Corticosterone blocks ovarian cyclicity and the LH surge via decreased kisspeptin neuron activation in female mice

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Stress elicits activation of the hypothalamic-pituitary-adrenal axis which leads to enhanced circulating glucocorticoids, as well as impaired gonadotropin secretion and ovarian cyclicity. Here we tested the hypothesis that elevated, stress-levels of glucocorticoids disrupt ovarian cyclicity by interfering with the preovulatory sequence of endocrine events necessary for the LH surge. Ovarian cyclicity was monitored in female mice implanted with a cholesterol or corticosterone pellet. Corticosterone, but not cholesterol, arrested cyclicity in diestrus. Subsequent studies focused on the mechanism whereby corticosterone stalled the preovulatory sequence by assessing responsiveness to the positive feedback estradiol signal. Ovariectomized mice were treated with an LH surge-inducing estradiol implant, as well as corticosterone or cholesterol, and assessed several days later for LH levels on the evening of the anticipated surge. All cholesterol females showed a clear LH surge. At the time of the anticipated surge, LH levels were undetectable in corticosterone-treated females. *In situ* hybridization analyses revealed that corticosterone robustly suppressed the percentage of AVPV/PeN *Kiss1* cells coexpressing *cfos*, as well as reduced the number of *Kiss1* cells and amount of *Kiss1* mRNA per cell, compared to expression in control brains. In addition, corticosterone blunted pituitary expression of the genes encoding the GnRH receptor and LHβ, indicating inhibition of gonadotropes during the blockage of the LH surge. Collectively, our findings support the hypothesis that physiological stress-levels of corticosterone disrupts ovarian cyclicity, in part, through disruption of positive feedback mechanisms at both the hypothalamic and pituitary levels which are necessary for generation of the preovulatory LH surge.

Physical and emotional stress can inhibit reproduction in males and females. For example, restraint and/or isolation, a model of psychosocial or emotional stress, reduces mean plasma LH concentrations in numerous species, including in rats, sheep and monkeys (1–6). In females, stress has been shown to disrupt ovulatory cyclicity, which is dependent on proper feedback control of gonadotropin synthesis and secretion (7, 8). One such mode of gonadotropin secretion is the pulsatile release of LH which dominates during the period of follicular development. Stressors such as restraint or endotoxin, a model of immune or inflammatory stress, reduce the frequency of LH pulses in rats and sheep (3, 9, 10). Decreased GnRH secretion, as well as diminished pituitary responsiveness to GnRH, underlies the reduction in LH pulsatility (11, 12). In addition to the suppression of LH pulses, the surge mode of LH secretion, which governs ovulation in females, is vulnerable to the effects of stress. Isolation-restraint stress can block the preovulatory LH surge in rats, and in sheep, the LH surge can be delayed or blocked by transport in a truck or by infusion of endotoxin (11, 13, 14). A delay in the LH surge has been observed in rhesus monkeys exposed to the combined stress of exercise, food restriction, and movement to a novel environment (8). Despite evidence that stress interferes with ovarian cyclicity in females, the fundamental mechanisms that result in

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Abbreviations:

disrupted pulsatile and surge LH secretion are not well understood.

In response to stress, the body reacts with a host of chemical mediators, such as endogenous opioid peptides, cytokines, excitatory and inhibitory amino acids, and hormones of the hypothalamic-pituitary-adrenal (HPA) stress axis, each of which could participate in mediating the effects of stress on reproduction. A few lines of evidence implicate enhanced release of glucocorticoids from the adrenal cortex in mediating suppression of gonadotropin secretion. First, impairment of reproductive function by various stressors, including those perturbing psychosocial, metabolic, and immune function, have all reported to be accompanied by a rise in circulating levels (4, 5, 15–17). In addition, exogenous administration of elevated glucocorticoids, in the absence of stress, disrupts gonadotropin secretion and estrous cyclicity in species ranging from rodents to domestic animals to primates and humans (18–31). Finally, evidence that RU486, a well-utilized antagonist of both GR and the progesterone receptor, attenuates the inhibitory effect of immobilization stress on LH secretion in male rats, or psychosocial stress on pituitary responsiveness to GnRH in female sheep, implicates a physiologic role for glucocorticoids in mediating the inhibitory effects of stress on LH secretion (9, 32, 33).

We recently demonstrated that the period between successive estrus phases was lengthened in gonad-intact female mice exposed to daily restraint stress, suggesting a disruption in the preovulatory events necessary to generate the LH surge, ovulation and subsequent stage of estrus (34). Much of the ovulatory cycle is dominated by negative feedback effects of estradiol and progesterone which restrain the secretion of LH pulses. As the cycle progresses, rising estradiol production by maturing ovarian follicles evokes a positive feedback action upon the hypothalamus and pituitary gland that culminates in generation of a GnRH and subsequent LH surge. Mechanistically, stress could block the LH surge by impairing the pulsatile release of LH which is required to drive the preovulatory increase in estradiol secretion. Alternatively, stress could directly interfere with the neuroendocrine response to this positive feedback estradiol signal at either the hypothalamic or pituitary level. Based on the type of stress and inhibitory mediators involved, it is possible that both modes of secretion are targets for reproductive suppression, although stress-specific pathways are still under investigation.

In this study, we tested the hypothesis that a stress-like elevation in plasma corticosterone interferes with the ovulatory cycle by disrupting neuroendocrine generation of the LH surge. We first demonstrate that an elevation in glucocorticoids, mimicking the endogenous level induced by psychosocial stress, arrests intact female mice in diestrus and prevents successive preovulatory events. Based on this response, we elucidated whether there were significant impairments in the neuroendocrine mechanisms necessary for generation of the LH surge by investigating the effect of glucocorticoids on both hypothalamic *Kiss1* circuits and anterior pituitary gonadotrope cells during estradiol positive-feedback induction of the LH surge.

Materials and Methods

Animals

Experiments were conducted on adult female C57Bl/6 mice (11–12 weeks old) housed in a UCSD vivarium animal facility under standard conditions. Mice were provided food and water ad libitum and housed in groups of 3–4 during all experiments under a 12 hours light-dark cycle (lights off at 1800). All experiments were performed in agreement with the National Institutes of Health Animal Care and Use Guidelines and with authorization from the Institutional Animal Care and Use Committee at the University of California, San Diego.

Corticosterone pellet preparation

Cholesterol or corticosterone-releasing pellets were manufactured in our laboratory by coating silicone-filled Silastic tubing of 1.0 cm length (0.040 in. I.D. x 0.085 in. O.D.) with molten steroid, as per Meyer et al, 1979 (35). Unlike gonadal steroids, corticosterone release from Silastic capsules is insufficient to elevate circulating levels of steroid to physiologic levels. Rather, implantation of solid or fused corticosteroids pellets have been effective in maintaining viability of adrenalectomized animals (36). Pilot studies were conducted to establish an effective corticosterone pellet composition (42.5 mg corticosterone : 7.5 mg cholesterol; Sigma, St. Louis, MO) that would produce a continuous elevation in circulating corticosterone which would mimic the high physiologic endogenous corticosterone level we observe in response to isolation/restraint stress in female mice (34). An equivalent weight of pure cholesterol was used to manufacture vehicle pellets for implantation in control animals.

Evaluation of estrous cyclicity

At 8 weeks of age, vaginal lavage was performed daily (between 0900 and 1100) with distilled H_2O and collected smears were mounted on glass slides for microscopic examination of cell type (37). Smears were classified into one of four phases of the estrous cycle: proestrus, estrus, metestrus or diestrus by two investigators, blinded to treatment groups. Female mice exhibiting a 4–6 day estrous cycle, including positive classification of all four estrous stages, were utilized in animal experiments. On the morning of metestrus, following demonstration of three consecutive estrous cycles, female mice were implanted subcutaneously, under isoflurane anesthesia, with a cholesterol or corticosterone pellet. Estrous cycle length was calculated as the number of days between successive occurrences of estrus. The time spent in each cycle stage was calculated as the proportion of days classified in each cycle stage relative to the length of the baseline or implant period.

LH surge induction

Mice were ovariectomized under isoflurane anesthesia and implanted subcutaneously with an estradiol implant that produces a physiological level of circulating estradiol which establishes positive feedback and reliably initiates the GnRH and LH surge (38). Estradiol-containing implants consisted of Silastic tubing of 1.2 cm length (0.125 in. I.D. x 0.078 in. O.D.) filled with 0.625 μg 17-β estradiol (Sigma) dissolved in sesame oil (39, 40). 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 (39, 41). At the time of ovariectomy, animals were also subcutaneously implanted with either a corticosterone or cholesterol pellet. Mice were sacrificed two days after surgery either in the morning (between 1000 and 1100 hours) or in the evening within 30 minutes of lights off (1800 hours). The LH surge was defined as a rise in serum LH concentration exceeding 10-fold above the mean ($\pm$ SEM) morning LH concentration.

Blood, brain and pituitary gland collection

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 by centrifugation (5000 rpm x 15 minutes) and stored at –20°C. Brains and pituitary glands were collected at sacrifice and immediately frozen on dry ice before being stored at –80°C.

Single- and double-label in situ hybridization (ISH)

Frozen brains were sectioned on a cryostat into five sets of 20-μm sections and thaw mounted on Superfrost-plus slides that were stored at –80°C until assay. Single-label ISH for *Kiss1*, double-label ISH for *cfos*/*Kiss1*, and double-label ISH for *ER*α/*Kiss1* were performed as previously described (39, 41–44). For the single-label *Kiss1* ISH, one set of slides containing the entire AVPV/PeN was fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2× SSC (sodium citrate, sodium chloride), delipidated in chloroform, dehydrated in ethanol, and air dried. Radiolabeled (^{33}P) *Kiss1* (0.04 pmol/mL) antisense riboprobes were combined with tRNA, heat-denatured, added to hybridization buffer, and applied to each slide (100 μL/slide). Slides were then placed in a 55°C humidity chamber overnight. On the second day, slides were washed in 4× SSC (sodium citrate, sodium chloride), treated with RNAse for 30 minutes at 37°C, then in in RNAse buffer without RNAse at 37°C for 30 minutes. Following a room-temperature wash in 2× SSC, slides were washed in 0.1× SSC at 62°C for 1 hour, dehydrated in ethanols, and air dried. Slides were then dipped in Kodak NTB emulsion, air dried, and stored at 4°C for 3 days before being developed and coverslipped.

For double-label *cfos*/*Kiss1* and *ER*α/*Kiss1* ISH, a similar protocol was used, with slight modifications, using separate slide sets for each ISH. Briefly, antisense radiolabeled (^{33}P) *cfos* (0.04 pmol/mL) or *ER*α (0.05 pmol/mL) riboprobe was combined with digoxigenin (DIG)-labeled *Kiss1* (1:500) riboprobe and tRNA, heat-denatured, dissolved together in hybridization buffer, and applied to each slide (100 μL/slide) prior to hybridization at 55°C overnight. Following the 62°C washes on day 2, slides were incubated in 2× SSC with 0.05% Triton X-100 containing 3% normal sheep serum for 1 hour at room temperature. Slides were then incubated overnight at room temperature with anti-DIG antibody conjugated to alkaline phosphatase (Roche; diluted 1:500 in buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100). On the third day, slides were washed with buffer 1, incubated with Vector Red alkaline phosphatase substrate (Vector Labs) for 1 hour at room temperature, and then air dried. Once dry, slides were dipped in emulsion, stored at 4°C, and developed 5 or 7 days later (*cfos*/*Kiss* or *ER*α/*Kiss1*, respectively).

Quantification and analysis of ISH data

ISH slides were analyzed by a person blinded to treatment groups using an automated image processing system used widely for evaluation of *Kiss1* cells (Dr. Don Clifton, University of Washington). For the single-label *Kiss1* slides, an individual blinded to treatments identified all silver grain clusters. Following identification of cells, the automated software program counted the number of silver grain clusters, representing cells, as well as the number of silver grains over each cell (a semiquantitative index of mRNA content per cell) (45). Cells were categorized as *Kiss1* positive when the number of silver grains in a cluster exceeded that of background by 3-fold (44, 46). A relative measure of total mRNA in the AVPV/PeN region was calculated by multiplying the total number of cells by the number of silver grains per cell (43, 44, 47). For double-label assays, a person blinded to treatments identified red fluorescent DIG-containing cells (*Kiss1* cells) under fluorescence microscopy. Once DIG cells were identified, the automated grain-counting software quantified silver grains (representing *cfos* or *ER*α mRNA) overlying each DIG cell. Signal-to-background ratios for individual cells were calculated by the program, and a cell was categorized as double-labeled if its ratio was greater than 3 (42–44, 46); signal-to-background ratios of less than 3 identified the cell as single-labeled.

Hormone assays

Serum LH levels were measured by the Center for Research in Reproduction Ligand Assay and Analysis Core at the University of Virginia by two-site sandwich immunoassay using monoclonal antibodies against bovine LH (no. 581B7) and the human LH-beta subunit (no. 5303), as described previously (48–50). Mouse LH reference prep (AFP5306A; provided by Dr. A.F. Parlow and the National Hormone and Peptide Program) is used as a standard. The limit of detectability for the mouse LH assay is 0.04 ng/ml. Circulating serum estradiol levels were also measured by the Ligand Assay and Analysis Core using a commercially available Calbiotech Mouse/Rat Estradiol ELISA (Cat. ES180S; Calbiotech, Spring Valley, CA) validated for use in mouse (51). Assay sensitivity for this estradiol ELISA was determined to be 3.0 pg/ml. Serum corticosterone levels were measured using DetectX Corticosterone EIA kit (Cat. K014; Arbor Assays, Ann Arbor, MI), per the manufacturer's instructions. Assay sensitivity for this corticosterone ELISA was determined to be 18.6 pg/ml.

Quantitative real-time PCR

Preparation of cDNA from mouse pituitary was performed as previously described (52). Briefly, RNA was extracted with Trizol reagent (Invitrogen/GIBCO, Carlsbad, CA) according to the manufacturer's instructions, treated to remove contaminating genomic DNA (DNA-free; Ambion, Austin, TX) and reverse

transcribed using iScript First-Strand Synthesis System (BioRad, Hercules, CA). Quantitative real-time PCR was performed on a CFX Real-Time PCR instrument (Bio-Rad) and utilized iQ SYBR Green Supermix (Bio-Rad) with specific primers for *Gapdh*, *Lhβ* or *Gnrhr*.

Lhβ forward: CTGTCAACGCAACTCTGG
Lhβ reverse: ACAGGAGGCAAAGCAGC
Gnrhr forward: GCCCCTTGCTGTACAAAG C
Gnrhr reverse: CCGTCTGCTAGGTAGATCATCC
Gapdh forward: TGCACCACCAACTGCTTAG
Gapdh reverse: GGATGCAGGGATGATGGTTC

The CFX real-time PCR program was as follows: 95°C for 15 minutes, followed by 40 cycles at 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Threshold cycle (CT) values for the genes of interest, *Lhβ* or *Gnrhr*, were compared to the reference gene, *Gapdh*, by the $2^{-\Delta\Delta Ct}$ method. Data are represented as mean fold change compared with control ± SEM.

Statistical analyses

Data are expressed as the mean ± SEM for each group. Differences were analyzed by one- or two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or by a chi square analysis. Specific details of individual analyses can be found in each figure legend. All statistical analyses were performed by JMP© 10.0.0 (SAS Institute, Cary, NC) and significance was established as $P < .05$.

Results

Elevated corticosterone blocks estrous cyclicity

This experiment tested whether a continuous exposure to stress-like levels of glucocorticoids, mimicking the maximal level of corticosterone that occurs in response to stress [~700 ng/ml, (53–57)], impairs ovarian cyclicity in female mice. On the morning of metestrus, female mice were implanted with a subcutaneous pellet containing either cholesterol or corticosterone (50 mg/pellet; n = 5–6/group). Circulating levels of corticosterone remained significantly elevated during the implant period in corticosterone-treated (Cort) females compared to cholesterol-treated vehicle (Veh) controls ($P < .05$, Figure 1A). Figure 1B illustrates profiles of vaginal histological classification during the baseline control period (Day 1–22) and implant period (Day 23–38) in one female mouse exposed to cholesterol and two exposed to corticosterone. In mice implanted with cholesterol, neither the average cycle length nor the percent time documented in individual cycle stages significantly differed between the baseline and implant periods (Figure 1C-D, Baseline Veh vs. Implant Veh). In contrast, corticosterone rapidly and robustly disrupted the pattern of cyclicity in females, resulting in a significant increase in cycle length during the implant period (Figure 1C, Baseline Cort vs. Implant Cort, $P < .05$). More specifically, females treated with corticosterone remained in diestrus for 90% of the implant period and failed to progress through estrus (Figure 1D), demonstrating a complete obstruction of ovarian cyclicity in the presence of chronically elevated glucocorticoids.

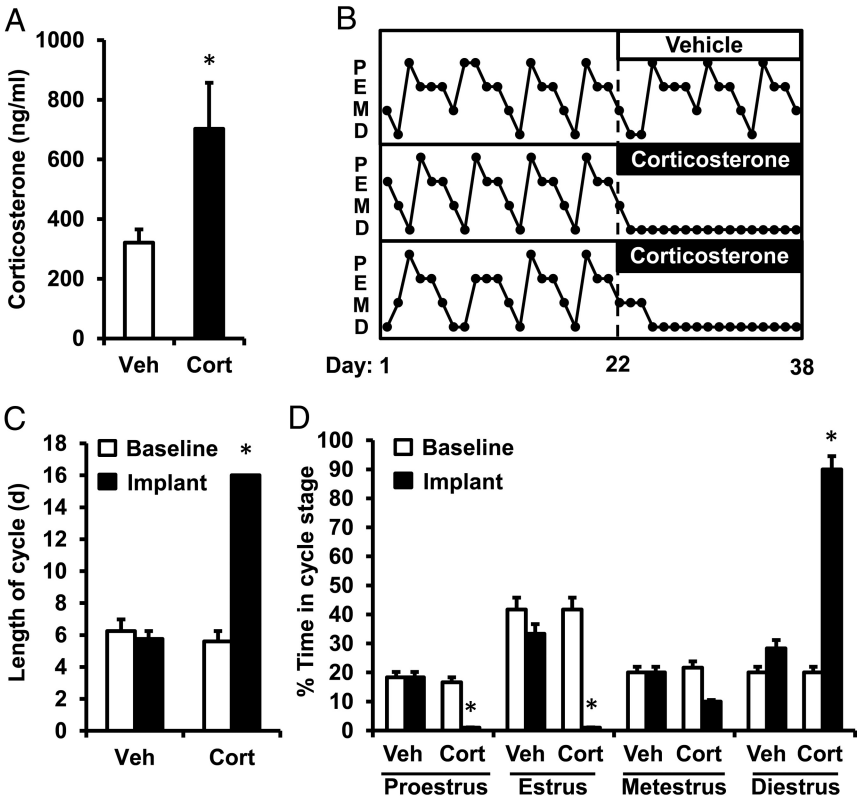

Figure 1. Corticosterone disrupts estrus cyclicity in the female mouse. A, Mean serum corticosterone measured on the final day of implantation in vehicle (Veh)- and corticosterone (Cort)-treated female mice. B, Representative profiles depicting estrous cyclicity in female mice, as measured by vaginal cytology, prior to (Days 1–22, Baseline) and during the period of implantation (Days 23–38, Implant) with a cholesterol (Vehicle, top panel) or corticosterone pellet (Corticosterone, two lower panels). P, proestrus, E, estrus, M, metestrus, D, diestrus. C, Average estrous cycle length during the Baseline and Implant periods in female mice treated with Veh or Cort. D, Average time spent in each stage of the cycle during the Baseline and Implant periods in Veh and Cort mice; n = 6 animals/group. Values were analyzed by one- or two-way ANOVA with group (Veh vs. Cort) and time (baseline period vs. implant period) as factors. *, indicates $P < .001$, Veh vs. Cort.

Corticosterone blocks the estradiol-induced LH surge

We postulate that impairment of the preovulatory GnRH and LH

surge is one mechanism whereby corticosterone could disrupt estrous cyclicity in female mice. Generation of the LH surge is not only dependent on the increase in estradiol secretion during proestrus, which constitutes the ovarian positive feedback signal, but also on neuroendocrine processing of this positive feedback estradiol signal (ie, the responsiveness of brain and pituitary cells to elevated estradiol). In this experiment, we directly tested the hypothesis that glucocorticoids interfere with the neuroendocrine response to the estradiol positive feedback signal. We utilized a well-established estradiol-induced LH surge paradigm in the presence or absence of elevated corticosterone (39, 40). As expected, estradiol-treated females implanted with a vehicle pellet displayed low circulating corticosterone levels in both the morning (AM) or evening (PM) of the anticipated LH surge (Figure 2A, open bars), whereas circulating levels of corticosterone in estradiol-primed females implanted with corticosterone were significantly elevated at both time points ($P < .05$; Figure 2A, closed bars). Morning levels of LH were low in estradiol-primed females treated with either vehicle or corticosterone, as expected since this is the period of estradiol-induced negative feedback (Figure 2B). All estradiol-treated vehicle controls displayed robust LH surges in the evening (Figure 2B-C). In contrast to this unanimous surge response in estradiol-treated vehicle females, none of the estradiol-treated females given corticosterone displayed an LH surge (Figure 2C). Indeed, LH values in these corticosterone-treated females were undetectable on the evening of the expected LH surge (Figure 2B), coinciding with elevated circulating levels of corticosterone that mirror the increment induced endogenously by stress. Circulating levels of estradiol did not differ between estradiol-primed females treated with corticosterone or vehicle ($P > .05$, Veh vs. Cort, 18.6 ± 2.3 vs. 16.4 ± 3.4 pg/ml). Collectively, these data demonstrate that stress-like levels of corticosterone prevent the LH surge response to a positive feedback estradiol signal.

Corticosterone inhibits *Kiss1* expression and neuronal activation at the time of the LH surge

Kisspeptin signaling is obligatory for expression of the LH surge in females. The specific kisspeptin population considered important for processing the positive feedback estradiol signal during generation of the GnRH and LH surge resides in the AVPV and neighboring periventricular nucleus (AVPV/PeN) (58–60). To test the hypothesis that glucocorticoids disrupt kisspeptin (encoded by *Kiss1*) expression required for the LH surge, we used single-label ISH to investigate whether corticosterone alters *Kiss1* levels in the AVPV/PeN during the estradiol-induced LH surge. Brains collected from estradiol-treated vehicle female controls, demonstrated high levels of *Kiss1*-containing cells, robust *Kiss1* mRNA content per cell, and high levels of total *Kiss1* expression within the AVPV/PeN region (Figure 3A, 3C-E). In contrast, estradiol-treated females given corticosterone showed significantly fewer detectable *Kiss1* cells by 35%, reduced *Kiss1* mRNA content per cell by 30%, and diminished total *Kiss1* mRNA levels in the AVPV/PeN by 56%. compared to estradiol-treated controls given vehicle (Figure 3B, 3C-E).

Having found that corticosterone strongly suppressed *Kiss1* expression within the AVPV/PeN at the time of the LH surge, we next investigated whether corticosterone treatment also lowered levels of *Kiss1* neuronal activation, evaluated by coexpression with *cfos*. In addition to confirming the reduction in AVPV/PeN *Kiss1* cell number observed in the single-label analysis (Figure 4C), double-label ISH analysis also revealed that corticosterone induced a dramatic reduction in total number of AVPV/PeN *Kiss1* neurons expressing *cfos* (Figure 4A vs. 4B), resulting in a significant decrease in the percent of *Kiss1* neurons show-

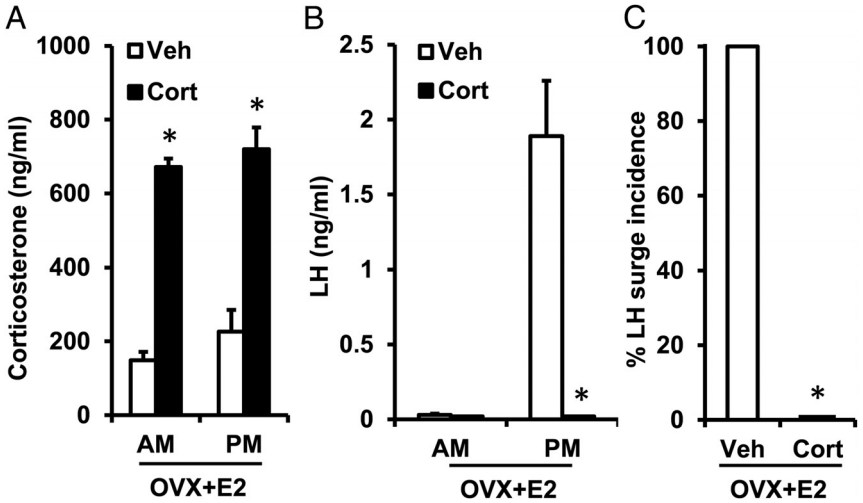

Figure 2. Corticosterone blocks the estradiol-induced LH surge. Mean (± SEM) serum corticosterone (ng/ml, A) and LH (ng/ml, B) in adult female mice ovariectomized and exposed to a positive feedback regimen of constant elevated estradiol along with either a cholesterol (Veh) or corticosterone (Cort) implant. Two days after surgery, blood was collected in the morning (AM, 1000 hours) or evening (PM, 1800 hours) of the expected LH surge in female mice. C, Percent of female mice, treated with either Veh or Cort, that responded to the estradiol stimulus with an LH surge; n = 6 animals/group. Values were analyzed by two-way ANOVA with group (Veh vs. Cort) and time (AM vs. PM) as factors or chi square analysis (LH surge incidence). *, indicates $P < .001$, Veh vs. Cort.

ing neuronal activation (Figure 4D; Veh vs. Cort, 65% vs. 14% colocalized with *cfos*). Collectively, these findings demonstrate that glucocorticoid-induced suppression of the estradiol-induced LH surge is associated with disrupted *Kiss1* neuronal activation and expression in the AVPV/PeN.

Corticosterone does not reduce the level of *ERα* mRNA expression in *Kiss1* cells

The presence of ERα (encoded by *ERα*) within AVPV/PeN *Kiss1* cells is necessary for enhanced *Kiss1* expression during the preovulatory GnRH and LH surge (42, 59, 61). Indeed, knockdown of ERα in *Kiss1* neurons prevents the estradiol-induced LH surge and results in complete infertility (62). Based on our finding that AVPV/PeN *Kiss1* neurons are less responsive to the estradiol positive feedback signal in the presence of elevated corticosterone, indicated by lower *Kiss1* mRNA expression and reduced neuronal activation (Figures 3 and 4), we next tested the hypothesis that glucocorticoids decrease *ERα* expression within *Kiss1* neurons. We conducted double-label ISH analysis for *ERα* expression within AVPV/PeN neurons expressing *Kiss1* in brains collected during the anticipated LH surge from estradiol-primed females treated with cholesterol or corticosterone. Steroid receptors are typically expressed at low levels in individual neurons and mRNA staining normally appears more diffuse compared to denser silver grain staining observed for neuropeptide mRNAs (41, 63). Again, the number of AVPV/PeN *Kiss1* cells identified in this double-label assay was significantly reduced by corticosterone as compared to vehicle-treated females (Figure 5A vs. 5B, 38% reduction in Figure 5C). However, the percent of *Kiss1* cells colocalized with *ERα* was not significantly different between the two groups (Veh vs. Cort; $P > .05$, Figure 5D), suggesting that the effect of corticosterone to suppress the *Kiss1* system during the LH surge is independent of suppressed *ERα* expression.

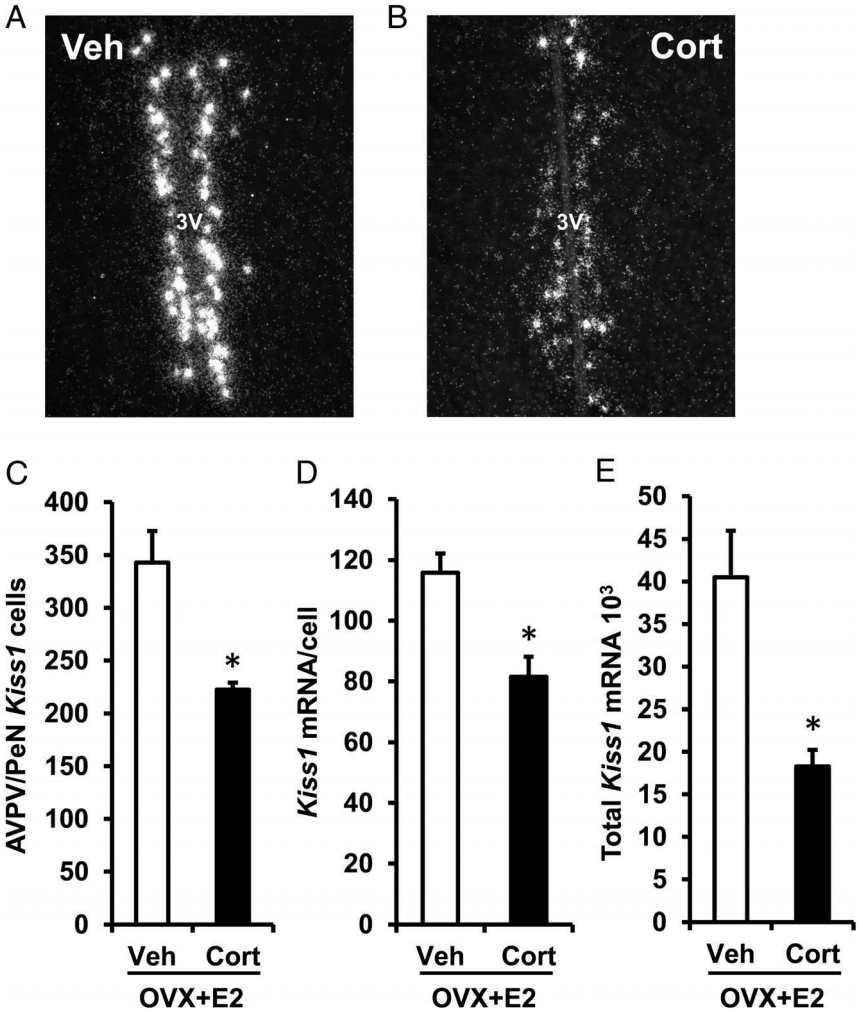

Figure 3. Corticosterone inhibits *Kiss1* expression at the time of the LH surge. Representative dark-field photomicrographs showing *Kiss1* mRNA (silver grain clusters) expressed in the AVPV/PeN at the time of the expected LH surge (1800 hours) in brains collected from female mice exposed to a surge-inducing estradiol stimulus, as well as a pellet containing cholesterol (A, Veh) or corticosterone (B, Cort). C, Mean (± SEM) number of *Kiss1* mRNA-containing cells in the AVPV/PeN of Veh- and Cort-treated females. D, Mean (± SEM) level of *Kiss1* mRNA per cell in the AVPV/PeN of Veh- and Cort-treated females. E, Mean (± SEM) total *Kiss1* mRNA in the AVPV/PeN of Veh- and Cort-treated females. n = 6 animals per group. Values were analyzed by one-way ANOVA. *, indicates $P < .01$, Veh vs. Cort; 3V, third ventricle.

Corticosterone inhibits pituitary gene expression at the time of the LH surge

In addition to actions at the central level, glucocorticoids have been shown to inhibit the response of the pituitary gonadotrope to GnRH. We analyzed LHβ and GnRH receptor gene expression (encoded by *Lhβ* and *Gnrhr*, respectively) in ovariectomized female mice exposed to the estrogen positive feedback LH surge paradigm with or without corticosterone. Females were sacrificed in the evening, at the time of the expected LH surge, and the pituitary gland collected for mRNA analysis by quantitative RT-PCR. Corticosterone reduced *Lhβ* transcript levels by 30% compared to vehicle controls ($P < .05$; Figure 6A, Veh vs. Cort). In addition, *Gnrhr* expression

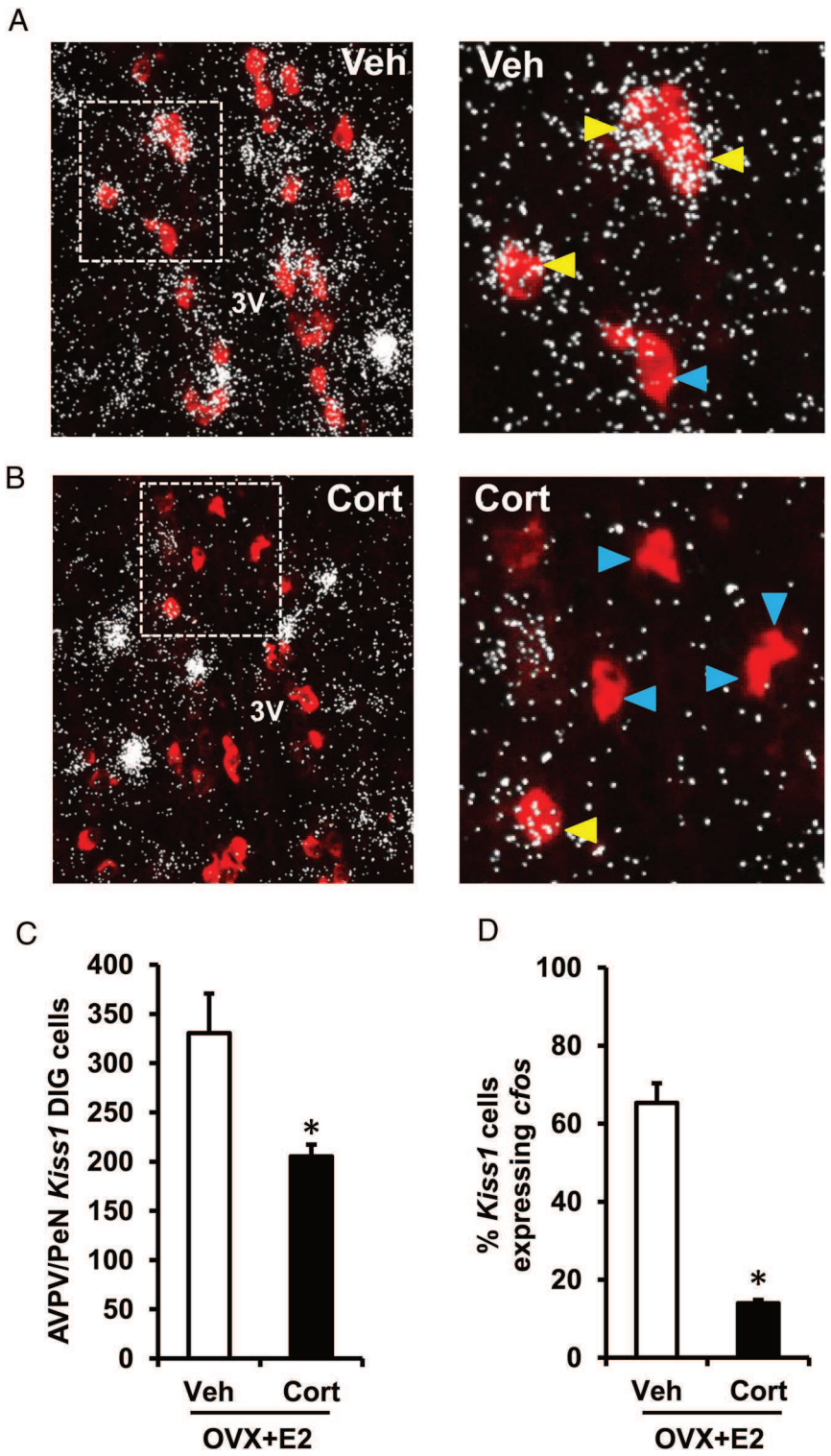

Figure 4. Corticosterone inhibits neuronal activation of AVPV/PeN *Kiss1* cells. Representative low- and high-power photomicrographs showing *Kiss1* mRNA-containing cells (red fluorescence) and *cfos* mRNA (silver grain clusters; representative of neuronal activation) in the AVPV/PeN at the time of the expected LH surge (1800 hours) in brains collected from female mice exposed to a surge-inducing estradiol stimulus, as well as a pellet containing cholesterol (A, Veh) or corticosterone (B, Cort). Dashed box indicates area of higher magnification. Yellow arrowheads denote examples of *Kiss1* cells with significant *cfos* coexpression, as determined by an automated image processing system. Blue arrowheads designate examples of *Kiss1* neurons that did not have notable *cfos* induction. C, Mean (± SEM) number of *Kiss1* mRNA-containing cells in the AVPV/PeN of Veh- and Cort-treated females. D, Mean (± SEM) percentage of *Kiss1*

was reduced by 50% in corticosterone-treated females ($P < .05$; Figure 6B). Collectively, these observations raise the possibility that stress can interfere with the preovulatory LH surge by disrupting the synthesis and secretion of LH at the level of the anterior pituitary gonadotrope cell, in addition to the suppression observed at the level of the hypothalamus.

Discussion

Here we utilize a mouse model to investigate mechanisms whereby elevated glucocorticoids suppress the reproductive neuroendocrine axis. We demonstrate that an elevation in the natural occurring glucocorticoid, which is normally enhanced in response to stress, prevents ovulatory cyclicity in intact female mice. Using a hormonal paradigm to generate the estradiol-induced LH surge in ovariectomized female mice, we demonstrate that multiple aspects of the positive feedback response to estradiol are disrupted by elevated glucocorticoids. At the central level, corticosterone decreased transcriptional activation of AVPV/PeN *Kiss1* neurons, as measured by neuronal *cfos* induction, and prevented enhanced AVPV/PeN *Kiss1* expression, including both a reduction in the *Kiss1* cell population and mRNA expression level per cell. At the pituitary level, corticosterone suppressed expression of the genes encoding the GnRH receptor, as well as the LHβ subunit, suggesting impaired pituitary responsiveness to GnRH. Collectively, these experiments indicate multiple mechanisms in which glucocorticoids disrupt the positive feedback action of estradiol to induce the preovulatory LH surge, either of which could contribute to the suppressive effect of corticoste-

rone on ovulatory cyclicity in female mice.

Early work revealed that the synthetic glucocorticoid, dexamethasone, could block both the natural preovulatory surge and the estradiol-induced LH surge in ovariectomized rats (25, 26, 64), but the neuroendocrine site and mechanism of action was not identified. Our results, using female C57BL/6 mice, are consistent with these previous findings and confirm the physiologic relevance of the natural glucocorticoid, corticosterone, as a potential inhibitory intermediate of the effects of stress. Furthermore, our study expands those initial observations by identifying the AVPV/PeN *Kiss1* neuron as a central target of corticosterone-induced suppression of the LH surge. *Kiss1* cells in the AVPV/PeN remain quiescent at the time of the anticipated LH surge, indicated by the 80% reduction in *cfos* expression and 50% suppression in total *Kiss1* expression. This population of AVPV/PeN *Kiss1* neurons expresses *ERα* and is thought to relay the estradiol signal to GnRH neurons, resulting in GnRH and LH surge release. In mouse models in which *ERα* is eliminated from AVPV/PeN *Kiss1* neurons, females fail to elicit a surge, identifying this neuronal population as a critical pathway during positive feedback surge generation. Taken together, our data support a mechanism whereby elevated glucocorticoids in female mice inhibit the preovulatory LH surge by impairing neuroendocrine responsiveness of AVPV/PeN *Kiss1* neurons to elicit the estradiol-induced surge release of GnRH and LH. It remains to be determined whether the *Kiss1* cell population in the arcuate nucleus is also suppressed by glucocorticoids. Elevated estrogen is well-known to inhibit arcuate *Kiss1* levels (59). Thus, the positive feedback paradigm employed in the current study would be expected to strongly lower *Kiss1* expression in the arcuate nucleus in all groups, precluding the accurate ability to assess further potential suppression by glucocorticoids in these females. Future studies employing nonestradiol treated females will be useful to elucidate any contribution of glucocorticoid suppression of the arcuate *Kiss1* system.

Evidence in rats demonstrates that a majority of *Kiss1* neurons in the AVPV/PeN contain GR (65), the receptor which mediates neuroendocrine actions of stress levels of glucocorticoids, positioning *Kiss1* neurons as a direct target for the suppressive actions of glucocorticoids. Our findings do not exclude, however, that cells upstream or downstream (ie, SCN or GnRH, respectively) of the AVPV *Kiss1* neurons are also inhibited by corticosterone during blockade of the surge. Questions regarding the role of other neurons impacted by corticosterone during blockade of the surge remain exciting, but purely speculative, as the animals models to piece together this pathway do not yet exist. Importantly, the mouse is an extremely useful model to genetically probe the pathways whereby corticosterone or stress alters reproductive neuroendocrine function. With regard to GnRH neurons, GR is expressed in a population of GnRH neurons (66) and glucocorticoids have been shown to inhibit expression in GT1–7 cells (67), a cell model of GnRH neurons. As for SCN involvement, VIP or Vasopressin neurons feed into the surge system and are an additional cell population whereby glucocorticoids may alter estradiol-induced responsiveness of the *Kiss1* neuron (42). Since the percent *Kiss1*/*ERα* coexpression did not differ with treatments, we interpret this to mean that corticosterone's ability to suppress the kisspeptin system is not due to a reduction of ER expression within *Kiss1* cells. However, we cannot exclude that ER signaling within AVPV/PeN Kiss1 neurons may be impaired by glucocorticoids. Further, ERα is highly expressed throughout the anterior hypothalamus and preoptic area, in many neuronal cell types in addition to AVPV/PeN kisspeptin neurons (see Figure 5A), and changes in this steroid receptor may be involved in suppression of the surge via another cell system, such as a neuron population sensing circadian input. Further work is clearly warranted to determine whether the disruptive influence of corticosterone is expressed at other neuroendocrine sites and the contribution of estradiol or its receptor to the suppression by corticosterone.

The anterior pituitary gonadotrope cell is a downstream neuroendocrine site in which corticosterone could prevent the LH surge is by reducing LH synthesis and secretion. Evidence in numerous species that glucocorticoids impair pituitary responsiveness to a single GnRH bolus or repeated GnRH pulses supports an inhibitory action of glucocorticoids upon the gonadotrope. Consistent with a direct action upon the gonadotrope, GR is expressed within gonadotrope cells of the mouse and rat (34, 68). Glucocorticoids have been shown to modulate signaling mechanisms downstream of the GnRH receptor, including protein kinase C and cyclic AMP, and blunt transactivation of the *Lhβ* promoter, either of which may lead to a reduction in gonadotropin gene expression or hormone release (34, 69, 70). The present study demonstrates that both *Lhβ* as well as *Gnrhr* mRNA levels are lowered by corticosterone and that this reduction is associated with undetectable levels of LH at the time of the expected LH surge, consistent with glucocorticoid-induced sup-

Legend to Figure 4 Continued. . .
mRNA-containing cells that coexpress *cfos* mRNA in the AVPV/PeN of Veh- and Cort-treated females; n = 6 per group. Values were analyzed by one-way ANOVA. *, indicates $P < .01$, Veh vs. Cort; 3V, third ventricle.

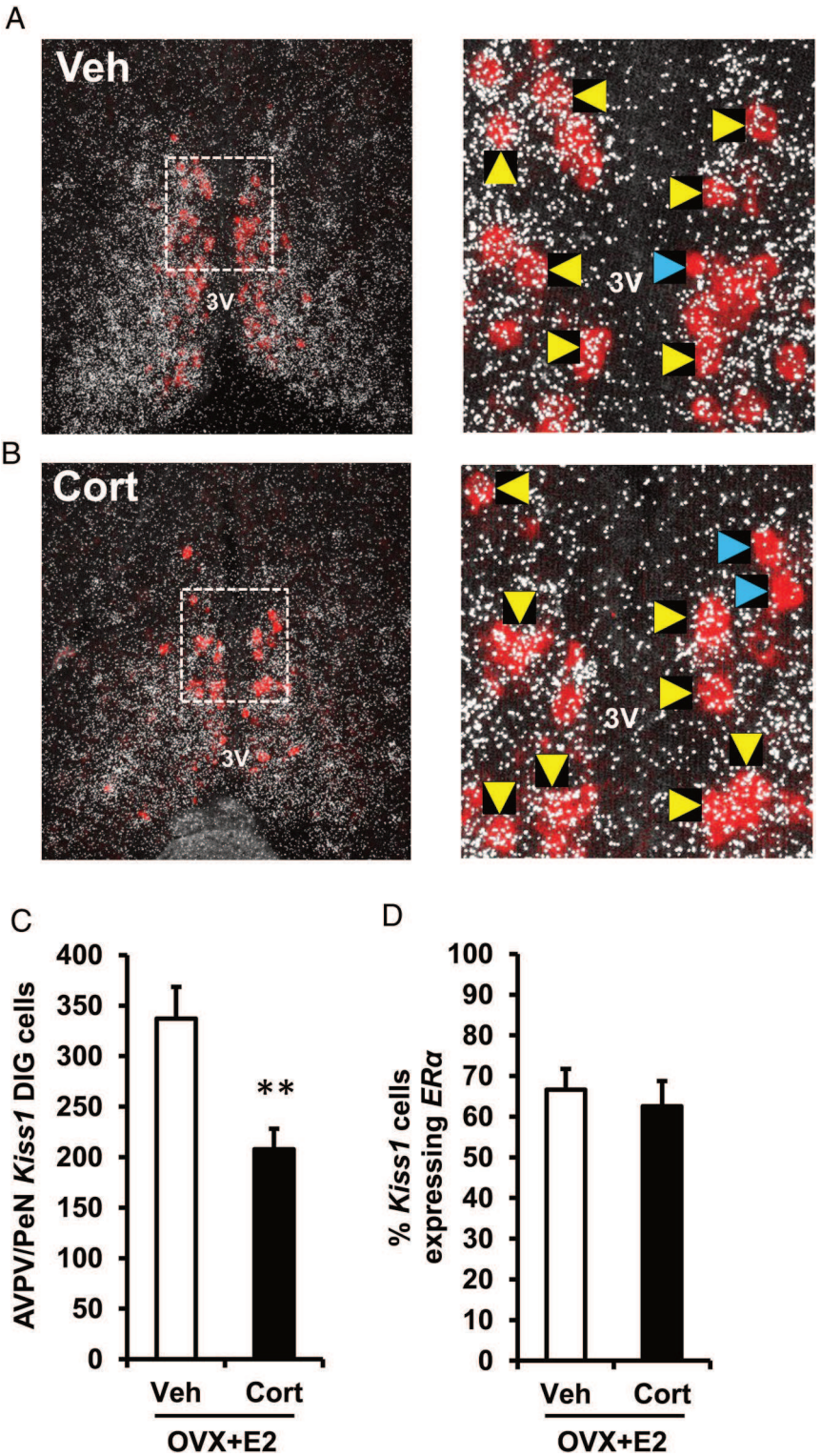

Figure 5. Corticosterone does not reduce the level of *ERα* mRNA expression in *Kiss1* cells. Representative photomicrographs of brains collected at 1800 hours from female mice exposed to a surge-inducing estradiol stimulus, as well as a pellet containing cholesterol (A, Veh) or corticosterone (B, Cort). Low- and high-power photomicrographs show *Kiss1* mRNA containing cells (red fluorescence) and *ERα* mRNA (silver grain clusters) in the AVPV/PeN. Dashed box indicates area of higher magnification presented. Yellow arrowheads denote examples of *Kiss1* cells with significant *ERα* coexpression. Blue arrowheads designate examples of *Kiss1* neurons that did not have *ERα* induction. C, Mean (± SEM) number of *Kiss1* mRNA-containing cells in the AVPV/PeN of Veh- and Cort-treated females. D, Mean (± SEM) percentage of *Kiss1*

pression of gonadotrope responsiveness during the surge.

Our findings suggest that stress levels of glucocorticoids alter multiple aspects of endocrine control necessary for maintenance of cyclicity and fertility. Specifically, our data suggest that corticosterone 1) suppresses basal LH levels causing females to be arrested in diestrus, preventing proestrus from occurring, and 2) inhibits neural kisspeptin circuits from properly being activated by elevated estradiol (which normally occurs in proestrus). Support for the first point is based on the response of ovarian-intact corticosterone-treated females to remain in diestrus and not transition to proestrus. Thus, we hypothesize that stress levels of glucocorticoids can impair the basal pulsatile release of LH which is required to drive the preovulatory increase in estradiol secretion needed to transition to proestrus, when the LH surge would occur. Indeed, this finding that has been shown in corticosterone-treated female sheep (71, 72). At this time, investigating pulsatile LH in mice remains very difficult and technically challenging, precluding analysis of this directly. Support for the second point comes from our present findings that glucocorticoids directly interfere with the neuroendocrine response to a positive feedback estradiol signal at both the hypothalamic or pituitary levels. Thus, glucocorticoids appear to provide inhibition of multiple aspects of female neuroendocrine reproductive axis by preventing both the transition into proestrus and proper activation of neuroendocrine circuits by proestrus-level estradiol signals.

Our finding that corticosterone impairs central as well as pituitary mechanisms important for expression of the LH surge raises the question of whether suppression of pituitary responsiveness account for the

full blockade of the LH surge? Two lines of evidence suggest that suppression of pituitary responsiveness may not be the sole neuroendocrine site for suppression. First, in studies in sheep where glucocorticoids inhibit pituitary responsiveness to both endogenous and exogenous GnRH pulses, the suppression in LH is maximally reduced by no more than 50% (73, 74). Second, evidence in ovariectomized female mice in which corticosterone induces acute suppression of mean LH, the reduction in circulating levels again reaches, at most, a 45% reduction (34). Therefore, the lack of any detectable LH leads us to speculate that suppression of the LH surge is due to combined effects at the hypothalamic and pituitary levels.

An important question raised by the current study is in regard to the relevance of elevated glucocorticoids to stress induced suppression of reproduction. In this regard, adaptations to stress comprise an initial autonomic activation and release of catecholamines from the sympathetic nervous system, followed by a more delayed and prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis and enhanced secretion of CRH, arginine vasopressin, ACTH, and the glucocorticoids (12–14). Each of these factors potentially mediates reproductive suppression. In actuality, the integrated actions of all of them are likely involved, with the relative importance of each depending on the nature of the stressor (12). In this study, we chose to focus on the natural glucocorticoid in rodents. Importantly, the corticosterone pellet used in the current study produces a constant elevation in circulating corticosterone, which differs from an endogenous stress-induced corticosterone rise that produces negative feedback upon the HPA axis which returns circulating corticosterone to basal levels. This corticosterone paradigm produces a defined circulating level of the natural glucocorticoid in mice, in the absence of stress, allowing us to test the mechanisms whereby this steroid alone disrupts reproductive function. Future studies will utilize this mouse model not only for investigating mechanisms of suppression, but the relevance of these glucocorticoid mediated mechanisms to the suppression of reproduction and fertility associated with stress.

In summary, we developed a mouse model to test the effects of stress levels of glucocorticoids on ovulatory cyclicity in female mice. Our results definitively show that corticosterone alone can inhibit reproductive function in female mice, in part, by interrupting generation of the LH surge response. Our findings point to two important mechanisms which could account for the disruption in reproductive neuroendocrine function. At the central level, glucocorticoids impair responsiveness of the AVPV kisspeptin system to a positive feedback estradiol signal. Coinciding with failure of female mice to respond with an estradiol-induced LH surge, gonadotropin expression is significantly reduced. Either of these inhibitory effects of glucocorticoids could by themselves prevent the LH surge response and may represent mechanisms contributing to menstrual cycle disturbances and infertility that are associated with physical and emotional stress.

Legend to Figure 5 Continued. . .
mRNA-containing cells that coexpress *ERα* mRNA in the AVPV/PeN of Veh- and Cort-treated females; n = 6 per group. Values were analyzed by one-way ANOVA. *, indicates $P < .05$; **, indicates $P < .01$; Veh vs. Cort; CC, corpus callosum; LV, lateral ventricle; LS, lateral septum; 3V, third ventricle.

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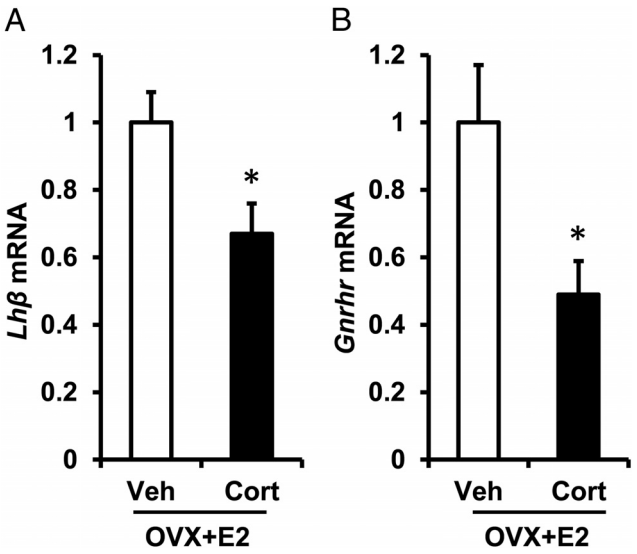

Figure 6. Corticosterone inhibits pituitary gene expression at the time of the LH surge. Quantitative RT-PCR analysis of *Lhβ* (A) and *Gnrhr* (B) mRNA was performed on individual mouse pituitary glands collected at the time of the expected LH surge (1800 hours) from female mice exposed to a surge-inducing estradiol stimulus, as well as a pellet containing cholesterol (Veh) or corticosterone (Cort) n = 6 per group. Ct values for *Lhβ* and *Gnrhr* were compared to *Gapdh* using the $2^{-\Delta\Delta Ct}$ method and analyzed by one-way ANOVA. Data is represented as mean fold change ± SEM. *, indicates $P < .05$, Veh vs. Cort.

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