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The preovulatory GnRH/LH surge depends on the presence of estradiol (E2) and is gated by a circadian oscillator in the suprachiasmatic nucleus (SCN) that causes the surge to occur within a specific temporal window. Although the mechanisms by which the clock times the LH surge are unclear, evidence suggests that the SCN is linked to GnRH neurons through a multisynaptic pathway that includes neurons in the anteroventral periventricular nucleus (AVPV). Recently, Kiss1 neurons in the AVPV have been implicated in the surge mechanism, suggesting that they may integrate circadian and E2 signals to generate the LH surge. We tested whether Kiss1 neurons display circadian patterns of regulation in synchrony with the temporal pattern of LH secretion. Mice housed in 14 h light, 10 h dark were ovariectomized, given E2 capsules (or nothing), and transferred into constant darkness. Two days later, the mice were killed at various times of day and their LH and Kiss1 levels assessed. In E2-treated females, LH levels were low except during late subjective day (indicative of an LH surge). Similarly, AVPV Kiss1 expression and c-fos coexpression in Kiss1 neurons showed circadian patterns that peaked coincident with LH. These temporal changes in Kiss1 neurons occurred under steady-state E2 and constant environmental conditions, suggesting that Kiss1 neurons are regulated by circadian signals. In the absence of E2, animals displayed no circadian pattern in LH secretion or Kiss1 expression. Collectively, these findings suggest that the LH surge is controlled by AVPV Kiss1 neurons whose activity is gated by SCN signals in an E2-dependent manner. (Endocrinology 150: 3664–3671, 2009)

In female mammals, ovulation is induced by a surge of LH secretion from the pituitary, an event which is itself stimulated by a surge of GnRH secretion from neurons in the forebrain (1). It is well established that estradiol (E2) is critical for triggering the preovulatory GnRH/LH surge; in rodents, the surge normally occurs only on proestrus, as E2 levels are peaking, and females that are ovariectomized (OVX) do not display LH surges (2, 3). Although E2 is an absolute prerequisite for generating the LH surge, in rodents, the surge event is also gated by a circadian oscillator in the suprachiasmatic nucleus (SCN). This gatekeeper ensures that the LH surge is precisely timed to occur within a 2- to 4-h window, near the onset of darkness (and coincident with the initiation of locomotor activity in nocturnal animals). Intact female rodents display an LH surge in the late afternoon of proestrus but not at other times of that day, and OVX females treated with constant steady-state E2 display a late afternoon LH surge, which repeats daily at the same time (4). In addition, lighting paradigms that alter the natural period of the circadian clock, or phase-shift the clock’s temporal output, modify the timing of the LH surge (5–8); in all cases, the surge onset remains tightly coupled to the onset of daily locomotor activity, which is also timed by the SCN, implying that the timing of the surge and activity rhythms are both gated by the SCN. Finally, lesions of the SCN or genetic disruptions of the circadian molecular clockwork disrupt estrous cyclicity and prevent the E2-induced LH surge (9–15). Collectively, these observations argue that proper functioning of the SCN’s circadian clockwork is essential for the timing and generation of

Abbreviations: AVPV, Anteroventral periventricular nucleus; CT, circadian time; DIG, digoxigenin; E2, estradiol; ER, estrogen receptor-α; OVX, ovariectomized; RNase, ribonuclease; SCN, suprachiasmatic nucleus; SSC, saline sodium citrate.
the GnRH/LH surge in rodents, but precisely how this occurs is poorly understood.

There are two possible routes by which the SCN could regulate GnRH neurons and hence the LH surge. One pathway involves direct innervation of GnRH neurons by the SCN (16–18); however, anatomical and physiological evidence suggests that a second route is perhaps more critical (4). This second pathway involves an indirect circuit linking the SCN with GnRH neurons through a relay station in the anteroventral periventricular nucleus (AVPV), a hypothalamic region that is critical for the GnRH/LH surge in rodents (reviewed in Refs. 3, 19, and 20). Some neurons in the AVPV express estrogen receptor-α (ERα), the primary receptor implicated in mediating positive feedback effects of E2 on the LH surge (3); moreover, some ERα-expressing cells in the AVPV receive direct neural input from the SCN (16, 21). Thus, an E2-sensitive population of neurons within the AVPV could represent the cellular conduit linking the SCN to GnRH neurons, and recent evidence suggests that these particular AVPV cells may be Kiss1 neurons.

The Kiss1 gene encodes kisspeptin, a neuropeptide that plays a critical role in the neuroendocrine regulation of reproduction. In rodents, sheep, and primates (including humans), treatment with kisspeptin elicits a rapid increase in gonadotropin secretion, mediated by kisspeptin’s direct stimulation of GnRH neurons (reviewed in Refs. 19 and 22). Kiss1 neurons are located in several regions of the hypothalamus (23), but in rodents, the population of Kiss1 cells in the AVPV has been implicated as the critical component that drives the preovulatory GnRH/LH surge. First, virtually all Kiss1 neurons in the AVPV express ERα, and Kiss1 mRNA in this region is strongly up-regulated by E2 (24, 25). Second, Kiss1 expression in the AVPV of cycling female rats is induced, along with Fos in Kiss1 neurons, at the time of the preovulatory LH surge (26). Third, infusions of antiserum to kisspeptin prevent the LH surge in intact and E2-treated female rats (27, 28). Finally, the expression of Kiss1 in the AVPV is sexually differentiated, with females having significantly more Kiss1 neurons than males (29, 30), corresponding with the ability of females (but not males) to display an LH surge.

Although these observations provide compelling evidence that Kiss1 neurons in the AVPV participate in the E2-mediated induction of the LH surge, it remains ambiguous whether Kiss1 neurons also represent part of the circadian circuit linking the SCN to GnRH neurons. If Kiss1 neurons in the AVPV mediate the circadian effects of the clock in the SCN on the daily LH surge, then Kiss1 gene expression or transcriptional activity within Kiss1 cells may display a circadian pattern of regulation. To address this possibility, we evaluated the temporal patterns of Kiss1 expression and induction of the immediate-early gene c-fos (as a marker of neuronal activation) within Kiss1 neurons in the AVPV of female mice. To eliminate the possible effects of fluctuating environmental conditions, all experiments were performed under constant environmental conditions (constant darkness and temperature). In addition, because animals lacking E2 do not display LH surges, we reasoned that this might reflect the absence of E2-dependent circadian activation of Kiss1 cells in the AVPV. As an alternative, we supposed that Kiss1 neurons could well maintain their daily circadian activation, regardless of the presence or absence of E2, but could generate a preovulatory LH surge only when E2-induced kisspeptin production was sufficiently high enough to drive GnRH neurons (e.g. on proestrus). To distinguish between these possibilities, we evaluated whether Kiss1 neurons display circadian activation in both the presence and absence of E2 and propose a model of GnRH/LH surge generation via an E2-dependent circadian activation of Kiss1 neurons in the AVPV.

Materials and Methods

Animals

Adult (2 month old) C57BL/6 female mice purchased from Charles River Laboratories (Wilmington, MA) were individually housed with access to a running wheel in a 14-h light, 10-h dark cycle (lights off at 2100 h). Mice had ad libitum access to standard rodent chow and water throughout the study. Running wheel activity was continuously recorded for the duration of the study using Clocklab (Actimetrics, Wilmette, IL) and analyzed using El Temps software (http://www.el-temps.com/). All experiments were approved by the University of Washington Animal Care and Use Committee.

OVX and LH surge protocol

Ovaries were removed from isoflurane-anesthetized mice that were pretreated with Buprenex analgesic (1.5 μg, sc). Briefly, the anesthetized animal’s ventral surface was shaved and cleaned and the ovaries dissected out through midline incisions in the skin and abdominal musculature. After ovary removal, the muscle was sutured and the skin closed with sterile wound clips.

To induce a predictably timed daily LH surge, mice in experiments 1 and 2 were sc implanted at the time of OVX with a SILASTIC brand (Dow Corning, Midland, MI) capsule containing 0.625 μg E2 (in sesame oil, following the protocol of Ref. 31). This E2 treatment paradigm results in constant serum levels of E2 (~30 pg/ml) between 2 and 5 d after implantation and reliably induces daily afternoon LH surges (defined as ≥8-fold above baseline levels) beginning on d 2 (31, 32). All surgeries were performed in the morning during the first several hours after lights on.

Blood and tissue collection

At the time of killing (see specific experiments for details), mice were anesthetized with isoflurane and their blood collected via retroorbital bleeding. Blood was assayed for LH concentrations via a sensitive mouse LH RIA, performed by the University of Virginia Ligand Assay Lab as previously described (33, 34). After blood collection, animals were rapidly decapitated and their brains immediately collected and frozen on dry ice. Frozen brains were stored at −80 C until sectioning on a cryostat. Five sets of 20-μm sections were cut in the coronal plane, thaw-mounted onto Superfrost-plus slides, and stored at −80 C. One set was used for each in situ hybridization (ISH) assay.

Single-label ISH

Slide-mounted brain sections were processed for Kiss1 ISH, as previously described (23, 26, 30). Briefly, sections were fixed in 4% paraformaldehyde, rinsed in phosphate buffer, treated with acetic anhydride, rinsed in 2% saline sodium citrate (SSC), delipidated in chloroform, dehydrated in ethanol, and air dried. Antisense mouse Kiss1 probe, spanning bases 76–486 of the mouse cDNA sequence, was generated using 3P, as previously described (23, 24). The radiolabeled Kiss1 riboprobe was combined with 1/20 volume yeast tRNA (Roche Biochemicals, Indianapolis, IN) in TE (0.1 M Tris/0.01 M EDTA, pH 8.0), heat denatured, added to hybridization buffer at a ratio of 1:4, and applied to each slide (100 μl/slide; 0.03 pmol probe/ml). Slides were then coverslipped and
placed in a humidity chamber at 55°C for 16 h. After hybridization, the slides were washed in 4× SSC at room temperature and placed into ribonuclease (RNase) [37 mg/ml RNase A (Roche Biochemicals) in 0.15 M sodium chloride, 10 mM Tris, 1 mM EDTA, pH 8.0] for 30 min at 37°C and then into RNase buffer without RNase at 37°C for 30 min. Slides were then washed in 0.1× SSC at 62°C, dehydrated in ethanol, and air dried. Dry slides were dipped in Kodak NTB emulsion (VWR, West Chester, PA), air dried, and stored at 4°C for 10–12 d (depending on the assay), after which they were developed and coverslipped.

### Double-label ISH

For double-label ISH, slide-mounted brain sections were treated similarly to single-label ISH with the following modifications. Digoxigenin (DIG)-labeled antisense Kiss1 probe was synthesized along with radio-labeled c-fos riboprobe (labeled using 32P), and both riboprobes were dissolved in the same hybridization buffer along with tRNA and applied to slides for overnight hybridization. The radiolabeled c-fos riboprobe was generated by using 32P as described previously (35). After the 0.1× SSC washes on d 2, slides were incubated in 2× SSC with 0.05% Triton X-100 and 2% sheep serum for 1 h at room temperature and then washed in buffer 1 (100 mM Tris-HCl, pH 7.5, and 150 mM NaCl). Slides were then incubated overnight at room temperature with alkaline-conjugated anti-DIG antibody fragments (Roche Biochemical) that were diluted 1:35–400 in buffer 1 containing 1% sheep serum and 0.3% Triton X-100. The next day, slides were washed with buffer 1 and incubated with Vector Red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA) for 2 h. The slides were then air dried, dipped in NTB emulsion, stored at 4°C, and developed 10–13 d later (depending on the assay).

### Experiment 1: evaluation of the circadian regulation of Kiss1 mRNA in the AVPV of E2-treated animals

Kisspeptin signaling arising from the AVPV has been proposed to mediate the generation of the LH surge. Because the LH surge is regulated by the circadian clock, we hypothesized that Kiss1 neurons in the AVPV are themselves under circadian control. To test this hypothesis, we used single-label ISH to analyze levels of Kiss1 mRNA in female mice that were housed under constant lighting conditions and experiencing constant E2 exposure. After 13–20 d acclimation to a 14-h light, 10-h dark cycle, mice were OVX in the morning and implanted with E2-containing capsules (Roche Biochemical) that were diluted 1:35–400 in buffer 1 containing 1% sheep serum and 0.3% Triton X-100. The next day, slides were washed with buffer 1 and incubated with Vector Red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA) for 2 h. The slides were then air dried, dipped in NTB emulsion, stored at 4°C, and developed 10–13 d later (depending on the assay).

#### Materials and Methods

#### Experiment 2: circadian activation of Kiss1 neurons in the AVPV of E2-treated animals

Experiment 1 determined that there is a circadian pattern of Kiss1 mRNA and c-fos mRNA in the brains of E2-treated female mice housed in constant darkness. To address this question, we used double-label ISH to determine colocalization of Kiss1 mRNA and c-fos mRNA in the brains of animals from experiment 1. One set of brain tissue from E2-treated females that were housed in constant darkness was processed for double-label ISH and the percentage of AVPV Kiss1 neurons coexpressing c-fos was compared across the eight circadian time points.

#### Quantification of ISH assays and statistical analysis

Slides were analyzed with an automated image processing system by a person unaware of the treatment group of each slide. For single-label experiments, custom grain-counting software was used to unilaterally
count the number of radiolabeled cells and the number of silver grains in each cell (a semiquantitative index of mRNA content per cell) (23, 26) for all AVPV sections. Cells were considered Kiss1 positive when the number of silver grains in a cluster exceeded that of background by 3-fold. For double-label assays, DIG-containing cells (Kiss1 cells) were identified under fluorescence microscopy, and the grain-counting software was used to quantify silver grains (representing c-fos mRNA) over each cell. A cell was considered double labeled if it had a signal-to-background ratio of at least 3 (26, 30). For each animal, the amount of double labeling was calculated as a percentage of the total number of Kiss1 mRNA-expressing cells and then averaged across animals to produce a group mean.

All LH and ISH data are expressed as the mean ± SEM for each group. One-way ANOVAs were used to assess variation among experimental groups (time points) in each experiment, and differences in means were assessed by post hoc Fisher’s least significant difference tests (with Staview 5.0.1; SAS Institute, Cary, NC). Significance level was set at P < 0.05.

Results

Experiment 1: Kiss1 gene expression in the AVPV of E2-treated female mice is under circadian control

This experiment tested whether Kiss1 expression in the AVPV exhibits a circadian pattern under constant conditions and whether this pattern mirrors that of LH secretion. Female mice housed in constant darkness, coinciding with a circadian pattern of LH secretion, were used to assess variation among experimental groups (time points) in each experiment, and differences in means were assessed by post hoc Fisher’s least significant difference tests (with Staview 5.0.1; SAS Institute, Cary, NC). Significance level was set at P < 0.05.

Experiment 2: Kiss1 cells in the AVPV of E2-treated mice show a circadian pattern of neuronal activation

Experiment 1 identified a circadian pattern of Kiss1 expression in the brains of E2-treated female mice housed in constant darkness, coinciding with a circadian pattern of LH secretion. This experiment tested whether Kiss1 cells in E2-treated mice also undergo a circadian pattern of neuronal activation, as determined by c-fos induction in Kiss1 cells. We found that the expression of c-fos in Kiss1 neurons in the AVPV was low or nondetectable in the subjective morning and late subjective night, despite the presence of c-fos-expressing cells in other brain regions at these times (e.g. thalamus and paraventricular nucleus). In contrast, c-fos expression in Kiss1 neurons was prevalent in subjective late afternoon/early evening, coincident with the occurrence of LH surges at these times (see experiment 1) and indicative of a circadian regulation (Fig. 4). Quantitatively, the percentage of Kiss1 neurons expressing c-fos was no more than 10% at CT 0, CT 4, and CT 8, elevated to 40% at CT 11 and CT 12, and roughly 10% again at CT 20 (P < 0.01; Fig. 4). Similar to the LH data in these E2-treated females (experiment 1), the percentage of c-fos/Kiss1 coexpression was intermediate at CT 9 and CT 16 (Fig. 4), reflecting high coexpression in one animal in each of these groups (the same animals with high LH).

Experiment 3: Kiss1 neurons in the AVPV of OVX mice do not exhibit circadian patterns of regulation

Because OVX mice are incapable of displaying an LH surge, this experiment tested whether the circadian patterns of Kiss1 gene expression and neuronal activation observed in experiments 1 and 2 are dependent on the presence of E2. Unlike our findings in E2-treated animals, serum levels of LH in OVX mice lacking E2 and housed in constant darkness showed no evidence of a circadian pattern. Although the baseline concentrations of LH were higher in all OVX animals (reflecting absence of negative feedback after OVX), there was no obvious circadian pattern or significant change in LH levels across the 24-h cycle (P > 0.95; Fig. 5). Likewise, neither Kiss1 cell number in the AVPV nor Kiss1 mRNA/cell were different among any of the circadian time points (P > 0.90 for each; Fig. 6), nor was there any significant difference in the percentage of c-fos coexpression in Kiss1 neurons across the circadian day (P > 0.90; Fig. 6D). Collectively, these observations demonstrate that there is little or no
circadian pattern of activation of Kiss1 neurons in the AVPV of mice lacking E2 and housed in constant conditions.

Discussion

Since the pioneering work of Everett and Sawyer in 1950 (36–39), we have known that the LH surge (and subsequently, ovulation) is dependent not only on E2 but also on a neural signal that reflects the time of day, so that the LH surge occurs only within a narrow temporal window (typically late afternoon in nocturnal rodents). Despite the fact that this LH surge phenomenon has been extensively studied over the intervening years (4), the neuronal circuitry and molecular mechanisms that represent the point of convergence between the E2 and circadian signals have remained a mystery. In the present study, we show that a population of Kiss1 neurons in the AVPV, previously implicated in the LH surge mechanism, is under circadian regulation. Moreover, we show that the ability of Kiss1 neurons to exhibit circadian regulation of both gene expression and neuronal activation is contingent on the presence of E2, perhaps explaining why an LH surge is absent in females with low or absent E2. Collectively, our findings suggest that Kiss1 neurons in the AVPV could receive and integrate both hormonal and temporal cues to generate and time the LH surge.

Considerable evidence suggests that the precisely timed pattern of LH release in female rodents reflects the interaction of E2 with an obligatory signal that emanates from the circadian clock in the SCN. First, intact female rodents normally display an LH surge only in the late afternoon of proestrus; however, barbiturate treatment during a critical window (2–3 h in the late afternoon) not only blocks the LH surge (and ovulation) but also delays its next appearance by exactly 24 h (36–39). Second, in OVX rodents, treatment with constant high levels of E2 elicits a daily LH surge, which occurs at the exact same time every day (40–42). However, females lacking a functional SCN (achieved by creating discrete lesions of SCN or genetic disruptions of the molecular clockwork in the SCN) are incapable of displaying an LH surge (at any time) in response to an E2 challenge (9, 11, 14, 15, 43). This establishes the absolute requirement of circadian signaling from the SCN to produce an LH surge. Finally, experimental paradigms that modify the circadian clock’s period or phase shift the clock’s temporal output correspondingly alter the timing of the LH surge in rodents. However, in all such cases, the onset of the LH surge remains tightly coupled to the onset of daily locomotor rhythm (5–8), indicating that the temporal gating of the surge and behavioral rhythms share a common circadian pacemaker, presumably the SCN. Although these findings argue persuasively that the SCN regulates the LH surge, the neural mechanisms by which this occurs are poorly understood. Because the AVPV plays a key role in generating the LH surge (reviewed in Refs. 3, 19, and 44), it seems plausible that the SCN signals to GnRH neurons through intermediaries in the AVPV. In support of this, tract tracing experiments demonstrate that SCN neurons innervate a subset of AVPV neurons (16) and these specific AVPV neurons also express ERα, which mediates the E2-induced LH surge.

The phenotypic identity of the E2-responsive neurons in the AVPV that are targets for SCN-derived projections is unknown. However, our results suggest that these neurons are Kiss1 neurons. In E2-treated female mice, we found that Kiss1 gene expression in the AVPV varied across the circadian day. The ex-
pression of Kiss1 increased (by ~25%) during the subjective late afternoon and early evening and then diminished in late subjective night. Because the animals were housed in constant darkness, these changes in Kiss1 gene expression were unlikely to have been produced by an hourglass mechanism that might be initiated each day through a daily resetting of a light-dark cycle. Furthermore, because all animals in all time points had the same constant E2 treatment, it is unlikely that these temporal changes in Kiss1 expression were caused by group differences or temporal changes in circulating levels of E2. These data indicate that Kiss1 gene expression is regulated by both sex steroid signals (up-regulated Kiss1 expression compared with OVX levels, as previously determined) (24, 30) and circadian signals (up-regulated Kiss1 expression at specific times) (present study). Moreover, transcriptional activation of Kiss1 neurons, as measured by neuronal c-fos induction, also exhibited a strong circadian pattern in constant conditions (in the presence of E2). Thus, in addition to a timed increase in Kiss1 gene transcription, Kiss1 neurons are also temporally induced by afferent circadian signals to increase the expression of other genes (or perhaps alter neuronal firing). Presumably, this temporal activation of Kiss1 neurons is related to a timed stimulation of GnRH neurons, resulting in the circadian-gated LH surge. Whether the observed circadian pattern of Kiss1 gene expression and Kiss1 neuronal activation are causally related is unknown, but it is conceivable that the pattern of increased Kiss1 gene expression reflects a replenishing of Kiss1 kisspeptin stores after the neuron has fired to release kisspeptin (denoted by high neuronal c-fos induction).

FIG. 4. A, Representative photomicrographs of Kiss1 mRNA and c-fos mRNA coexpression in the AVPV of OVX, E2-treated female mice housed in constant conditions and killed at one of eight times throughout the circadian day. Kiss1-containing neurons were visualized with Vector Red substrate, and c-fos mRNA was marked by the presence of silver grains. White arrows denote example Kiss1 cells lacking c-fos; yellow arrows denote example Kiss1 cells coexpressing c-fos. B, Mean (±SEM) percentage of Kiss1 mRNA-containing neurons in the AVPV that coexpress c-fos in OVX, E2-treated female mice killed at one of eight times throughout the circadian day. There was a significant effect of time (P < 0.01) with increased coexpression of Kiss1 and c-fos in the late afternoon/early evening. Values with different letters differ significantly from each other. n = 4–6 animals per group.

FIG. 5. Mean (±SEM) plasma LH in OVX mice housed in constant conditions and killed at one of eight times throughout the circadian day. There was no significant difference in LH levels between any of the time points. n = 4–5 animals per group.

FIG. 6. Lack of a circadian pattern in Kiss1 gene expression in OVX females. A, Representative dark-field photomicrographs showing Kiss1 mRNA-expressing cells in the AVPV of OVX mice housed in constant conditions and killed at different time points throughout the circadian day. B, Mean (±SEM) number of Kiss1 mRNA-expressing neurons in the AVPV of OVX female mice housed in constant conditions and killed at one of eight time points throughout the circadian day. C, Mean (±SEM) number of silver grains per Kiss1 cell in the AVPV of OVX mice killed across the circadian day. D, Mean (±SEM) percentage of Kiss1 mRNA-containing neurons in the AVPV that coexpress c-fos in OVX female mice. In A–C, there was no significant difference in the measures between any of the circadian time points (P > 0.05 for all measures). n = 3–5 animals per group.
gesting that a multisynaptic neuronal circuit links the SCN with GnRH neurons through the AVPV. Although neuroanatomical evidence documenting a direct SCN-Kiss1 circuit has yet to be reported, preliminary observations in mice suggest a direct, monosynaptic innervation of Kiss1 neurons in the AVPV by vasopressin signaling arising from the SCN (49). These investigations also implicate vasopressin as the key neurotransmitter up-regulating Kiss1 neurons, a conjecture supported by findings that vasopressin can stimulate GnRH/LH secretion in animals lacking a functional SCN (50, 51). It is also worth noting that Kiss1 cells themselves may be circadian oscillators and that the observed temporal changes in Kiss1 activation could be generated intrinsically by intracellular clocks. This possibility derives support from the fact that many non-SCN cells throughout the body (e.g. GnRH or liver cells) express clock genes and in some cases exhibit endogenous circadian rhythmicity in vitro, independent of SCN input (52, 53). However, given the strong evidence that SCN signaling is critical for both the generation and timing of the LH surge, and the fact that the SCN projects to the AVPV (and likely Kiss1 cells), the most parsimonious explanation is that the circadian pattern of Kiss1 neurons is controlled by the SCN.

Animals lacking E2 cannot display an LH surge. The inability to surge in the absence of E2 could reflect the absence of circadian input to the surge-generating circuitry and/or some other missing E2-dependent aspect of the surge-generating mechanism. Here, we show that the circadian pattern of Kiss1 regulation observed in E2-treated mice was completely absent in OVX animals. Thus, despite a lower, yet significant, number of detectable Kiss1 neurons in the OVX condition, these Kiss1 cells were not temporally activated in the absence of E2. This finding argues against a model in which the SCN activates Kiss1 neurons every day but generates an LH surge only when there is sufficient Kiss1 mRNA and kisspeptin release to drive a GnRH surge. Instead, our results imply that either 1) the SCN does not automatically signal to Kiss1 neurons every day but, instead, does so only in the presence of E2 or 2) the SCN sends temporal cues to Kiss1 neurons every day, but the ability of Kiss1 neurons to receive/decode the circadian signal is E2 dependent. Both the SCN and Kiss1 neurons express ERs (24, 54, 55), rendering either of these models plausible. It should also be noted that E2 may also act upstream of the surge-generating circuitry and/or some other missing E2-dependent aspect of the surge-generating mechanism. Here, we show that the circadian pattern of Kiss1 regulation observed in E2-treated mice was completely absent in OVX animals. Thus, despite a lower, yet significant, number of detectable Kiss1 neurons in the OVX condition, these Kiss1 cells were not temporally activated in the absence of E2. This finding argues against a model in which the SCN activates Kiss1 neurons every day but generates an LH surge only when there is sufficient Kiss1 mRNA and kisspeptin release to drive a GnRH surge. Instead, our results imply that either 1) the SCN does not automatically signal to Kiss1 neurons every day but, instead, does so only in the presence of E2 or 2) the SCN sends temporal cues to Kiss1 neurons every day, but the ability of Kiss1 neurons to receive/decode the circadian signal is E2 dependent. Both the SCN and Kiss1 neurons express ERs (24, 54, 55), rendering either of these models plausible. It should also be noted that E2 may also act upstream of the SCN at other E2-responsive neurons that project to the clock (56). Regardless of mechanism, any of these E2-dependent models would result in Kiss1 neurons being up-regulated by circadian input only when there is E2 present, thus explaining why the LH surge occurs only during proestrus or conditions of elevated E2. Notably, these models indicate that E2 has a dual role for inducing the LH surge: 1) to stimulate Kiss1 gene expression, presumably to generate abundant kisspeptin, regardless of time of day (i.e. fewer AVPV Kiss1 neurons in OVX than E2-treated animals) and 2) to permit the SCN to activate Kiss1 neurons, thereby triggering them to signal to GnRH neurons. Although complementary and synergistic in their effects on the hypothalamo-pituitary-gonadal axis, these two actions of E2 on the Kiss1 system may involve independent processes.

In conclusion, we report a significant circadian regulation of Kiss1 gene expression and transcriptional activation of Kiss1 neurons in the AVPV of female mice housed in constant conditions and exposed to steady-state E2. These results implicate Kiss1 neurons in the AVPV as critical integrators of both sex steroids and circadian signals and suggest that the timing of the LH surge involves temporal activation of Kiss1 neurons. We also determined that this circadian pattern of Kiss1 neuron regulation is E2 dependent, implying that the SCN is incapable of activating Kiss1 neurons in the absence of the molecular/cellular effects of E2.

Acknowledgments

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