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Analysis of Multiple Positive Feedback Paradigms Demonstrates a Complete Absence of LH Surges and GnRH Activation in Mice Lacking Kisspeptin Signaling¹

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ABSTRACT

Kisspeptin stimulates gonadotropin-releasing hormone (GnRH) neurons via the kisspeptin receptor, *Kiss1r*. In rodents, estrogen-responsive kisspeptin neurons in the rostral hypothalamus have been postulated to mediate estrogen-induced positive feedback induction of the preovulatory luteinizing hormone (LH) surge. However, conflicting evidence exists regarding the ability of mice lacking *Kiss1r* to display LH surges in response to exogenous hormones. Whether the discrepancy reflects different mouse strains used and/or utilization of different surge-induction paradigms is unknown. Here, we tested multiple hormonal paradigms in one *Kiss1r* knockout (KO) model to see which paradigms, if any, could generate circadian-timed LH surges. *Kiss1r* KO and wild-type (WT) females were ovariectomized, given sex steroids in various modes, and assessed several days later for LH levels in the morning or evening (when surges occur). Serum LH levels were very low in all morning animals, regardless of genotype or hormonal paradigm. In each paradigm, virtually all WT females displayed clear LH surges in the evening, whereas none of the KO females demonstrated LH surges. The lack of LH surges in KO mice reflects a lack of GnRH secretion rather than diminished pituitary responsiveness from a lifetime lack of GnRH exposure because KO mice responded to GnRH priming with robust LH secretion. Moreover, high *cfos*-GnRH coexpression was detected in WT females in the evening, whereas low *cfos*-GnRH coexpression was present in KO females at all time points. Our findings conclusively demonstrate that WT females consistently display LH surges under multiple hormonal paradigms, whereas *Kiss1r* KO mice do not, indicating that kisspeptin-*Kiss1r* signaling is mandatory for GnRH/LH surge induction.

estradiol, GnRH, GPR54, *Kiss1*, *Kiss1r*, kisspeptin, LH surge, positive feedback

INTRODUCTION

In mammals, the preovulatory luteinizing hormone (LH) surge, which triggers ovulation, is governed by neurons in the forebrain and preoptic area that release gonadotropin-releasing

hormone (GnRH). Throughout the estrous cycle, low levels of ovarian-derived estradiol (E_2) inhibit GnRH secretion via negative feedback until proestrus, when increasing levels of E_2 exert positive feedback on hypothalamic neural circuits to induce a preovulatory GnRH/LH surge [1, 2]. In rodents, the E_2 -induced LH surge is timed by a circadian clock such that the surge occurs exclusively in the late afternoon/early evening of proestrus, around the time of lights off [3, 4]. The stimulatory effects of E_2 on GnRH and LH secretion have been shown to be dependent on estrogen receptor α ($ER\alpha$), as female mice with neuronal deletions in $ER\alpha$ but not $ER\beta$ fail to exhibit E_2 -induced LH surges [5]. However, these $ER\alpha$ -mediated effects on GnRH do not occur directly in GnRH neurons since these cells lack $ER\alpha$ [6, 7]. In addition, progesterone and its receptor (PR) have also been shown to be important contributors to the LH surge, as PR KO mice are unable to produce an E_2 -induced LH surge [8, 9].

The neuropeptide kisspeptin, encoded by the *Kiss1* gene, and its receptor, *Kiss1r* (formerly termed GPR54), are critical for puberty and fertility in mammals [10, 11]. Kisspeptin potently stimulates GnRH secretion and hence LH secretion via direct binding of *Kiss1r* in GnRH cells [12, 13]. Two separate populations of kisspeptin-synthesizing neurons have been characterized in the mammalian hypothalamus, one in the arcuate nucleus (ARC; infundibular nucleus in primates) and one more rostrally in the preoptic region. In rodents, the preoptic kisspeptin population lies specifically in the continuum comprising the anterior ventral periventricular nucleus and neighboring periventricular nucleus (AVPV/PeN) [14–16]. Sex steroids, such as E_2 , suppress the levels of *Kiss1* mRNA in the ARC but robustly elevate *Kiss1* expression in the AVPV/PeN [16–18]. Since kisspeptin can directly stimulate GnRH neuron electrical activity and GnRH secretion [19, 20] and virtually all *Kiss1* neurons in the AVPV/PeN express $ER\alpha$ [18], it is believed that E_2 's positive feedback effects on GnRH/LH secretion are mediated by AVPV/PeN *Kiss1* neurons [21]. Indeed, on the afternoon of proestrus in rats, there is an increase in the number of *cfos*-expressing *Kiss1* neurons in the AVPV/PeN that coincides with *cfos* induction in GnRH neurons [17, 22]. Similarly, *cfos* is induced in *Kiss1* neurons in the AVPV/PeN of mice exclusively at the time of the LH surge but not at other times [23]. Thus, *Kiss1* neuronal activation prior to or at the time of the LH surge may play a critical role in driving the surge. Supporting this, kisspeptin neurons in the AVPV/PeN are sexually dimorphic in cell number and *Kiss1* expression (both greater in females than males), correlating with the sexually dimorphic nature of the LH surge in rodents (occurs only in females) [16, 24].

Conflicting evidence currently exists concerning the vitality of kisspeptin-*Kiss1r* signaling in the initiation of an E_2 -induced LH surge. Dungan et al. [25] surprisingly reported that *Kiss1r* is not essential for generating an E_2 -induced LH surge, with adult *Kiss1r* KO females capable of displaying LH surges in response to exogenous E_2 treatment. However, in stark contrast, using both a different *Kiss1r* KO mouse model and

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a different E_2 paradigm for inducing LH surges, Clarkson et al. [26] reported that kisspeptin-Kiss1r signaling is necessary for GnRH neuronal activation and the LH surge. These latter results were more consistent with the presumption that E_2 exerts its positive feedback effects on GnRH/LH secretion via ER α in AVPV/PeN Kiss1 neurons. The contradictory findings might be attributed to differences in the transgenic mouse lines and/or the estrogen paradigms employed by the two groups, though this issue has not been addressed, leaving the issue unresolved. The controversy was heightened by a more recent finding that selective ablation of kisspeptin cells in transgenic mice did not prevent females from sexually maturing or being fertile in adulthood [27].

In order to further define the roles of kisspeptin-Kiss1r signaling in the preovulatory LH surge, we sought to resolve the controversy regarding the ability of Kiss1r KO mice to exhibit LH surges. Using the Kiss1r KO mouse strain that was reported to display LH surges, our overall goal was 2-fold: 1) to determine whether Kiss1r KO mice can in fact display GnRH neuronal activation and an LH surge under various surge-inducing paradigms, including the different paradigms used by each of the previous two studies as well as additional paradigms not previously tested in Kiss1r KO mice and 2) if Kiss1r KO females do in fact surge, begin to identify novel neural LH surge circuits underlying this kisspeptin-independent process. Indeed, several other brain factors have been suggested to perhaps play a role in the LH surge mechanism [3, 28, 29], but whether they are sufficient to drive the surge without kisspeptin signaling is unclear. Despite this latter initial goal, our cumulative results conclusively indicate that Kiss1r is in fact essential for producing an LH surge under each and every hormonal paradigm examined.

MATERIALS AND METHODS

Animals

Experiments were conducted on adult (10–12 wk old) female Kiss1r KO and wild-type (WT) littermates generated from Kiss1r heterozygous breeders from a Kiss1r KO mouse line originally created by Omeros, Inc. (Seattle, WA), and kindly provided to us by Dr. Robert Steiner (University of Washington, Seattle, WA). This Kiss1r KO mouse line was previously shown by us to be completely hypogonadal and infertile and to have undetectable reproductive hormone levels [25, 30, 31]. All females were genotyped after weaning via PCR analysis of tail DNA. Mice were provided food and water ad libitum and housed in groups of two to three under a 12L:12D cycle (lights off at 1800 h). All experiments were performed in agreement with the National Institutes of Health Animal Care and Use Guidelines and with authorization from the Animal Care and Use Committee of the University of California, San Diego. For all surgeries, mice received Buprenex analgesic (1.5 μ g, s.c.) following surgery.

Blood and Brain Collection and Hormone Assays

At specific circadian times (see below), mice were anesthetized with isoflurane and blood drawn via retro-orbital bleeding just prior to killing via rapid decapitation. Serum was isolated from the blood samples and stored at -20°C . Serum LH levels were measured by the University of Virginia's Ligand Assay Core (Charlottesville, VA) by a sensitive sandwich RIA assay. Specifically, serum LH is measured by a sensitive two-site sandwich immunoassay using monoclonal antibodies against bovine LH (no. 581B7) and the human LH-beta subunit (no. 5303), as described previously [32–34]. Mouse LH reference prep (AFP5306A; provided by Dr. A.F. Parlow and the National Hormone and Peptide Program) is used as standard. The limit of detectability for the mouse LH assay is 0.04 ng/ml. Circulating serum E_2 levels were also measured by University of Virginia's Ligand Assay Core, using Calbiotech ELISA. This mouse E_2 assay has a sensitivity of 3 pg/ml. For animals in experiment 1, brains were collected at killing and immediately frozen on dry ice before being stored at -80°C . Frozen brains were sectioned on a cryostat into five sets of 20- μ m sections and thaw-mounted on Superfrost-plus slides that were stored at -80°C until assaying via in situ hybridization.

Double-Label In Situ Hybridization

For double-label in situ hybridization (ISH) of *cfos* in GnRH neurons (experiment 5), slide-mounted brain sections encompassing the entire forebrain and preoptic area were fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2 \times SSC (sodium citrate, sodium chloride), delipidated in chloroform, dehydrated in ethanol (70%, 95%, 100%), and air-dried. Radio-labeled (^{33}P) antisense *cfos* (0.04 pmol/ml) and digoxigenin-labeled *Gnrh* (1:500) riboprobes were combined with tRNA, denatured, dissolved together in hybridization buffer, and applied to each slide (100 μ l/slide). Slides were coverslipped and placed in a humidity chamber at 55 $^\circ\text{C}$ for 16 h. Following hybridization, slides were washed in 4 \times SSC and then placed into RNase (37 mg/ml RNase A in 0.15 M sodium chloride, 10 mM Tris, 1 mM EDTA, pH 8.0) for 30 min at 37 $^\circ\text{C}$, then in RNase buffer without RNase at 37 $^\circ\text{C}$. After a wash in 2 \times SSC at room temperature, slides were washed in 0.1 \times SSC at 62 $^\circ\text{C}$ for 1 h and then incubated in 2 \times SSC with 0.05% Triton X-100 containing 2% sheep serum (NSS) for 1 h at room temperature. The slides were then washed in buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl) and incubated overnight at room temperature with anti-DIG antibody conjugated to alkaline phosphatase (Roche) diluted 1:500 in buffer 1 containing 1% NSS and 0.3% Triton X-100. The following day, slides were washed with buffer 1 and incubated with Vector Red alkaline phosphatase substrate (Vector Labs, Burlingame, CA) for 1 h at room temperature. Slides were then dried, dipped in emulsion, stored at 4 $^\circ\text{C}$, and developed and coverslipped 9 days later.

ISH slides were analyzed with an automated grains imaging processing system (Dr. Don Clifton, University of Washington) by a person unaware of the treatment group of each slide. Red fluorescent DIG-containing (GnRH) cells were identified under fluorescence microscopy and the grain-counting software used to quantify the number of silver grains (*cfos* mRNA) overlying each cell. Signal-to-background ratios for individual cells were calculated by the program, and a cell was considered double labeled if its ratio was >3 .

Hormonal Paradigm 1: LH Surge Induction by Constant Elevated Estradiol

Mice were anesthetized with isoflurane and then ovariectomized (OVX) and given E_2 implants. Silastic brand (Dow Corning, Midland, MI; inner diameter = 0.20 cm, outer diameter = 0.318 cm) capsules containing 0.625 μ g of 17- β E_2 dissolved in sesame oil were subcutaneously implanted. This hormone paradigm typically produces constantly elevated serum E_2 levels of ~ 20 –30 pg/ml, resembling mouse proestrus levels [35]. Under this hormonal milieu, female mice will normally produce a daily circadian-timed LH surge, occurring each evening exclusively around the time of lights off (1800 h) [23, 35]. Adult WT and Kiss1r KO females treated with this E_2 regimen were killed 2 days after surgery either in the morning (between 1000 and 1100 h) or in the evening just after lights off (1810–1830 h), and their blood was collected for LH and E_2 analysis ($n = 7$ –8/group).

Hormonal Paradigm 2: LH Surge Induction by Rising Estradiol Levels

Mice were anesthetized with isoflurane and OVX and subcutaneously given low-dose E_2 implants (Silastic tubing; inner diameter 0.10 cm, outer diameter 0.21 cm) containing crystalline 17- β E_2 dissolved in Silastic medical adhesive at 1 μ g/20 g body weight (BW) [26, 36, 37]. Five days after this low-estrogen treatment, mice were subcutaneously injected in the morning with 1 μ g/20 g BW estradiol benzoate (EB) in sesame oil (100 μ l) to produce elevated proestrus-like E_2 levels on the following day [36, 37]. This hormone paradigm produces low diestrus levels of serum E_2 for several days and then increases serum E_2 to proestrus levels over the last 2 days, mirroring the natural cyclical rise in E_2 during a female rodent's typical estrous cycle. Under this hormonal regimen, female mice will normally produce a robust circadian-timed LH surge on the day after EB injection, occurring around the time of lights off [36, 37]. Adult WT and Kiss1r KO females treated with this E_2 +EB paradigm were killed the day after the EB injection either in the morning (between 1000 and 1100 h) or in the evening just after lights off (1810–1830 h), and their blood was collected for LH and E_2 analyses ($n = 7$ –10/group).

Hormonal Paradigm 3: LH Surge Induction by a Combination of Estradiol and Progesterone

Anesthetized mice were OVX and subcutaneously given low-dose E_2 implants (Silastic tubing; inner diameter 0.04 inch, outer diameter 0.85 inch) containing crystalline 17- β E_2 dissolved in Silastic medical adhesive at 1 μ g/20 g BW, as with the previous hormonal paradigm above. Five days after this low-

estradiol treatment, mice were subcutaneously injected in the morning with 1 µg/20 g BW EB in sesame oil (100 µl). On the next day, mice were injected subcutaneously with progesterone (P) dissolved in sesame oil and given at a dose of 300 µg/20 g BW [36, 37]. This hormone paradigm produces low diestrous levels of serum E₂ for several days, followed by elevated proestrus-like levels of E₂ and elevated serum P, resembling a female rodent's typical estrous cycle. As with the other hormonal regimens, female mice treated with this E₂+P paradigm will normally produce a circadian-timed LH surge on the day of the P injection, occurring in the evening around the time of lights off. Adult WT and *Kiss1r* KO females treated with this E₂+P paradigm were killed the day of the P injection, either in the morning (between 1130 and 1200 h) or in the evening just after lights off (1810–1830 h), and their blood was collected for LH analysis (n = 7–8/group).

Hormonal Paradigm 4: GnRH Priming Prior to LH Surge Induction by Elevated E₂

This regimen combined GnRH priming with the hormonal paradigm 1 above. In *Kiss1r* KO mice, an inability of females to produce an LH surge might be attributed to a chronic lack of gonadotrope exposure (and hence responsiveness of the pituitary) to GnRH. Thus, adults were first primed with exogenous GnRH to stimulate the pituitary. Mice received a single daily subcutaneous GnRH injection (200 ng) every other day for a total of three injections. On the last day of priming, blood was drawn 15 min after the GnRH injection to assess pituitary's ability to secrete LH in response to GnRH. Two days later, all mice were OVX and implanted subcutaneously with a Silastic E₂ implant producing elevated proestrus levels of E₂, as in hormonal paradigm 1. Two days later, blood was collected prior to killing at either 1100 or 1830 h, and the serum was analyzed for LH levels (n = 7–11/group).

LH Surge Criteria

There is no universally accepted criterion to define an LH surge; in experimentally E₂-treated female mice, we typically define an LH surge as being at least 0.60 ng/ml or greater (most LH surges are above 1.00 ng/ml). However, for the present study, wherever possible, we used the criterion delineated by Dungan et al. [25] in their *Kiss1r* KO paper, so that our data could better be compared with theirs. That study used a value of the mean LH for all morning mice plus two times the SD as a threshold for identifying an LH surge. This criterion is experiment specific (since morning values may differ between experiments); in their own experiment, it equated to an LH surge being greater than 0.53 ng/ml. Of note, in experiment 1 of the present study, all morning mice had identical LH values (all below the limit of detection), and, as such, no SD could be calculated. Thus, for experiment 1, we used our typical LH surge criteria of 0.60 ng/ml. For experiments 2, 3, and 4, the Dungan et al. [25] criteria could also be used and were 0.45, 0.53, and 0.16 ng/ml, respectively. Regardless, we note that in all these experiments, the number of mice classified as displaying an LH surge was identical using either of the two criteria (0.60 ng/ml or morning mean + 2 SD).

Statistical Analyses

All data are expressed as the mean ± SEM for each group. In all experiments, differences were analyzed by analysis of variance, followed by post hoc comparisons via Fisher (protected) LSD test. For all comparisons, statistical significance was set at $P < 0.05$. All analyses were performed in Statview 5.0.1 (SAS Institute, Cary, NC).

RESULTS

Experiment 1: LH Surge Induction by Constant Elevated E₂ in *Kiss1r* KO and WT Mice

A previous report [25] suggested that *Kiss1r* KO females (same strain as our mice) could display LH surges in response to constant elevated proestrus levels of E₂ (hormonal paradigm 1). This experiment tested if, in fact, adult *Kiss1r* KO female mice were capable of displaying a circadian-timed LH surge in response to elevated levels of E₂. After treatment with hormonal paradigm 1, all WT females displayed robust LH surges at the evening time point (range: 0.60–9.10 ng/ml), whereas LH levels were extremely low in WT females in the morning (~0.08 ng/ml; Fig. 1), consistent with a circadian

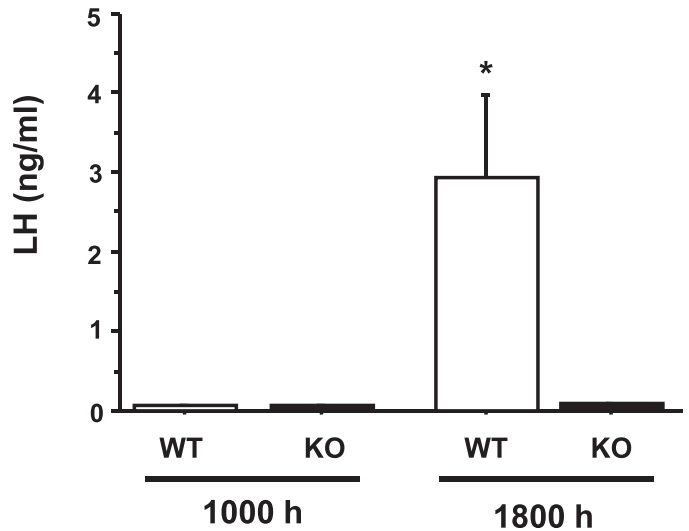


FIG. 1. Mean serum LH levels in adult female WT and *Kiss1r* KO mice exposed to a positive feedback regimen of constant elevated E₂. Two days after E₂ implantation, blood was collected from mice at either a morning (approx. 1000 h) or an evening (lights off; 1800 h) time point. *Significantly different from all other groups ($P < 0.01$). All data are expressed as the mean ± SEM for each group.

induction of the LH surge in the evening. In contrast, no *Kiss1r* KO females exhibited any hint of LH surges in the evening (range: 0.08–0.11 ng/ml; Fig. 1). Indeed, LH levels of all *Kiss1r* KO mice were extremely low at both time points (at or below the limit of detectability; Fig. 1), significantly lower than those of evening WT females ($P < 0.001$) and similar to levels of morning WT females.

Experiment 2: LH Surge Induction by Rising E₂ Levels in *Kiss1r* KO and WT Females

This experiment tested if adult *Kiss1r* KO female mice could display an LH surge in response to rising levels of E₂ that mimic those of a normal estrous cycle (hormonal paradigm 2). This E₂+EB paradigm failed to elicit LH surges in a different *Kiss1r* KO line [26] but was not previously tested in our *Kiss1r* KO mouse line (which had been reported by Dungan et al. [25] to be capable of displaying surges with a different paradigm). We found that all WT females exposed to this E₂+EB paradigm displayed robust LH surges at the evening time point (range: 2.03–8.30 ng/ml), whereas LH levels in the morning were extremely low in WT females (Fig. 2). Conversely, no *Kiss1r* KO females exhibited LH surges in the evening (range: 0.04–0.09 ng/ml; Fig. 2). As in experiment 1, LH levels of *Kiss1r* KO mice were extremely low at both morning and evening time points, significantly lower than those of evening WT females ($P < 0.001$) and similar to levels of morning WT females.

Experiment 3: LH Surge Induction by a Combination of E₂ and P in *Kiss1r* KO and WT Females

In gonadal-intact female rodents, the LH surge is induced by rising E₂ on proestrus that is accompanied by rising P levels. P has been proposed to modulate the surge onset, duration, and/or magnitude. Here, we used an E₂+EB+P paradigm (hormonal paradigm 3) to test whether P may be required for *Kiss1r* KO female mice to produce an LH surge. We found that under this E₂+EB+P paradigm, most (75%) WT females

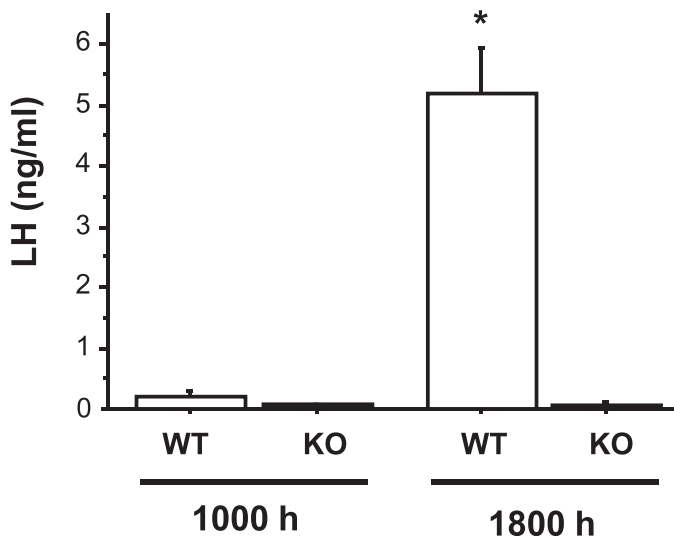


FIG. 2. Mean serum LH levels in female WT and *Kiss1r* KO mice exposed to a positive feedback regimen of low E_2 followed by an injection of EB to induce rising E_2 . The day after EB injection, blood was collected from mice at either a morning (1000 h) or an evening (lights off; 1800 h) time point. *Significantly different from all other groups ($P < 0.01$). All data are expressed as the mean \pm SEM for each group.

displayed an LH surge at the evening time point (range of surging females: 0.70–1.48 ng/ml; Fig. 3). LH levels were very low in all WT females killed in the morning. As before, and in contrast to the WTs, *Kiss1r* KO females treated with E_2 +EB+P did not generate any semblance of an LH surge at the evening time point (Fig. 3). All evening values of LH were extremely low in KO females (being at the limit of detection in all mice) and were significantly different from WT evening levels ($P < 0.001$) and similar to morning levels of both genotypes.

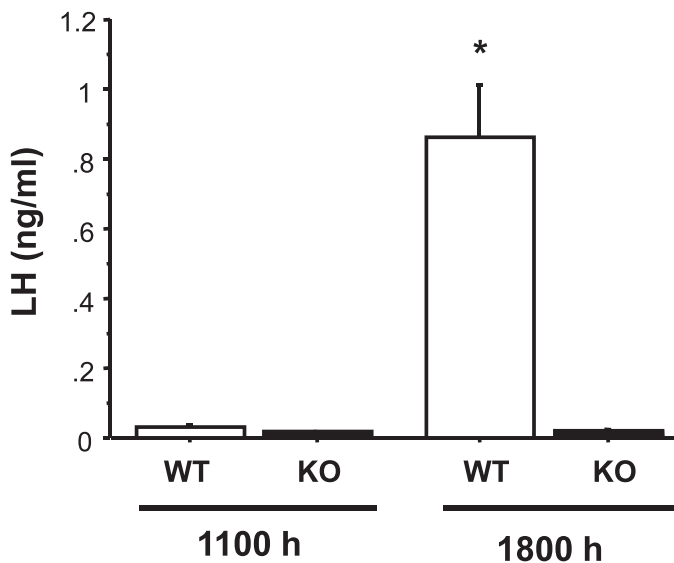


FIG. 3. Mean serum LH levels in female WT and *Kiss1r* KO mice exposed to a positive feedback paradigm of low E_2 followed by morning injections of EB and then, 1 day later, P. The day of the P injection, blood was collected from mice at either a morning (approx. 1100 h) or an evening (lights off; 1800 h) time point. *Significantly different from all other groups ($P < 0.01$). All data are expressed as the mean \pm SEM for each group.

Experiment 4: GnRH Priming of the Pituitary Prior to LH Surge Induction by E_2

The inability of *Kiss1r* KO females to produce an LH surge under the three different hormonal paradigms above might possibly be attributed to a lifetime lack of pituitary exposure to sufficient GnRH and hence reduced gonadotrope responsiveness to a GnRH surge signal. If so, a positive feedback paradigm might trigger a GnRH surge but not an accompanying LH surge. To assess this possibility, adult females of both genotypes were first primed with GnRH injections over the course of several days before being subjected to an LH surge-induction protocol. After the final GnRH priming injection, blood levels were analyzed for LH levels. Unlike control mice not given GnRH, both WT and *Kiss1r* KO mice displayed large elevations in serum LH 15 min after GnRH treatment (Fig. 4A; $P < 0.01$ relative to controls not receiving GnRH), indicating that the pituitaries of both genotypes were highly responsive to GnRH stimulation. All GnRH-primed mice were then treated with constant E_2 to see if an LH surge could now be induced in *Kiss1r* KO mice. As in the previous experiments, WT mice showed robust LH surges in the evening (range 0.96–1.65 ng/ml), whereas none of the previously GnRH-primed *Kiss1r* KO mice displayed an LH surge in response to elevated E_2 (Fig. 4B). LH levels of GnRH-primed *Kiss1r* KO mice were extremely low (below limit of detectability) at both time points, similar to morning WT levels and significantly lower than WT evening levels ($P < 0.01$).

Experiment 5: Positive Feedback Induction of *cfos* in GnRH Neurons in WT but Not *Kiss1r* KO Females

The lack of LH surges in *Kiss1r* KO mice in the previous experiments suggested an absence of a preceding GnRH surge. To assess whether positive feedback can or cannot activate GnRH neurons in *Kiss1r* KO mice, we measured *cfos* induction in GnRH neurons of WT and *Kiss1r* KO females that had been treated with hormonal paradigm 1. GnRH neurons were analyzed in three regions of the GnRH neural network: the medial septum, the OVLT, and the anterior POA. There were no statistical differences between genotypes or groups in the number of GnRH neurons counted in each area or overall (~100 total cells/animal on average; not shown). Mirroring the LH results in experiment 1, WT females had high *cfos*-GnRH coexpression in the evening but not in the morning (Figs. 5, 6), indicating that their GnRH neurons were activated in the evening, in line with a GnRH/LH surge. In WT evening females, elevated *cfos* induction was detected in GnRH neurons in all three brain regions, with the highest level of *cfos*-GnRH coexpression in the OVLT and POA. Conversely, *Kiss1r* KO females did not have elevated *cfos*-GnRH coexpression in the evening in any area of the GnRH neuronal network (Figs. 5, 6). The level of *cfos* induction in GnRH neurons of KO mice was low (<10%) in both the morning and the evening, similar to WT morning levels. This outcome suggests that the lack of LH surges in *Kiss1r* KO mice is because the GnRH system is not activated rather than the pituitary being unresponsive to a GnRH surge signal.

E_2 Levels in the Different Paradigms

Circulating serum E_2 levels were measured in mice of both genotypes from each of the experiments. There were no significant differences in E_2 levels between WT and KO mice at either morning or evening time points in any of the hormonal paradigms examined (Table 1). In addition, Table 1 summa-

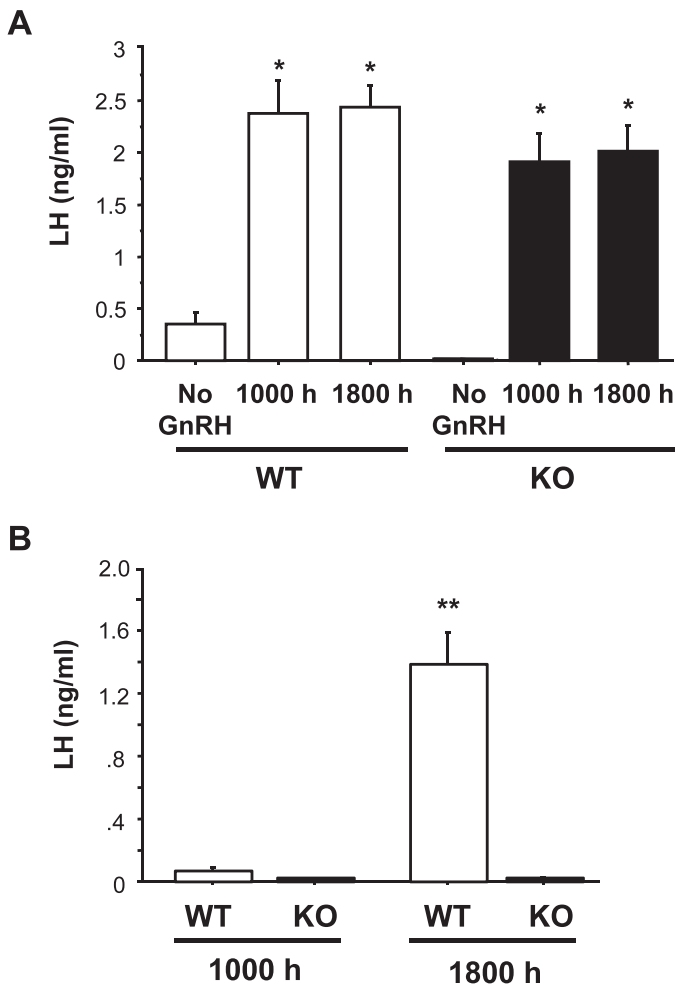


FIG. 4. Mean serum LH levels in female WT and *Kiss1r* KO mice exposed to a positive feedback regimen of constant elevated E_2 after several days of GnRH priming. **A**) LH levels in mice 15 min after the final GnRH priming injection. GnRH stimulated robust LH secretion in both genotypes, indicating a responsive pituitary. *Significantly different from non-GnRH treated group ($P < 0.01$). **B**) Two days after the last GnRH priming treatment, mice were OVX and given E_2 , and blood was collected 2 days later at a morning (1000 h) or an evening (lights off; 1800 h) time point. **Significantly different from all other groups. All data are expressed as the mean \pm SEM for each group.

rizes the percent of animals in each group that displayed an LH surge under each hormonal paradigm.

DISCUSSION

A wealth of evidence accumulated over the past decade has implicated the kisspeptin system in directly mediating the positive feedback effects of E_2 on the GnRH and LH surges that drive ovulation [3]. However, despite this, several prominent reports have cast doubt on whether this kisspeptin positive feedback model is in fact correct and whether other neural circuits may instead be involved. Indeed, the first published analysis of the ability of *Kiss1r* KO females to display LH surges in response to exogenous E_2 surprisingly reported that these females can in fact show LH surges after E_2 supplementation, suggesting that kisspeptin-Kiss1r signaling is not necessary for mice to generate LH surges [25]. However, soon thereafter, another report argued the opposite: that *Kiss1r* KO mice cannot display LH surges when given E_2 , supporting the developing dogma that kisspeptin-Kiss1r signaling is

essential for positive feedback [26]. The reasons for these opposite and discrepant findings were not addressed, though it could have been due to technical differences, as the two studies used both different *Kiss1r* KO strains and different E_2 paradigms. The mouse model that surged was not tested with the E_2 paradigm that failed to elicit surges, and the E_2 paradigm that did elicit surges was never tested in the mouse model failing to surge with the other protocol. Thus, direct comparison between the two studies was limited and lacking. Here, we sought to directly address this controversy to establish which E_2 paradigms, if any, could in fact elicit LH surges in *Kiss1r* KO females. We convincingly demonstrate that *Kiss1r* KO females are incapable of generating LH surges under any hormonal paradigm tested, including even after GnRH priming.

Unlike WT mice, which consistently displayed LH surges in the evening, *Kiss1r* KO females did not produce a single steroid-induced LH surge under any of the three hormonal paradigms tested. Indeed, *Kiss1r* KO females did not even demonstrate a semblance of an LH surge or any elevation of LH of any kind: in all cases, LH levels in *Kiss1r* KO mice were extremely low and typically undetectable, regardless of time of day. Combining all experiments, the overall percent of evening WT females displaying an LH surge was 94% (30 of 32 mice), whereas the overall percent of evening KO females that surged was 0% (0 of 33 mice). The complete lack of surges in the KO mice could not be attributed to deficits in E_2 , as serum E_2 levels were similar between genotypes in all experiments. These data indicate quite clearly that mice cannot generate LH surges in the absence of kisspeptin signaling.

In the absence of any other data, the lack of detectable LH surges in the *Kiss1r* KO mice could, in theory, be attributable to defects at several levels: the inability of GnRH neurons to be activated and release a surge of GnRH and/or an inability of the pituitary to respond to an incoming GnRH signal and secrete a surge of LH. The latter possibility could stem from a lifetime lack of gonadotrope exposure to GnRH, resulting in a severely reduced responsiveness to the first GnRH signal. Indeed, in normal rodents, the LH surge magnitude increases over the end of pubertal maturation, eventually producing full magnitude surges in adulthood [1]. Given that *Kiss1r* KO mice are essentially prepubertal, even at adult ages, their gonadotrope system may, in theory, resemble that of a prepubertal animal, perhaps explaining the lack of a robust LH surge on first-time exposure to GnRH in an experimental positive feedback paradigm. We tested this possibility by priming the pituitaries of adult *Kiss1r* KO mice with GnRH injections; after the third GnRH injection, LH was robustly elevated in *Kiss1r* KO and WT mice, indicating a responsive pituitary. However, when subsequently challenged with an E_2 surge paradigm, these GnRH-primed *Kiss1r* KO mice, unlike WT mice, failed to produce any semblance of a LH surge. Thus, the lack of an LH surge likely reflects lack of a GnRH surge rather than diminished pituitary responsiveness to incoming GnRH. This conclusion was supported by our finding of high *cfos*-GnRH coexpression in WT females at the evening time point but low *cfos*-GnRH coexpression in evening *Kiss1r* KO mice, indicating that GnRH neurons are not properly activated by E_2 positive feedback paradigms in *Kiss1r* KO mice.

Our findings support those of Clarkson et al. [26], who found that another line of *Kiss1r* KO mice do not surge after E_2 +EB treatment. Moreover, we extend those findings to show that several additional positive feedback paradigms not tested in that study, including one involving constant elevated E_2 and another involving P supplementation, also do not elicit LH surges in *Kiss1r* KO mice. Moreover, this is the first study to

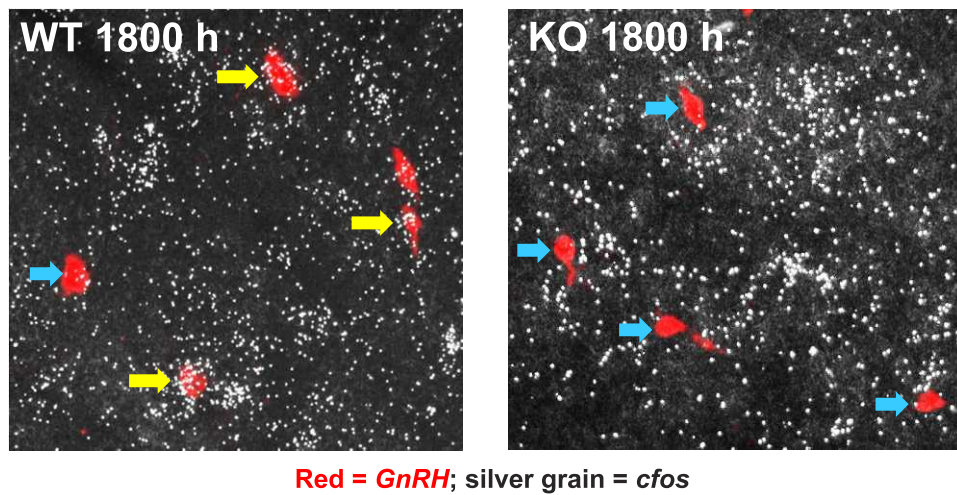


FIG. 5. Representative photomicrographs of *cfos* mRNA coexpression in GnRH neurons in WT and *Kiss1r* KO female mice exposed to an LH surge paradigm of constant elevated E_2 and sacrificed in the evening. Yellow arrows denote examples of GnRH cells (red fluorescence) with significant *cfos* (silver grains) coexpression. Blue arrows designate example GnRH neurons that did not have *cfos* induction.

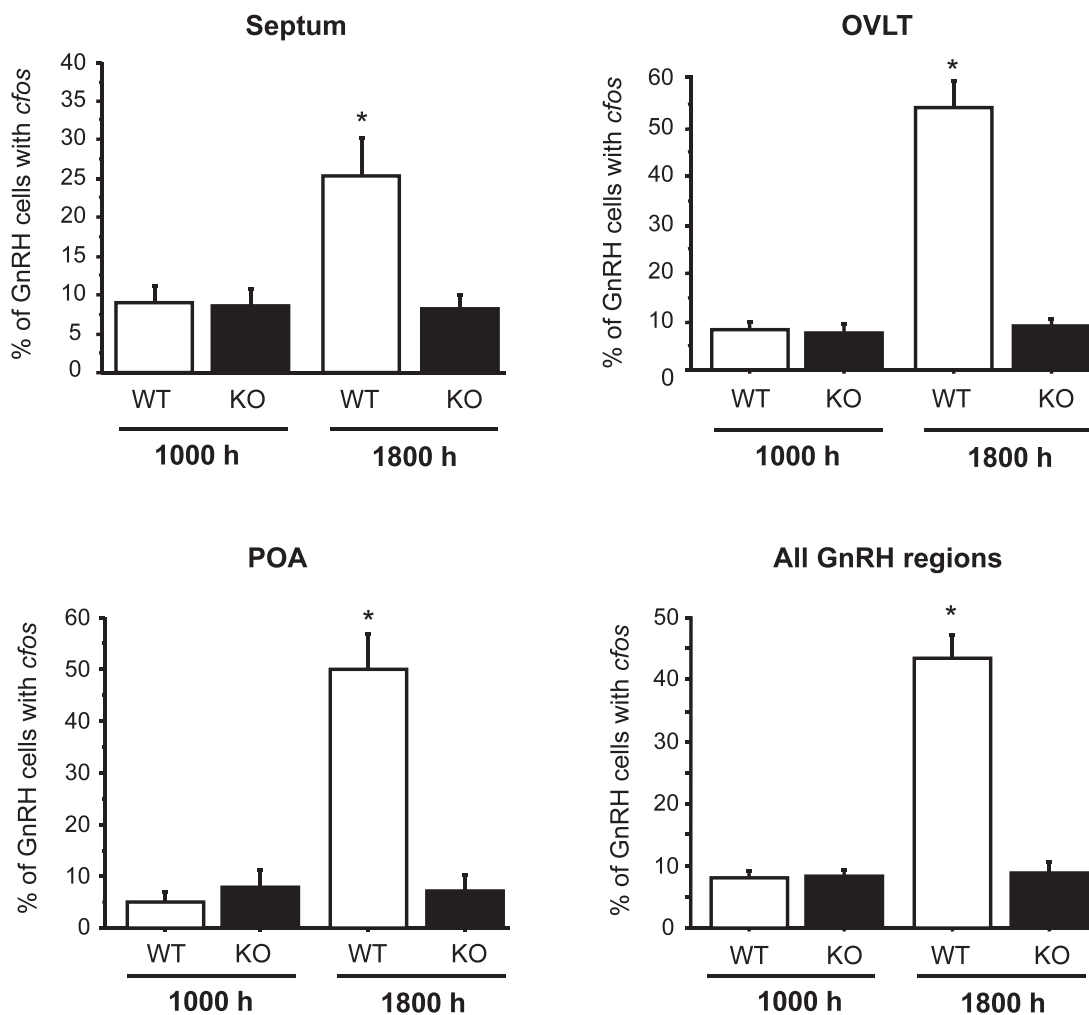


FIG. 6. Mean percent of GnRH neurons coexpressing *cfos* in female mice exposed to an LH surge paradigm of constant elevated E_2 . Two days after E_2 implantation, brains were collected from mice at either a morning (1000 h) or an evening (1800 h) time point. Brains were assayed for *cfos* induction in GnRH cells using double-label ISH. Mean levels of *cfos*-GnRH coexpression in mice from different time-points were quantified in three brain regions within the GnRH neural system as well as the entire GnRH population. *Significantly different from all other groups ($P < 0.01$). All data are expressed as the mean \pm SEM for each group.

TABLE 1. Serum estradiol (E_2) levels and percentage of mice surging in WT and *Kiss1r* KO mice treated with different hormonal paradigms to induce evening LH surges.

LH surge paradigm	E_2 regimen	Serum E_2 levels (pg/ml) ^a				Mice surging ^b	
		WT morning	KO morning	WT evening	KO evening	WT evening	KO evening
1	Elevated E_2	25.4 ± 1.7	25.9 ± 3.1	20.6 ± 0.9	22.1 ± 1.3	100%	0%
2	Low E_2 + EB	39.5 ± 1.7	41.9 ± 9.4	32.3 ± 5.1	33.9 ± 7.6	100%	0%
3	Low E_2 + EB + P	25.9 ± 3.5	23.0 ± 2.9	23.3 ± 2.5	20.9 ± 2.0	75%	0%

^a Serum E_2 levels did not differ significantly between genotypes for any hormonal paradigm.

^b Unlike WT mice, no KO mice surged in any of the three paradigms.

document the inability of *Kiss1r* KO mice to surge even after GnRH priming. Thus, our conclusions that Kiss1r is necessary for GnRH activation and the LH surge are in agreement with those of the Clarkson et al. [26] study. However, the reason for the discrepancy between our present findings and the previous finding by Dungan et al. [25] regarding the ability of elevated E_2 (hormonal paradigm 1) to induce LH surges and GnRH neuronal activation in the identical *Kiss1r* KO strain as ours is unknown. Our mice were the same age, housed in the same conditions (though at different universities), exposed to the same E_2 paradigm, and had similar E_2 levels as in the Dungan study. Thus, there is unlikely to be any major technical differences between our two studies. We do note that our *Kiss1r* KO mouse line was originally on a mixed 129/BL6 background; our lab has further backcrossed our colony several generations into C57Bl6, though it still remains a mixed 129/BL6 strain. We therefore cannot rule out a subtle “strain” difference between our mice and those used in the Dungan study, but we do not believe this would be enough to fully induce or prevent GnRH activation or an LH surge, as there is no indication of gross disparities in the ability of strains to generate LH surges. We do note that the mean LH surge levels for the *Kiss1r* KO mice reported in the Dungan study were of lower magnitude than in our study, and not all of those mice surpassed the designated threshold level to qualify as an LH surge, indicating that a decent proportion of those *Kiss1r* KO mice may not have actually “surged.” Moreover, the morning values for GnRH neuronal activation of both WT and KO were uncharacteristically high in the Dungan study (25–30%), nearly reaching typical evening “surge” levels (~40–50%). In our mice, morning levels of GnRH neuron activation were below 9% in both genotypes, which is more consistent with known low levels of activation at nonsurge times. We do not have a clear reason for this discrepancy between studies but note that our study assessed *cfos* and GnRH mRNA using ISH, whereas Dungan et al. [25] assessed Fos and GnRH protein using IHC.

Our study utilized three different hormonal paradigms to induce LH surges in female mice, providing a context to compare the efficacy of different experimental models of positive feedback. Overall, all three paradigms were efficient at inducing LH surges in WT females. The percent of WT females surging and the magnitude of the surge were not different between constant elevated E_2 (paradigm 1) and low E_2 +EB (paradigm 2), though the latter method produced slightly higher circulating E_2 levels than the former. If anything, this may suggest that the lower E_2 levels achieved in paradigm 1, having full efficacy for surge induction, may be slightly more physiological, though this is likely not a huge difference. Interestingly, the paradigm including P (paradigm 3) yielded slightly lower mean LH surge levels and had two WT animals that failed to reach the surge criteria. This was surprising, as in rats, P is thought to increase the magnitude of the LH surge. However, P is also known to advance and/or shorten duration of the surge in rats [2]. Thus, it is likely that in

our mice treated with P, the LH surge either began earlier or had a shorter duration than in the other non-P hormonal paradigms. If so, we may have been sampling toward the end of the surge event right after lights off and catching the latter part of the surge rather than its peak. Alternatively, the LH surge system in mice is quite easily perturbed by stress, and it is possible (though probably unlikely) that handling and injections of P earlier that day increased stress levels, which reduced the LH surge later that evening. Regardless of the observed slightly lower surge magnitude, the fact remains that a surge was still present. Indeed, our data clearly and convincingly show that the majority (75%) of WT mice show an LH surge under this P paradigm, whereas none of the KO mice displayed any evidence of LH elevation at all, demonstrating the necessity of Kiss1r for this surge process.

In conclusion, our results suggest that kisspeptin-Kiss1r signaling is essential for the sex steroid-induced activation of GnRH neurons and LH surge in mice. The inability of *Kiss1r* KO mice to surge is likely to inactivation of GnRH neurons, presumably by kisspeptin signaling arising from the anterior hypothalamus. Although one of our initial goals when embarking on this project was to potentially identify novel neural circuits that may be able to generate an LH surge in the absence of kisspeptin-Kiss1r signaling, our findings rather indicate that kisspeptin-Kiss1r signaling is a necessary and essential component of the positive feedback induction of the LH surge and hence ovulation. While several other brain factors, such as GABA/glutamate and RFRP-3, have been suggested to perhaps play a role in the LH surge mechanism [3, 28, 29], these or other factors do not appear to be sufficient to drive the surge without kisspeptin signaling and may therefore be modulatory to the surge rather than essential. Indeed, our findings suggest that there are not additional parallel pathways independent of kisspeptin that are sufficient for the LH surge process, at least in rodents.

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