH surge (22). AVPV/PeN kisspeptin neurons receive input from the suprachiasmatic nucleus (SCN), the central circadian clock, and can be stimulated exogenously by neuropeptides expressed in the SCN (23, 24).

In contrast to kisspeptin, the neuropeptide RFRP-3 has potent inhibitory actions on LH secretion and is considered the mammalian ortholog of the avian gonadotropin-inhibiting hormone (25–30). In rodents, RFRP-3 may directly inhibit GnRH neuron activity (29) and/or GnRH secretion (31), or may act indirectly via kisspeptin neurons (32), thereby inhibiting LH secretion (33, 34). In the rodent brain, RFRP-3 cell bodies are found exclusively in and around the dorsal-medial nucleus of the hypothalamus (DMN), as determined by immunohistochemistry and *in situ* hybridization (11, 26, 33, 35, 36). With regard to the preovulatory LH surge, in female hamsters, RFRP-3 neurons show decreased immunoreactivity and decreased neuronal activation (assessed with c-fos induction) during the late afternoon of proestrus, coinciding with increased GnRH neuronal activation and the endogenous LH surge (37). Thus, it has been speculated that the increase in GnRH neuronal activation driving the LH surge could be due, in part, to an attenuation of upstream suppressive influence by RFRP-3. This possibility is supported by the finding that high estrogen levels, as would occur during estrogen positive feedback and the LH surge, moderately suppress *Rfrp* mRNA expression in rodents (11) and RFRP-3 receptor knockout mice have larger litters (34).

In rodents, the LH surge mechanism is sexually differentiated, with only females being able to generate LH surges in response to sex steroid–priming in adulthood. Indeed, adult male rodents given appropriate estradiol or estradiol plus progesterone paradigms fail to generate LH surges, indicating their reproductive brain circuitry is different in one or more ways from that of females. However, the underlying mechanisms of this sex difference in LH surge capability are still poorly understood. Importantly, as with the LH surge, the AVPV/PeN population of *Kiss1* neurons is sexually dimorphic, with female rodents having considerably more *Kiss1* neurons and more *Kiss1* mRNA in each cell than males (38). Thus, one common postulation is that the sexually dimorphic ability to generate LH surges simply reflects the total amount of kisspeptin being greater in females than males. Although this is likely a key facet of the LH surge sex difference, it remains to be determined if males demonstrate additional sex differences in other parameters relating to the LH surge, such as circadian-timed increases in *Kiss1* levels or kisspeptin neuronal activation, circadian-timed decreases in the RFRP-3 system, or coexpression levels of sex steroid receptors.

This study was designed to investigate changes in kisspeptin and RFRP-3 neurons in mice with regard to the sexually dimorphic induction of the LH surge. We compared male and female mice that were treated with a similar exogenous estrogen regimen known to elicit LH surges and determined (1) whether the circadian increase of AVPV/PeN *Kiss1* mRNA levels and/or *Kiss1* neuronal activation is sexually dimorphic; (2) whether sex differences in estrogen receptor α (ERα) or progesterone receptor (PR) levels in the AVPV/PeN or coexpression in AVPV/PeN *Kiss1* neurons exist that may account, in part, for sex differences in *Kiss1* neuron circadian changes; and (3) whether hypothalamic *Rfrp* expression and/or *Rfrp* neuronal activation in mice exhibit temporal changes coincident with the circadian-timed LH surge and, if so, does this occur similarly in both sexes.

Materials and Methods

Animals

Young adult (7 to 8 weeks old) C57BL6 mice of both sexes were singly-housed on a 12-12 light-dark cycle (lights off at 5:00 PM) with food, water, and in-cage running wheel available *ad libitum* for the entire study, matching the housing setup of our previous study (22). The running wheels were hooked up to computers to continuously measure daily circadian changes in locomotor activity (not shown). All experiments were conducted in accordance with the National Institutes of Health Animal Care and Use Guidelines and with approval of the Animal Care and Use Committee of the University of California, San Diego.

Hormonal treatment and tissue collection

After 7 to 10 days of acclimatization to the cages with running wheels, mice were anesthetized with isoflurane and bilaterally gonadectomized. Mice were then subcutaneously implanted with a SILASTIC brand (Dow Corning, Midland,

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MI; inner diameter, 0.20 cm; outer diameter, 0.318 cm) capsule containing either 0.750 μg (for females) or 0.885 μg (for males, because of their higher body weight) of 17-β estradiol (E_2) dissolved in sesame oil (22, 39). Previous studies in female mice by our laboratory and others, and pilot experiments in male mice, determined that this hormone treatment produces similar constant elevated E_2 levels of ~18 to 24 pg/mL in both sexes, similar to female mouse proestrus levels, and induces a robust circadian-timed LH surge in females 2 days later, around the time of lights off (22, 39, 40).

Two days after E_2 capsule implantation, all mice were briefly anesthetized with isoflurane and immediately euthanized by rapid decapitation at one of five circadian time (CT) points: CT4, CT10.5, CT12, CT13.5, and CT18 (n = 7 to 10 mice/sex/time point), with CT 12 representing the time of lights off (5:00 PM) by standard chronobiology convention. In this context, CT4 and CT12 correspond to baseline morning LH and peak LH surge levels, respectively. CT10.5 and CT13.5 bracket 90 minutes on either side of the peak of the LH surge to study the onset and offset periods of the surge event. CT18 represents a late-night time period after the LH surge has ended, when LH levels and GnRH neuronal activity have returned to baseline morning values. At euthanization, blood and brains were collected. Brains were immediately frozen on dry ice and stored at −80°C until being processed with *in situ* hybridization (ISH). Blood was centrifuged 90 minutes after collection and the serum stored at −20°C until assaying for hormone levels. Euthanizations that occurred at time points during lights off (*e.g.,* CT13.5, CT18) were performed under red-light illumination.

Hormone measurements

All hormone measurements were run in singlet and performed by the University of Virginia Ligand Assay Core. Blood serum collected was measured for LH levels using a sensitive mouse LH radioimmunoassay, as in previous studies (22, 39–42). Serum was also tested for E_2 levels to confirm elevated circulating E_2 in both sexes. Serum E_2 was measured by enzyme-linked immunosorbent assay, as in previous reports (39).

Single-label ISH

Five coronal series of 20-μm brain sections were cut on a cryostat, thaw-mounted onto Superfrost-plus slides, and stored at −80°C until use in ISH. The *Kiss1* (20), *Rfrp* (11), *ERα* (11), *PR* (42), *Gnrh* (39), and *cfos* (43) complementary RNA ISH riboprobes have been described and validated previously. Single-label ISH was performed as previously described (11, 39). In brief, slide-mounted sections encompassing the entire AVPV/PeN or DMN were fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2× sodium citrate, sodium chloride (SSC), delipidated in chloroform, dehydrated in ethanols, and air-dried. Radiolabeled (^{33}P) *Kiss1*, *Rfrp, ERα*, or *PR* antisense riboprobe (0.04 pmol/mL) was combined with transfer RNA, heat-denatured, added to hybridization buffer, and applied to each slide (100 μL/slide). Slides were cover-slipped and placed in a 55°C humidity chamber overnight. The slides were then washed in 4× SSC, treated with RNAse A treatment at 37°C, washed in 2× SSC at room temperature, and washed in 0.1× SSC at 62°C before being dehydrated in ethanols and air-dried. Slides were then dipped in Kodak NTB emulsion, air-dried, and stored at 4°C for 3 to 4 days (depending on the assay) before being developed and cover-slipped.

Double-label ISH

Double-label ISH was performed as previously described (32). Briefly, slide-mounted brain sections encompassing the preoptic area, AVPV/PeN, or DMN were fixed in 4% paraformaldehyde; pretreated with acetic anhydride, rinsed in 2× SSC; delipidated in chloroform; dehydrated in ethanols; and air-dried. Radiolabeled (^{33}P) antisense *cfos* or *ERα* (0.05 pmol/mL) and digoxigenin (DIG)-labeled *Gnrh, Kiss1,* or *Rfrp* riboprobes (Roche Digoxigenin labeling kit, 1:500) were combined with transfer RNA, heat denatured, dissolved together in hybridization buffer applied to slides (100 μL/slide), and hybridized at 55°C overnight. The next day, slides were washed in 4× SSC, treated with RNAse A at 37°C, and subsequently washed in 0.1× SSC at 62°C. Slides were then incubated in 2× SSC with 0.05% Triton X-100 containing 3% normal sheep serum for 75 minutes at room temperature and then incubated overnight at room temperature with anti-DIG antibody conjugated to alkaline phosphatase (1:500; Roche). The next day, slides were washed and then incubated with Vector Red alkaline phosphatase substrate (Vector Laboratories, CA) for 1 hour at room temperature. Slides were then air-dried, dipped in Kodak NTB emulsion, stored at 4°C, and developed and cover-slipped 7 to 9 days later (depending on the assay).

ISH quantification and analyses

ISH slides were analyzed with an automated image processing system (Dr. Don Clifton, University of Washington) by a person unaware of the treatment group of each slide (44). For single-label assays, the software counted the number of ISH silver grain clusters representing *Kiss1*, *Rfrp, ERα,* or *PR* cells as well as the number of silver grains in each cell (a semi-quantitative index of *Kiss1*, *Rfrp, ERα,* or *PR* mRNA expressed per cell) (45–47). Cells were considered *Kiss1* or *Rfrp* positive when the number of silver grains in a cluster exceeded that of background by threefold. For double labels, DIG-containing cells (*Gnrh*, *Kiss1,* or *Rfrp* cells) were identified under fluorescence microscopy, and the grain-counting software was then used to quantify silver grains (representing *cfos* or *ERα* mRNA) overlying each cell in dark field. Signal-to-background ratios for individual cells were calculated, and a cell was considered double-labeled if its ratio was >3.

Statistical analysis

All data are expressed as the mean ± standard error of the mean for each group. In all experiments, differences were analyzed by Student *t* test or two-way analysis of variance (ANOVA), followed by post hoc comparisons for individual groups via Fisher's (protected) least significant difference. Statistical significance was set at $P < 0.05$. All analyses were performed in Statview 5.0.1 (SAS Institute, Cary, NC).

Results

Circadian increases in LH levels and GnRH neuronal activation in females but not males

ANOVA analysis determined that serum LH levels showed significant main effects for both CT ($P < 0.01$) and sex ($P < 0.01$) and a significant CT-by-sex interaction ($P < 0.01$). In E_2-treated females, serum LH values were nearly undetectable in the morning at CT4, increased significantly

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in the late afternoon at CT10.5 and CT12, and then fell again in the early evening at CT13.5 (Fig. 1). LH was again near undetectable baseline levels by the night at CT18. In contrast, as expected, E_2-treated males showed no circadian changes in serum LH (Fig. 1), with LH values at all time points being similar to morning CT4 values.

The pattern of *cfos* induction (a marker of neuronal activation) in GnRH neurons matched serum LH values, as expected. ANOVA revealed that GnRH-*cfos* coexpression showed significant main effects for both CT ($P < 0.01$) and sex ($P < 0.01$), and also a significant CT-by-sex interaction ($P < 0.01$). E_2-treated females displayed high

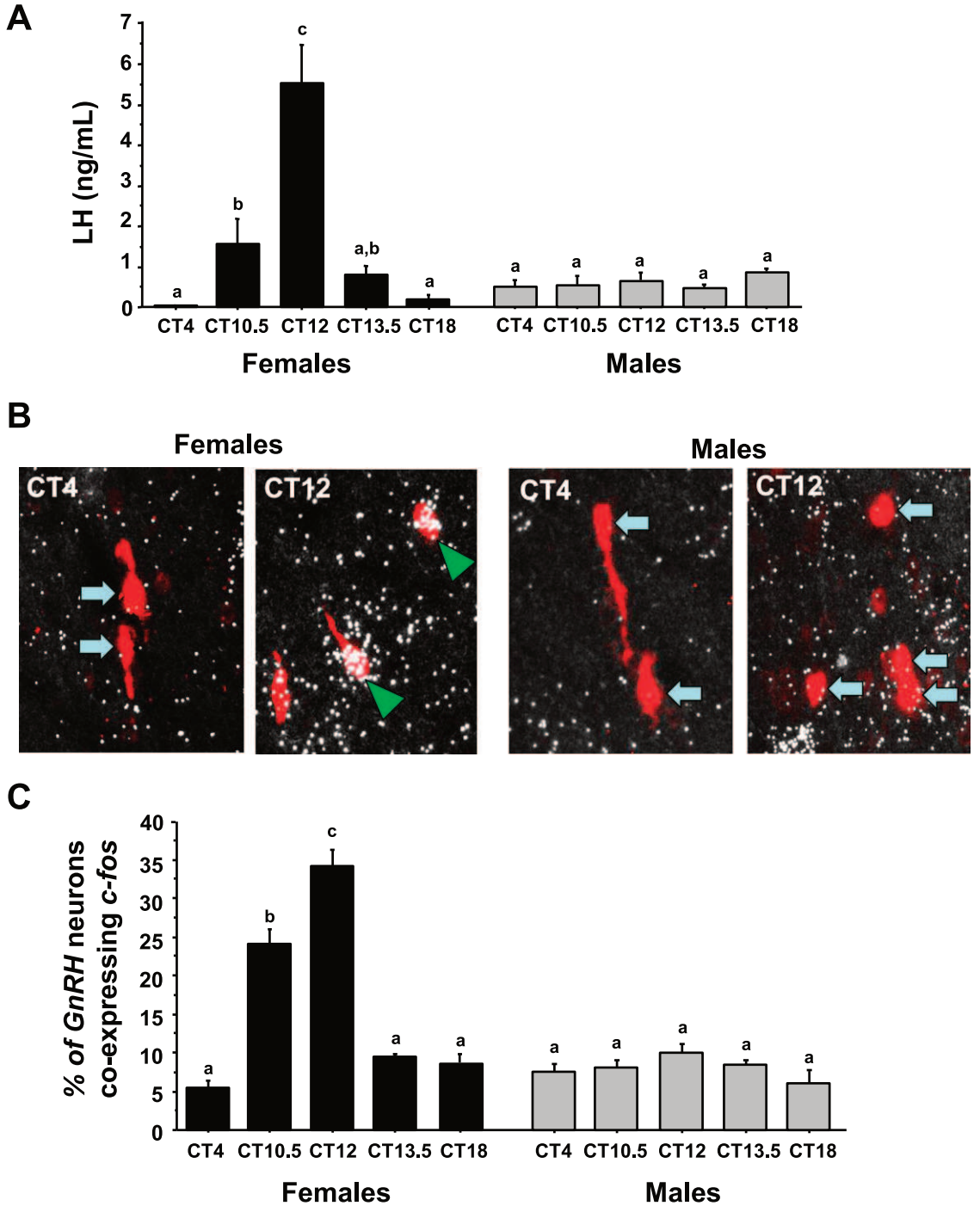

Figure 1. Circadian-timed changes in LH and *cfos* induction in GnRH neurons of gonadectomized and estrogen-replaced animals. (A) Serum LH of gonadectomized and estrogen-replaced female and male mice. Female mice show statistically significant circadian-timed increase in serum LH, occurring shortly before and during the dark phase, whereas males show no differences in serum LH at any time. (B) Representative photomicrographs of *cfos* (a marker of neuronal activation, silver grains) colocalizing with *Gnrh* neurons (red fluorescence) of both males and females at two circadian time points (all mice are gonadectomized and had estrogen replaced). Green arrowheads, *Gnrh* cells with *cfos*; blue arrows, example *Gnrh* cells lacking *cfos* expression. (C) Quantification of the percent colocalization of *cfos* in *Gnrh* neurons. Different lowercase letters indicate significantly different groups ($P < 0.05$).

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levels of *cfos* coexpression in GnRH neurons at the height of the LH surge (CT12) but low *cfos*-GnRH expression at CT4 and CT18 when LH was virtually undetectable (Fig. 1B and 1C). Conversely, E_2-treated males showed no elevated *cfos*-GnRH coexpression at any time point and there were no CT-dependent changes in *cfos* induction in GnRH neurons of males (Fig. 1B and 1C).

Circadian increases in AVPV/PeN Kiss1 mRNA levels are sexually dimorphic

To determine if there is a sex difference in the circadian-timed upregulation of *Kiss1* expression in the AVPV/PeN during an LH surge, single-label ISH was used to measure *Kiss1* mRNA levels in the AVPV/PeN of E_2-treated males and females throughout the day, with

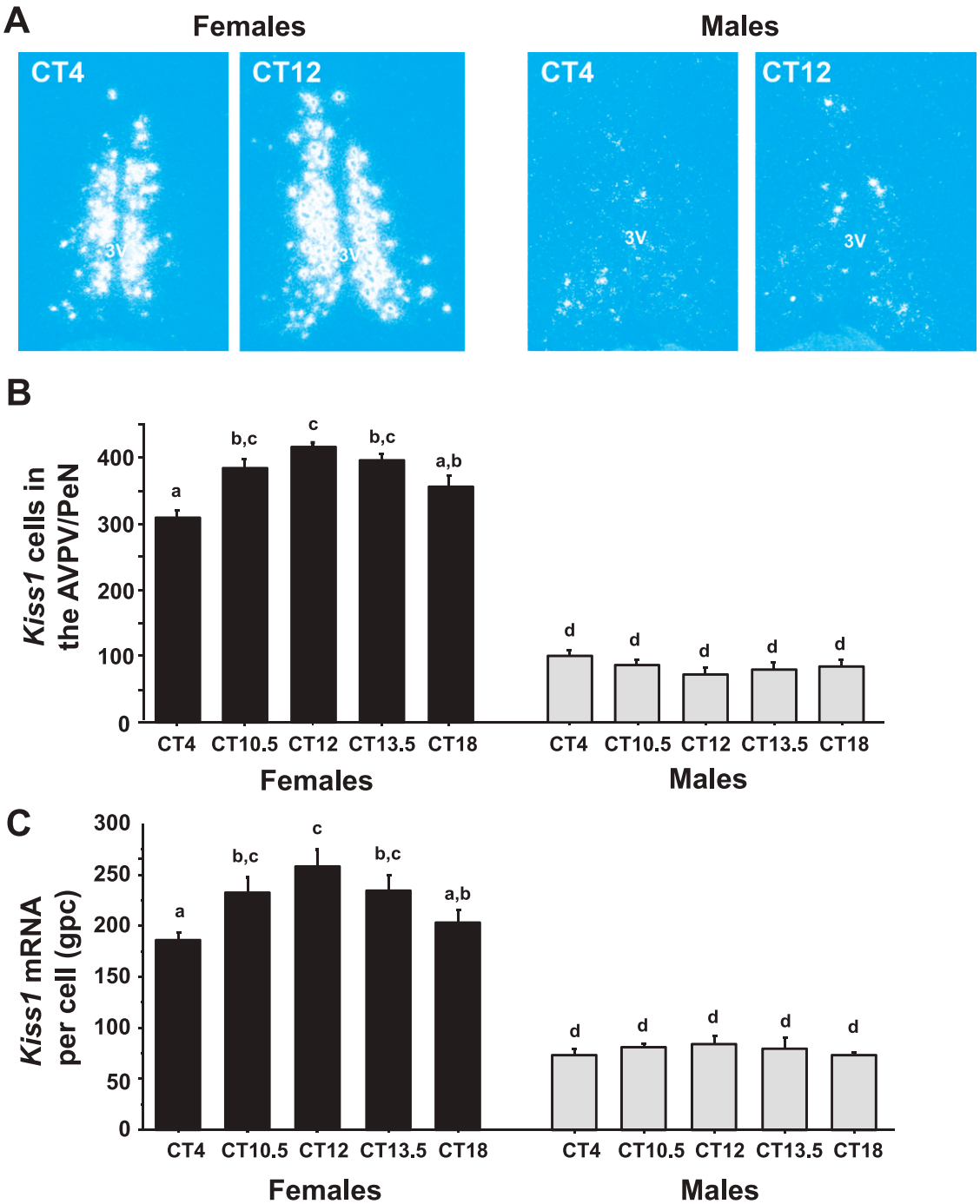

Figure 2. ISH for *Kiss1* mRNA in the AVPV/PeN during the time of the LH surge. (A) Representative photomicrographs of *Kiss1* mRNA in the AVPV/PeN in both males and females at two time points. Female mice euthanized at CT12 have considerably more *Kiss1* mRNA than CT4 females or males at either time point. All animals are gonadectomized and estrogen replaced. (B) Quantification of the number of *Kiss1* neurons in the AVPV/PeN. (C) Quantification of the grains per cell (a semiquantitative measure of mRNA) of *Kiss1* neurons in the AVPV/PeN. Different lowercase letters indicate significantly different groups. 3V, third ventricle; gpc, grains per cell.

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specific emphasis on times before and during the LH surge. ANOVA determined that AVPV/PeN *Kiss1* levels showed significant a main effect of sex, as previously reported (38), with E_2-treated females having more *Kiss1* cells ($P < 0.01$) and higher *Kiss1* mRNA per cell ($P < 0.01$) than E_2-treated males. ANOVA also revealed that AVPV/PeN *Kiss1* cell numbers showed a significant main effect of CT ($P < 0.01$) and a significant CT-by-sex interaction ($P < 0.01$). E_2-treated female mice showed a substantial temporal increase in the number of *Kiss1* neurons during the LH surge (CT12), relative to baseline values at CT4, whereas E_2-treated males showed no CT-dependent changes in *Kiss1* cell number (Fig. 2A–2C), $P < 0.01$]. Similarly, there was a significant main effect of CT for *Kiss1* mRNA levels per cell ($P < 0.05$), although there was no significant sex-by-CT interaction for this measure (Fig. 2A–2C).

Circadian increases in AVPV/PeN Kiss1 neuronal activation occur in females but not males

To determine if there is a sex difference in the circadian increase in neuronal activation of AVPV/PeN *Kiss1* neurons, double-label ISH was used to measure *cfos* induction in AVPV/PeN *Kiss1* neurons of E_2-treated males and females throughout the day. ANOVA revealed that *Kiss1-cfos* coexpression levels showed significant main effects for both sex ($P < 0.01$) and CT ($P < 0.01$), and also a significant CT-by-sex interaction ($P < 0.01$). E_2-treated female mice showed a significant CT-dependent increase in *Kiss1* neuronal activation (*cfos/Kiss1* coexpression) that occurred in synchrony with the timing of the LH surge and heightened GnRH neuronal activation (CT10.5 and CT12 vs CT4; $P < 0.01$; Fig. 3). In contrast, E_2-treated males failed to show marked increases in *Kiss1* neuronal activation at any circadian time point, with *cfos/Kiss1* coexpression being similar throughout the day.

ERα and PR levels in the AVPV/PeN are higher in females than males

To determine if the sex differences in CT-dependent *Kiss1* gene expression and *Kiss1* neuronal activation might be due, in part, to sex differences in E_2 signaling, single-label ISH for ER levels was performed on brains from both sexes of E_2-treated mice at the CT12 time point (when the LH surge is maximal). E_2-treated females

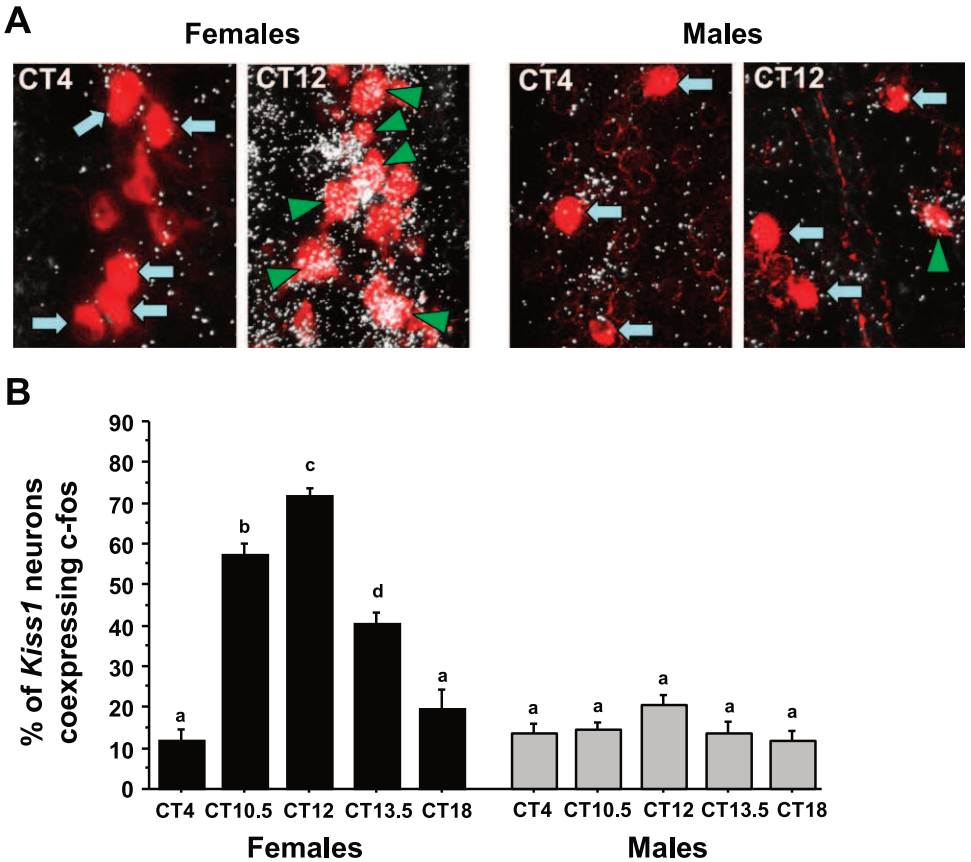

Figure 3. Double-label ISH for *cfos* colocalization in *Kiss1* neurons during the time of the LH surge. (A) Representative photomicrographs of *cfos* (silver grains) colocalizing with AVPV/PeN *Kiss1* neurons (red fluorescence) in E_2-treated males and females at two time points. *Kiss1* neurons in female mice euthanized at CT12 have substantially more *cfos* colocalization than at CT4; male *Kiss1* neurons have low *cfos* induction at both time points. Green arrowheads, example *Kiss1* cells with *cfos*; blue arrows, example *Kiss1* cells lacking *cfos* expression. (B) Quantification of the percent colocalization of *cfos* in AVPV/PeN *Kiss1* neurons in each sex across the circadian day. Different lowercase letters indicate significantly different groups ($P < 0.05$).

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expressed significantly greater numbers of cells expressing ERα mRNA in the AVPV/PeN than E_2-treated males (Fig. 4, $P < 0.05$). Furthermore, the mean relative amount of ERα mRNA in each AVPV/PeN neuron was significantly less in E_2-treated males than E_2-treated females (Fig. 4, $P < 0.05$).

Previous findings from our laboratory and others have demonstrated that PR signaling in the AVPV (48), including specifically in kisspeptin neurons (42), is critical for proper LH surge induction in female rodents. We therefore next tested whether E_2-treated males and females display different levels of PR in the AVPV region, because such a sex difference could be one potential mechanism underlying sex differences in circadian *Kiss1* levels or *Kiss1* neuronal activation. We found that, although both sexes expressed PR in the AVPV region, such expression was significantly greater in females than males (Fig. 5). Specifically, E_2-treated females had many more detectable *PR* neurons and greater *PR* mRNA levels per neuron in the AVPV than did E_2-treated males ($P < 0.05$ for both measures, Fig. 5).

To determine if the lower ERα levels in the AVPV/PeN in males were occurring in kisspeptin neurons specifically, double-label ISH for *ERα* levels in *Kiss1* neurons was performed on brains from both sexes of E_2-treated mice at the CT12 time point. We found that >80% of AVPV/PeN *Kiss1* neurons in E_2-treated females express ERα mRNA, whereas only ~40% of AVPV/PeN *Kiss1* neurons in E_2-treated males express ERα (Fig. 6, $P < 0.05$). Furthermore, the relative amount of ERα mRNA in each *Kiss1* neuron that showed coexpression was significantly less in males than females (Fig. 6, $P < 0.05$).

Circadian decreases in Rfrp neuronal activation occur in females but not males

A previous study in female hamsters showed a circadian-timed reduction in both the number of detectable RFRP-3 neurons and RFRP-3 neuronal activation at the time of the endogenous LH surge on proestrus. However, similar temporal changes in RFRP-3 levels or neuronal activation have not been assessed in other species or in males. We therefore hypothesized that *Rfrp* gene expression and/or *Rfrp* neuronal activation is similarly decreased in female mice before or during the LH surge, thereby permitting increased GnRH/LH release. We also hypothesized that part of the sexually dimorphic nature of the LH surge may include sex differences in circadian changes of RFPR-3 neurons. To test these possibilities, we used ISH to examine DMN *Rfrp* mRNA levels and *Rfrp-cfos* coexpression in the brains of E_2-treated mice of both sexes. ANOVA analysis revealed that *Rfrp* levels did not show a substantial main effect for either CT or sex or a substantial interaction. There were no CT-dependent differences in the number of *Rfrp* neurons or the amount of *Rfrp* mRNA per cell at any time point in E_2-treated mice of either sex (Fig. 7A–7C), nor any sex difference in *Rfrp* levels in these E_2-treated mice (Fig. 7A–7C).

Using double-label ISH to study *cfos-Rfrp* coexpression, we next assessed *Rfrp* neuronal activation in both sexes across the circadian day. We identified a substantial temporal decrease in *Rfrp* neuronal activation during the height of, and toward the end of, the LH surge in E_2-treated females (Fig. 8A) , $P < 0.05$]. In contrast, we found no temporal differences in *Rfrp* neuronal activation between any time point in E_2-treated males, with

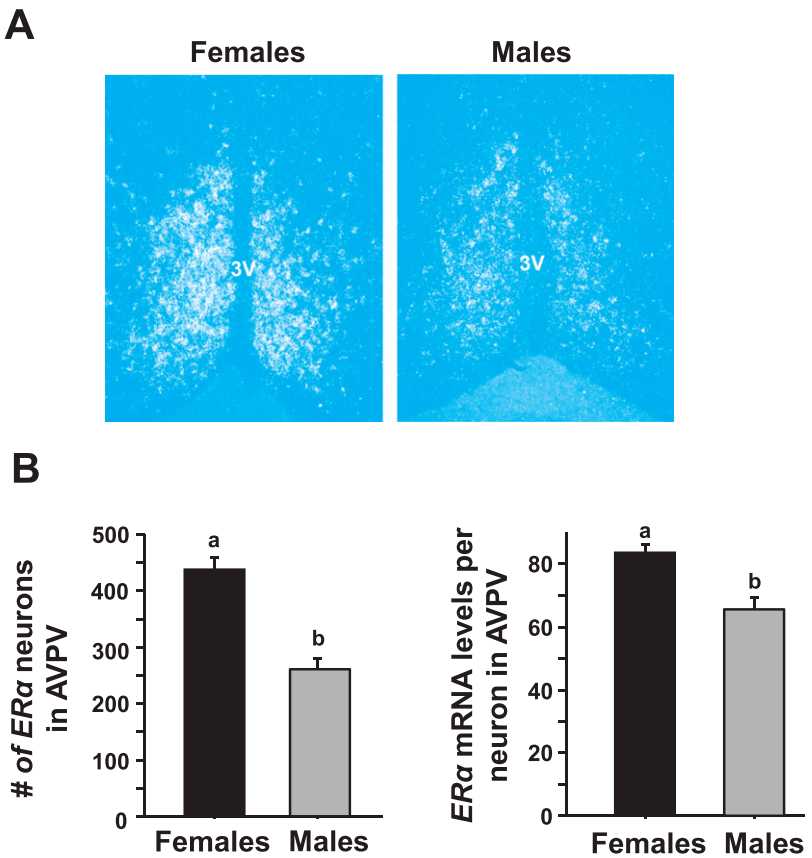

Figure 4. Single-label ISH for ERα mRNA expression in AVPV/PeN in male and female mice. (A) Representative photomicrographs of ERα in the AVPV/PeN in E_2-treated male and female mice at CT12. Female mice have a considerably higher degree of ERα in the AVPV/PeN than do males. (B) Quantification of the gene expression of ERα in the AVPV/PeN of E_2-treated males and females. Different lowercase letters indicate significantly different groups ($P < 0.05$).

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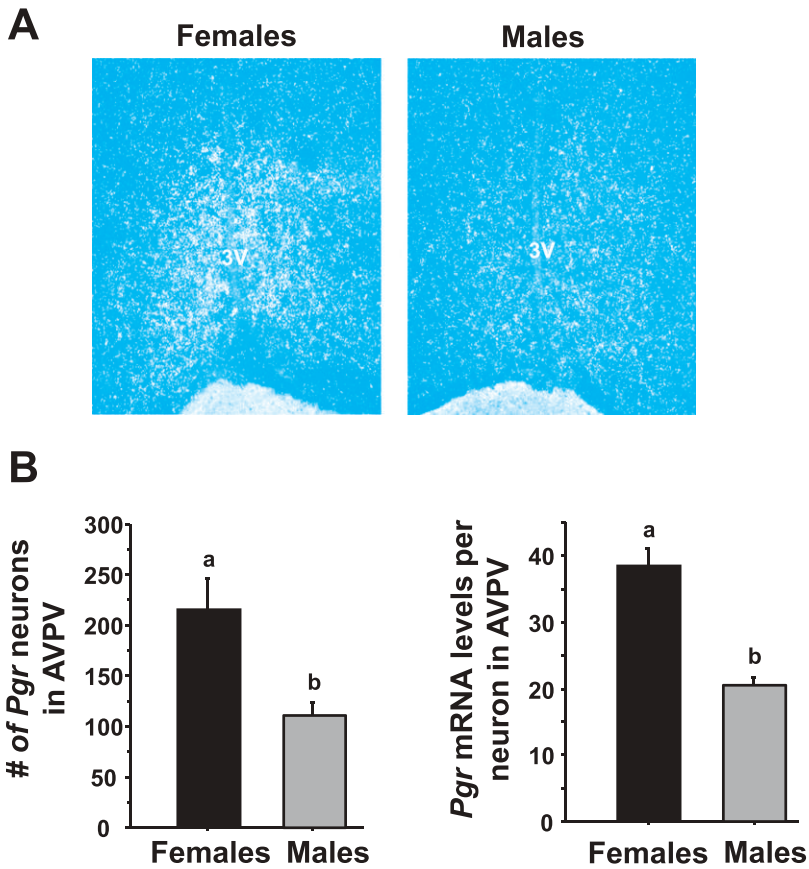

Figure 5. Single-label ISH for PR gene expression in AVPV/PeN in male and female mice. (A) Representative photomicrographs of progesterone receptor gene (*Pgr*) in the AVPV/PeN in E_2-treated male and female mice at CT12. Female mice have a considerably higher degree of *Pgr* in the AVPV/PeN than do males. (B) Quantification of the expression of *Pgr* in the AVPV/PeN of E_2-treated males and females. Different lowercase letters indicate significantly different groups ($P < 0.05$).

Rfrp neuronal activation remaining constant in these mice throughout the day (Fig. 8B).

Discussion

The LH surge in rodents is both circadian-timed and sexually differentiated, with only females being able to generate an LH surge in response to adulthood sex steroid priming; however, the neural and molecular mechanisms underlying the sexually dimorphic nature of the LH surge is not fully known. In this study, we demonstrated that, unlike in E_2-primed females, the AVPV/PeN kisspeptin population of E_2-primed males remains relatively unchanged throughout the day, despite similar elevated E_2 exposure. Specifically, E_2-treated female mice show substantial CT-dependent increases in both AVPV/PeN *Kiss1* mRNA levels and neuronal activation of *Kiss1* neurons during the late afternoon/early evening, coincident with the LH surge and GnRH neuronal activation, whereas E_2-treated male mice fail to show notable CT-dependent changes in *Kiss1* mRNA levels, *Kiss1* neuronal activation, GnRH neuronal activation, or LH secretion. Furthermore, we report that female mice have a higher degree of ERα coexpression in *Kiss1* AVPV/PeN neurons than do males, suggesting that the male AVPV/PeN *Kiss1* system may be less sensitive to E_2 signals. This sex difference in ERα correlates with a higher degree of expression of PR, a key player in the LH surge mechanism, in the AVPV/PeN of females than males. Last, we also show that, although there are no overt circadian changes in *Rfrp* mRNA expression in either sex, *Rfrp* neuronal activation is significantly diminished in female mice in the early evening, coincident with the peak of the LH surge. Conversely, *Rfrp* neuronal activation remained unchanged in male mice between the morning and evening. Collectively, these findings provide insights into the long-standing question of why the brains of adult females, but not males, are capable of generating a GnRH/LH surge in response to elevated E_2 exposure.

The role of kisspeptin in stimulating the LH surge is unequivocal; several experiments have demonstrated that *Kiss1* and *Kiss1r* null mice are unable to produce an LH surge under multiple hormonal paradigms (39, 49). Given that normal male rodents are unable to produce an LH surge, even after suitable sex steroid priming in adulthood, and that there is a striking sex difference in the number of *Kiss1* cells in the AVPV/PeN (38), many investigators have assumed that male mice are unable to produce an LH surge simply because they have less kisspeptin in the AVPV/PeN than females. Although this sex difference in absolute kisspeptin levels is likely to be an important aspect of the LH surge sex difference, this assumption excludes the possibility of other sexually dimorphic factors also being involved, and overlooks the large potency of kisspeptin stimulation of GnRH that might be achieved even with only a few kisspeptin cells (in males). Our present findings establish that the AVPV/PeN kisspeptin system is also sexually dimorphic in its circadian-dependent changes in *Kiss1* expression levels and *Kiss1* neuronal activation, because male mice fail to show a noteworthy circadian increase in either *Kiss1* mRNA levels or *cfos-Kiss1* coexpression in the early evening as do females. Thus, these male kisspeptin cells are not only fewer in number but also fail to become "activated" in the early evening as do female kisspeptin

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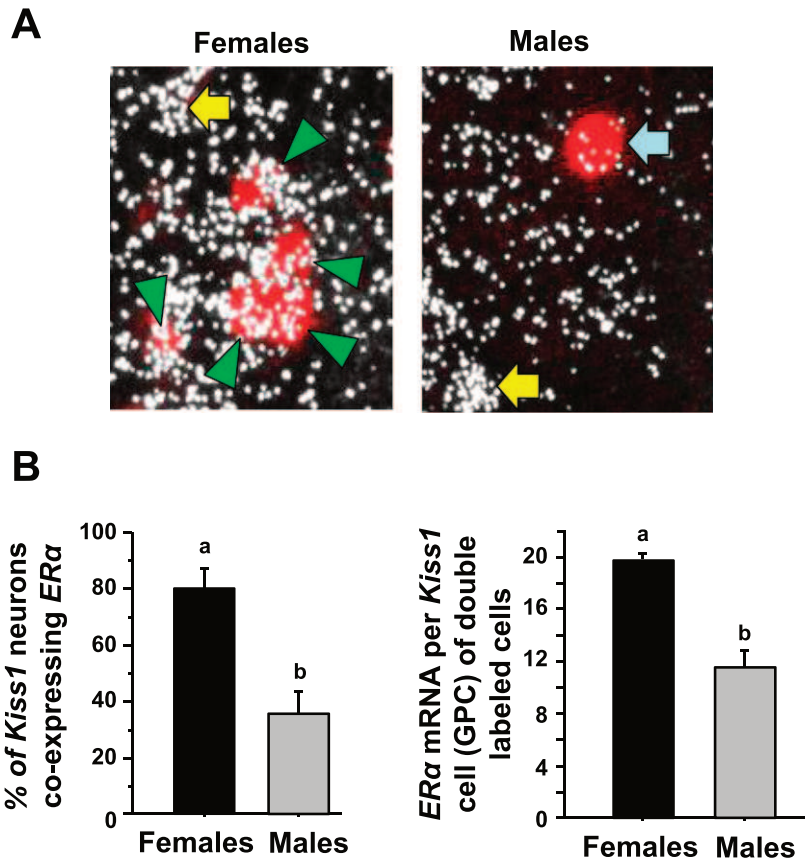

Figure 6. Double-label ISH for ERα coexpression in AVPV/PeN *Kiss1* neurons in male and female mice. (A) Representative photomicrographs of ERα mRNA colocalizing with AVPV/PeN *Kiss1* neurons in E_2-treated male and female mice at CT12. Green arrowheads, example *Kiss1* cells with ERα; blue arrow, example *Kiss1* cells lacking ERα expression; yellow arrow, ERα cell that is not a *Kiss1* cell. (B) Quantification of the percent coexpression of ERα in AVPV/PeN *Kiss1* neurons of E_2-treated males and females. Female mice have a considerably higher degree of *Kiss1* + *ERα* coexpression than do males. Different lowercase letters indicate significantly different groups ($P < 0.05$). GPC, grains per cell.

cells. Moreover, as noted later, sex steroid receptor levels are also higher in female AVPV/PeN kisspeptin cells than in males. Thus, several factors associated with the AVPV/PeN *Kiss1* system may be contributing to the sex difference in LH surge generation.

ERα necessity for LH surge generation is indisputable and is the primary estrogen receptor responsible for mediating the upregulation of *Kiss1* expression in the AVPV/PeN (50). Furthermore, mice with conditional deletion of ERα from *Kiss1* neurons fail to exhibit an LH surge (51). Previous reports have shown that 60% to 98% of female AVPV/PeN *Kiss1* neurons express ERα (49, 50, 52), depending on the species and method of detection. However, ERα levels in male AVPV neurons have not previously been measured with regard to the time of LH surge. Our present results show that E_2-treated male mice express ERα in their AVPV/PeN *Kiss1* cells, but to a lesser degree than E_2-treated females. Given ERα's essential role in inducing *Kiss1* expression and LH surge generation, the lower degree of ERα coexpression in male *Kiss1* neurons may explain, in part, why male AVPV/PeN *Kiss1* fail to show a large induction of *cfos* or a circadian upregulation of *Kiss1* mRNA. Furthermore, we also demonstrated a sex difference in PR levels in the AVPV, with these levels being lower in males than females. AVPV PR signaling is critical for LH surge generation (48, 53), and female mice lacking PR exclusively in *Kiss1* neurons fail to generate proper LH surges and show subfertility (42). Thus, the lower levels of AVPV PR observed in males may contribute to their lack of LH surge generation.

In both female mice and hamsters, arginine-vasopressin (AVP) fibers originating from the SCN innervate AVPV/PeN kisspeptin cell bodies (23, 24). Although this specific SCN-kisspeptin circuit has not yet been examined in male rodents, there are no reported sex differences in AVP fibers originating from the SCN (54–56); therefore, AVPV/PeN kisspeptin neurons of males may be innervated similarly as females. Female AVPV/PeN kisspeptin neurons express the AVP receptor, V1a, and increase their firing rate when stimulated with AVP, but again, similar experiments have not been reported for male AVPV/PeN kisspeptin cells (23, 24). Future studies addressing these issues in both sexes in detail will shed more light on potential sex differences in "upstream" circadian inputs into *Kiss1* neurons.

RFRP-3's known inhibitory effects on the reproductive axis led us to hypothesize that *Rfrp* expression would be lower during the LH surge, perhaps reflecting lessening of inhibition on GnRH secretion to permit a GnRH surge. Indeed, when given exogenously in rats, RFRP-3 can blunt the endogenous activation of GnRH neurons during E_2-induced LH surges (28). Moreover, in Syrian hamsters, the number of detectable RFRP-3 immunoreactive cell bodies is lower during the endogenous proestrus LH surge and increases again to baseline levels after the LH surge is over (37). However, in the current study, we found that E_2-treated female mice show no important CT-dependent changes in *Rfrp* mRNA expression before or during the LH surge, nor were there any CT-dependent changes in in *Rfrp* mRNA levels in similarly treated male mice. This suggests a possible species difference in the circadian control of *Rfrp*

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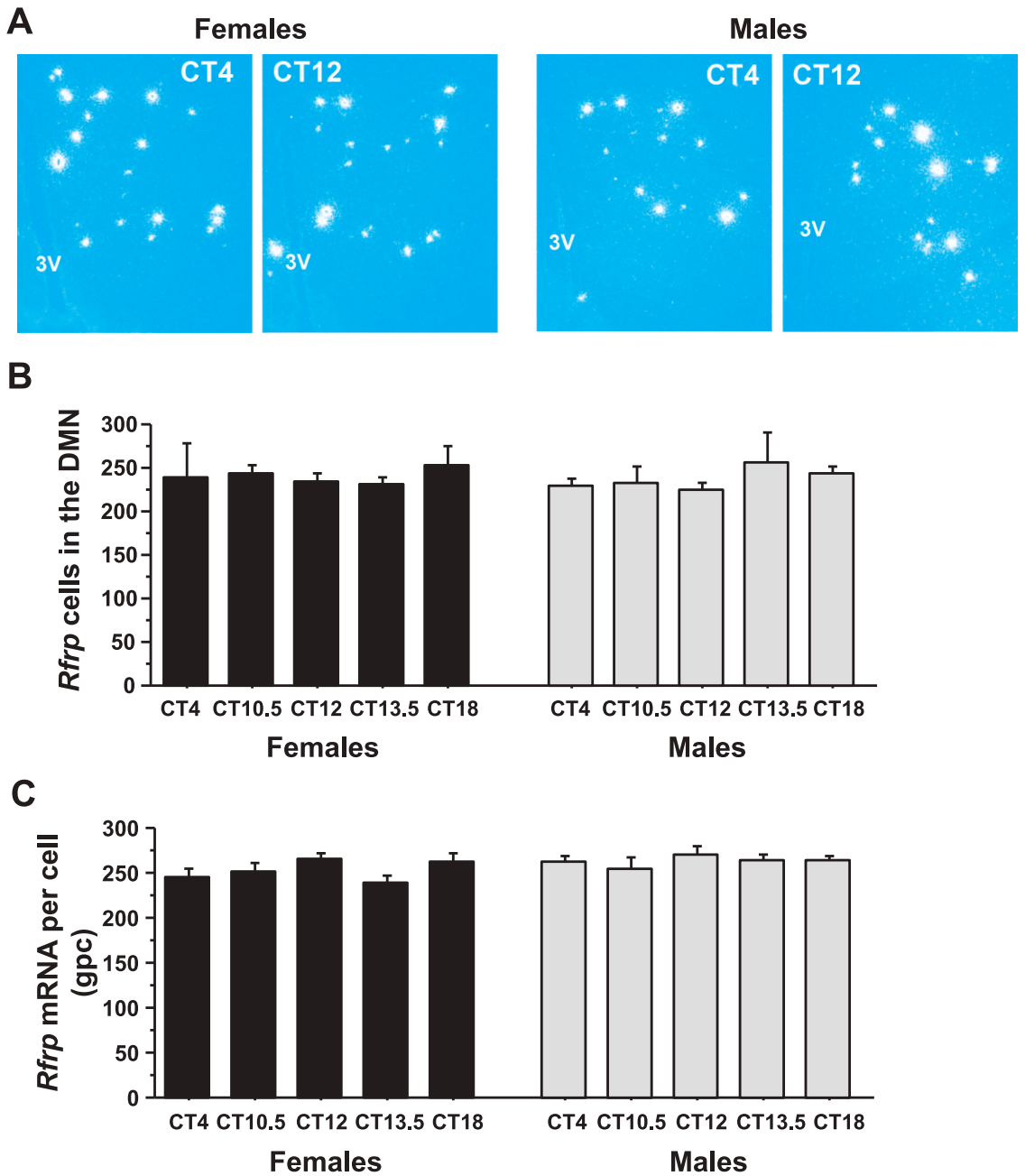

Figure 7. ISH for *Rfrp* mRNA in the DMN before and during the time of the LH surge. (A) Representative photomicrographs of *Rfrp* mRNA in the AVPV/PeN in E_2-treated males and females at two circadian times. (B) Quantification of the number of *Rfrp* neurons in the DMN of E_2-treated male and female mice at different circadian times. (C) Quantification of the relative *Rfrp* mRNA per cell in E_2-treated male and female mice across the circadian day. Different letters indicate significantly different groups ($P < 0.05$). 3V, third ventricle.

neurons. However, we did find that the activation status of *Rfrp* neurons, as measured by *cfos* coexpression, did show circadian changes in E_2-treated female mice, decreasing during the LH surge, similar to the temporal pattern previously seen in proestrus Syrian hamsters (37). Interestingly, both our study and the previous report in hamsters show that the onset of the LH surge precedes the maximal circadian decrease in *Rfrp* neuronal activation. It may be that RFRP-3 neurons are not involved in the initial triggering of the surge but rather play a role in modulating or timing the peak secretion of the surge or the overall duration of the surge. In contrast, kisspeptin neurons show simultaneous neuronal activation in synchrony with the LH surge onset, suggesting they play a key role in initial triggering of the LH surge. It is also possible that the observed circadian-timed decrease of *Rfrp* neuron activation is coincidental but unrelated to the LH surge mechanism, an issue that requires additional future studies to tease apart. Yet, as noted later, this CT-dependent decrease in *Rfrp* neuron activation is sexually dimorphic, perhaps linking it to the LH surge. Last, the observed CT-dependent changes in *Kiss1* gene expression

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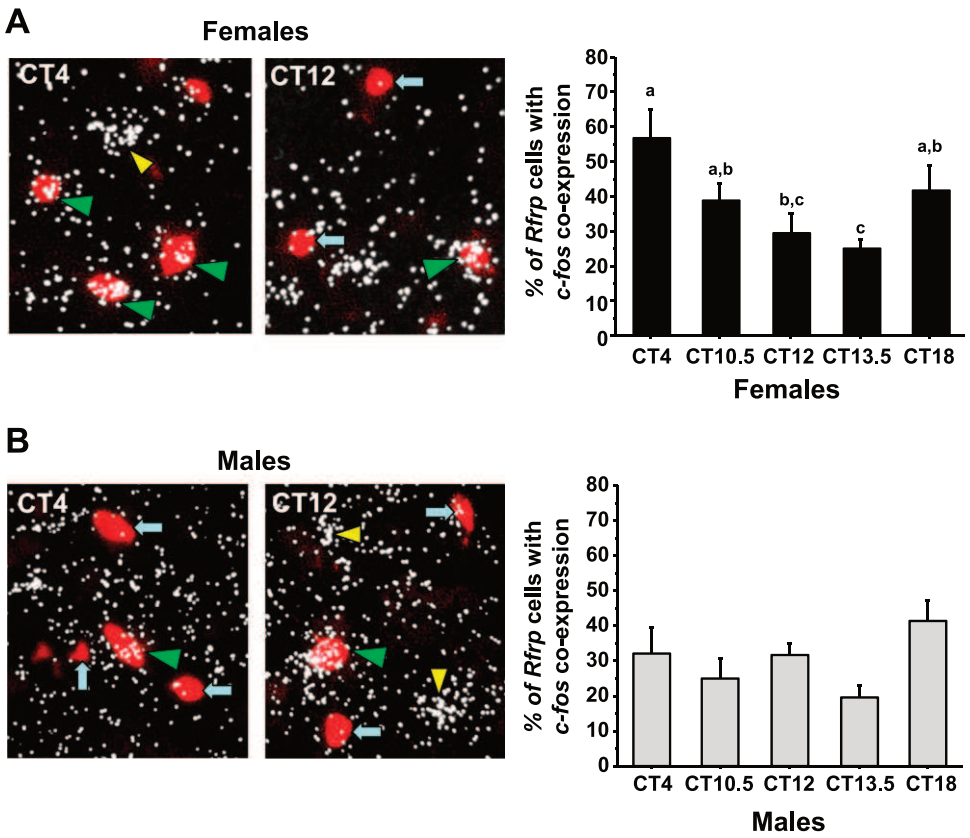

Figure 8. Double-label ISH for *cfos* colocalization in *Rfrp* neurons of mice during the time of the LH surge. (A) (Left) Representative photomicrographs of *cfos* (a marker of neuronal activation, silver grains) colocalizing with *Rfrp* neurons (red fluorescence) of E_2-treated females mice at two circadian times. Green arrowheads, example *Rfrp* cells with *cfos;* blue arrows, example *Rfrp* cells lacking *cfos* expression; yellow arrowhead, example *cfos*-expressing cell that is not an *Rfrp* cell. (Right) Quantification of *cfos* induction in female *Rfrp* neurons at various circadian times. Female mice euthanized at CT12 have notably less *cfos* colocalization in *Rfrp* neurons than females euthanized at CT4. Different lowercase letters indicate significantly different groups ($P < 0.05$). (B) (Left) Representative photomicrographs of *cfos* colocalizing with *Rfrp* neurons of E_2-treated male mice at two circadian times. See (A) for explanation of arrows and arrowheads. (Right) Quantitative analysis shows no major circadian differences in the degree of *Rfrp* neuronal activation in males.

in females may be compensatory mechanisms to replenish releasable kisspeptin peptide during and after the large surge release; in contrast, the absence of circadian changes in *Rfrp* levels may be because RFRP-3 release is being suppressed, rather than stimulated, and thus no compensatory mechanism is needed to replenish peptide stores.

We found that the circadian dampening of *Rfrp* neuron activation is sexually dimorphic because the *Rfrp* neurons of male mice, unlike females, showed no circadian changes in *cfos* coexpression. To date, this circadian change in *Rfrp* neuronal activation is the only known sex difference in *Rfrp* neurons because previous investigations have yielded no substantial effect of sex on Rfrp cell number, cell morphology, developmental pattern, or gene coexpression, including coexpression of ERα (11, 26, 32, 33). This *Rfrp* neuronal activation sex difference may be intrinsic to the *Rfrp* neuron itself, or *Rfrp* neurons may receive sexually dimorphic inputs from other brain regions, such as the SCN or interneurons linked to the SCN (57). The DMN is generally not considered a sexually dimorphic nucleus (56, 58), so an intrinsic sex difference in *Rfrp* neurons seems unlikely but remains possible. Further investigations will hopefully assess the presence or absence of sexually dimorphic inputs to *Rfrp* neurons.

In summary, the data presented here further our understanding of the sexually dimorphic nature of the circadian-timed LH surge. Although the sex difference in absolute AVPV/PeN kisspeptin levels has been assumed to be an important aspect of the LH surge sex difference, this assumption excludes the possibility of other sexually dimorphic factors also being involved. In fact, our present findings identify additional sexually dimorphic aspects of several reproductive neural populations involved in governing the LH surge. Unlike females, male mice, in addition to having fewer overall *Kiss1*-expressing neurons in the AVPV/PeN as previously shown, fail to show a circadian increase in *Kiss1* expression levels or *Kiss1* neuronal activation in response to E_2 priming at positive

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feedback levels. This may be due to our finding that males have less ERα coexpression in AVPV/PeN *Kiss1* neurons and less PR than do females. Moreover, although the amount of *Rfrp* mRNA produced in the DMN does not change before or during the LH surge in females or similarly treated males, the activation of *Rfrp* neurons is diminished during the peak of the LH surge in females, but not in males. Whether similar patterns, or lack thereof, occur with kisspeptin, RFRP-3, and sex steroid receptor protein levels was not determined and remains to be ascertained. Overall, our findings suggest that the inability of steroid-treated male rodents to demonstrate positive feedback induction of GnRH/LH secretion may reflect multiple neural parameters, including lower overall kisspeptin levels, absent circadian increases in *Kiss1* expression, minimal *Kiss1* neuron activation, lower levels of ERα and PR, and an absent circadian decrease in RFRP-3 neuron activation.

Acknowledgments

Financial Support: This research was supported by National Science Foundation Grants IOS-1025893 and IOS-1457226, National Institutes of Health (NIH) Grant R01 HD082567, and NIH Grants P50 HD012303 (University of California, San Diego) and P50 HD28934 (University of Virginia Ligand Assay Core).

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Disclosure Summary: The authors have nothing to disclose.

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