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# Estradiol enables chronic corticosterone to inhibit pulsatile LH secretion and suppress Kiss1 neuronal activation in female mice

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## Abstract

**Introduction:** Two common responses to stress include elevated circulating glucocorticoids and impaired luteinizing hormone (LH) secretion. We have previously shown that a chronic, stress-level of corticosterone can impair ovarian cyclicity in intact mice by preventing follicular-phase endocrine events.

**Objective:** Here, we sought to investigate if corticosterone can disrupt LH pulses and whether estradiol is necessary for this inhibition.

**Methods:** Our approach was to measure LH pulses prior to and following administration of chronic corticosterone or cholesterol in ovariectomized mice treated with or without estradiol, as well as assess changes in arcuate kisspeptin (Kiss1) neuronal activation, as determined by co-expression with c-Fos.

**Results:** In ovariectomized mice, a chronic 48h elevation in corticosterone did not alter the pulsatile pattern of LH. In contrast, corticosterone induced a robust suppression of pulsatile LH secretion in mice treated with estradiol. This suppression represented a decrease in pulse frequency without a change in amplitude. We show that the majority of arcuate Kiss1 neurons contain glucocorticoid receptor, revealing a potential site of corticosterone action. Although arcuate *Kiss1* and *Tac2* gene expression did not change in response to corticosterone, arcuate Kiss1 neuronal activation was significantly reduced by chronic corticosterone, but only in mice treated with estradiol.

**Conclusions:** Collectively, these data demonstrate that chronic corticosterone inhibits LH pulse frequency and reduces Kiss1 neuronal activation in female mice, both in an estradiol-dependent

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Author Contributions

M.J.K. designed and performed the experiments, interpreted and analyzed results, and contributed to the writing of the manuscript; R.B.M. helped with IHC studies, interpreted results, and contributed to the writing of the manuscript; K.T. contributed to acquisition of animal tissue for experiments, interpretation of results, and participated in critical discussions during manuscript revision; C.I.S. conducted preliminary GR/Kiss1 IHC studies, contributed to the interpretation of results, and participated in critical discussions during manuscript revision. K.M.B. designed the experiments, interpreted the results, and wrote the manuscript. All authors edited, reviewed and approved the current manuscript.

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manner. Our findings support the possibility that enhanced sensitivity to glucocorticoids, due to ovarian steroid milieu, may contribute to reproductive impairment associated with stress or pathophysiologic conditions of elevated glucocorticoids.

**Keywords**

kisspeptin; glucocorticoid receptor; luteinizing hormone; arcuate nucleus; GnRH

## Introduction

Stress profoundly alters physiologic function through activation of a host of chemical and hormonal pathways, including activation of the hypothalamic-pituitary-adrenal (HPA) axis and the release of glucocorticoids. An increase in circulating glucocorticoids is a common physiologic response to many stressors and has been considered a potential mediator of reproductive suppression during stress. Evidence that a glucocorticoid receptor (GR) antagonist prevents the inhibition of gonadotropin secretion during stress in intact male rats and ovariectomized (OVX) female sheep treated with estradiol, supports a mediatory role of elevated glucocorticoids [1–3]. We have previously demonstrated that a chronic elevation of a stress-like level of corticosterone, the natural glucocorticoid in rodents, disrupts the ovulatory cycle of the female mouse [4]. Female mice exposed to elevated corticosterone remain in diestrus, comparable to the early follicular phase in humans, suggesting that a suppression in luteinizing hormone (LH) secretion contributes to ovulatory cycle disruption. Indeed, increased circulating glucocorticoids, administered in the absence of stress, have been shown to impair pulsatile LH secretion during the follicular phase in women [5] or ewes [6], but the neuroendocrine mechanisms underlying reduced LH secretion remains unclear.

A large body of *in vivo* and *in vitro* work supports the hypothesis that glucocorticoids can act centrally and at the pituitary gland to inhibit LH secretion. Corticosterone can suppress pituitary responsiveness to gonadotropin-releasing hormone (GnRH) and lower LH pulse amplitude in numerous species and under a variety of gonadal steroid regimens [7–14], which suggests a pituitary mechanism. On the other hand, data from castrated rhesus monkeys demonstrates that the glucocorticoid-induced suppression of LH can be reversed by exogenous GnRH, indicating a central mechanism of suppression [15]. A hypothalamic mechanism is also supported by data in which glucocorticoids alter LH pulse frequency, which is taken as evidence for an action upon the central GnRH pulse generator. For example, elevated corticosterone decreases the frequency of both GnRH and LH pulses in ovary-intact ewes during the follicular phase of the estrous cycle or in OVX ewes treated with estradiol and progesterone to simulate a follicular phase [6, 16]. Interestingly, corticosterone does not reduce GnRH or LH pulse frequency in OVX ewes devoid of gonadal steroids, indicating that this mode of LH suppression is dependent on ovarian steroid milieu [7]. Scant evidence for the presence of either GR or ovarian steroid receptors within GnRH neurons themselves [17–19] points attention to an afferent cell type expressing kisspeptin (encoded by the Kiss1 gene). Kisspeptin neurons in the arcuate nucleus also contain neurokinin B and dynorphin and are thus termed KNDy cells [20, 21]. Based on abundant anatomical, pharmacological, and electrophysiological data in rats, goats and

sheep, this neuron population was hypothesized to form the GnRH pulse generator [21–25]. More recently, arcuate kisspeptin neurons were confirmed to be the GnRH pulse generator in mice [26]. Thus, this cell population may form the pulse generator in numerous species. Importantly, KNDy cells express estradiol receptor alpha (ERα) [27, 28] and in sheep, also express GR [29]. Therefore, this cell population may be a cellular mediator of the suppressive effect of elevated glucocorticoids on GnRH and LH pulsatile secretion.

The goal of this study was to test the hypothesis that elevated corticosterone impairs ovarian cyclicity in females by inhibiting the GnRH pulse generator. For this purpose, we took advantage of our ability to monitor robust pulsatile LH secretion in female mice under OVX conditions, when the release of LH is unrestrained by gonadal steroids. As the results of our initial experiment implicated ovarian steroids as a potential factor influencing the response to corticosterone, subsequent experiments utilized a low physiologic estradiol treatment, approximating a diestrus level based on uterine weight, to test the effect of this ovarian steroid in modulating the response to chronic corticosterone. We pursued experiments to determine whether the reduction in pulsatile LH was mediated by a diminishment in gonadotrope responsiveness to GnRH or suppression of kisspeptin-containing cells within the arcuate nucleus.

## Materials and Methods

### Animals

Wild-type C57BL/6 (Envigo; Experiments 1–3 and 5A), Kiss1CreGFP (Experiment 4), or Kiss1hrGFP (Experiment 5B) mice were housed 2 per cage under standard conditions under a 12-h light, 12-h dark cycle with lights on at 0600 h. Kiss1CreGFP [30] transgenic mice express Cre-recombinase driven by Kiss1 regulatory elements which allows Kiss1 neurons to be identified by Cre-mediated expression of green fluorescent protein (GFP). Kiss1hrGFP [31] transgenic mice express humanized recombinant GFP (hrGFP) driven by Kiss1 regulatory elements which allows Kiss1 expression to be assessed with the GFP reporter. Animals had *ad libitum* access to water and Harlan irradiated chow #2920X at a University of California, San Diego vivarium. OVX was performed aseptically under isoflurane anesthesia on animals 8–14 wk old, unless otherwise stated. All surgeries and serial blood samplings were performed between 0800 h and 1200 h. All animal procedures were performed in accordance with the University of California, San Diego Institutional Animal Care and Use Committee regulations and were performed in accordance with the National Institutes of Health guidelines for the care and use of research animals.

### Experimental Details

**Experiment 1: Does chronic corticosterone reduce pulsatile LH secretion?—** C57BL/6 mice were handled daily for 5 wk prior to blood collection to acclimatize animals to the serial tail-bleed sampling required for pulsatile LH measurement as published previously [32, 33]. Mice were OVX and implanted subcutaneously with a silastic capsule containing oil (see Fig. 1A for experimental details). After 10 d, blood samples from the tail vein were collected with a pipette every 6 min for two 90-min intervals, pre: −1.5 – 0 h or post: 46.5 – 48 h, relative to the subcutaneous implantation of a pellet containing either

cholesterol or corticosterone (n=3/group) while briefly exposed to isoflurane anesthesia. Cholesterol or corticosterone-releasing pellets were manufactured in our laboratory as described previously [4], by coating silicone-filled (Liquid Nails, Butler County, PA) Silastic tubing of 1.0 cm length (0.040 in. I.D. × 0.085 in. O.D.) with molten steroid, as per Meyer et al., 1979 [34]. Pilot studies were conducted to establish an effective corticosterone pellet composition (25 mg corticosterone:25 mg cholesterol; Sigma, St. Louis, MO) that would produce a continuous elevation in circulating corticosterone which would mimic the peak level of endogenous corticosterone we observe in response to isolation/restraint stress in female mice [35]. Note, the unchanging corticosterone level produced in the present study likely exceeds the duration of an endogenous corticosterone response to acute stress. An equivalent weight of pure cholesterol (50 mg) was used to manufacture vehicle pellets for implantation in control animals. Tail blood (15 μL) was collected 2 d following implantation at 0700 h, prior to serial sampling, for analysis of circulating corticosterone levels in OVX animals.

### Experiment 2: Does estradiol enable chronic corticosterone to inhibit pulsatile LH secretion?

**Estradiol implants.:** Silastic tubing (0.125 in. inner diameter × 0.078 in outer diameter, DOW, Midland, MI) was cut to 2 cm in length. One end of the tubing was sealed with 0.3 cm silicon allowed to cure overnight. Next, 1.4 cm of the tubing was filled with sesame oil (0 ng control group, MilliporeSigma) or 20, 40, or 100 ng of 17-β estradiol (MilliporeSigma) dissolved in sesame oil. The open end of the tubing was then sealed with 0.3 cm silicon and allowed to cure. The implants were submerged in sterile saline for 14–18 hours prior to surgery.

**OVX+E treatment.:** Mice were OVX and implanted subcutaneously with a silastic capsule containing 0, 20, 40 or 100 ng estradiol (n=6–17/group for 0 or 100 ng; n=4/group for 20 or 40 ng estradiol). LH pulse frequency and body weight were measured 10 d post OVX. Tail blood (15 μL) was collected at 1700 h and the following day at 0700 h (11–12 d post OVX) to evaluate the diurnal rise of corticosterone which is observed in intact females, but not in OVX mice [36]. Corticosterone values were compared to values in regularly cycling females in diestrus, as determined by vaginal lavage. Finally, mice were euthanized, and the uterus was dissected and weighed (12 d post OVX). Uterine and body weight values were compared to a diestrus control group, which received an oil capsule during a sham OVX procedure to account for surgical influences 10 d prior to assessment (animal was anesthetized and the ovaries exteriorized through lumbar laparotomy prior to routine suture and staple closure).

To test the effect of estradiol on the response to corticosterone, mice were OVX and implanted with 100 ng estradiol, hereafter referred to as OVX+E. Ten d following corticosterone or cholesterol (n=8–9/group), blood samples were collected for measurement of pulsatile LH and corticosterone, as in Experiment 1 (Fig. 1B).

### Experiment 3: Does estradiol enable chronic corticosterone-induced suppression of pituitary gene expression or gonadotrope responsiveness to GnRH?

**A. Pituitary responsiveness to GnRH.:** Ten d after OVX+E, mice were implanted with cholesterol or corticosterone (n=8/group). Approximately 48 h later, tail blood for measurement of LH was collected every 6 min for 24 min prior to and 30 min following intraperitoneal (ip) injection with GnRH (800 ng/kg, MilliporeSigma, St. Louis, MO; Fig. 1C). Serial samples were collected to observe the LH pulse pattern prior to GnRH administration. The response to GnRH was calculated as the difference in LH concentration at time 0 compared to the peak LH value occurring within 12 min of GnRH (i.e, the higher value at either the 6- or 12-min time point).

**B. Pituitary gene expression.:** Ten d after OVX or OVX+E, animals received either a cholesterol or corticosterone implant (n=7–10/group). Approximately 48 h later, animals were euthanized under isoflurane via rapid decapitation. The brain and pituitary gland were immediately frozen on dry ice, and stored at −80°C for RNA extraction and gene expression analysis. Tissues were collected from one cohort of mice and analyzed in Experiment 3B (pituitary) and Experiment 5A (brain).

**Experiment 4: Is GR expressed in KNDy neurons?—**Ten d after OVX or OVX+E, Kiss1CreGFP mice (8–38 wk old, n=4/group) were euthanized with an overdose of pentobarbitol (Fatal Plus, MWI Animal Health, Boise, ID) and perfused by cardiac puncture with 4% paraformaldehyde in PBS. Once removed, brains were stored overnight in 4% paraformaldehyde and then transferred to 30% sucrose in phosphate buffer for at least 1 d.

### Experiment 5: Does chronic corticosterone inhibit KNDy gene expression or KNDy neuron activity?

**A. KNDy gene expression.:** Ten d after OVX or OVX+E, mice were implanted with cholesterol corticosterone (n=6–10/group) and brains were collected 48 h later, according to Experiment 3B.

**B. c-Fos in KNDy neurons.:** Ten d after OVX or OVX+E, Kiss1hrGFP mice were implanted with cholesterol or corticosterone (n=5–7/group). Approximately 48 h later, animals were perfused by cardiac puncture and fixed brain tissue was collected as in Experiment 4.

## RNA Isolation and Gene Expression Analysis

RNA was extracted from individual pituitaries via Trizol reagent (Life Technologies - Ambion, Waltham, MA) per manufacturer's directions. To isolate RNA from the arcuate nucleus, brains were sectioned coronally (250 μm) on a cryostat at −10°C. Sections were collected from −1.22 to −2.54 mm from bregma, according to a mouse brain atlas [37]. Semicircular micro-punches, 2 mm in diameter, which encompassed the whole arcuate nucleus were collected and stored at −80°C. RNA was isolated from arcuate punches using the RNAqueous Micro Kit (Life Technologies - Ambion), per manufacturer's instructions.

Following isolation, DNA contamination was removed with DNA-free DNase treatment (Life Technologies - Ambion) and RNA was quantified by nanodrop. RNA (1 μg) was reverse transcribed using the iScript Complementary DNA synthesis kit (Bio-RAD, Hercules, CA). For quantitative polymerase chain reaction (qPCR), complementary DNA and gene-specific primers were loaded according to Supplemental Table 1, along with SYBR green (Bio-RAD). Data were analyzed using the comparative cycle threshold ($^{\Delta\Delta}$Ct) method using *Gapdh* as a reference gene, as it displayed transcriptional stability across samples [38].

## Immunohistochemistry (IHC)

**IH C fo r GFP and GR—**Fixed tissue was sectioned (30 μm) on a cryostat in 3 series and stored in cryoprotectant solution [30% ethylene glycol, 30% sucrose, 1% polyvinyl pyrrolidone in phosphate buffer (PB)] at −20°C until processed for immunohistochemistry. The following steps were performed at room temperature with gentile agitation unless noted otherwise. Sections from one series, encompassing the entire arcuate nucleus, were processed from each animal. Tissue was rinsed in phosphate-buffer saline (PBS) with 0.1% Triton X-100 (PBST) 6 times, for 10 min each. Antigen retrieval was performed by incubating tissue in boiling Citra Buffer (Fisher Scientific, Waltham, MA) for 10 min, twice. Tissue was rinsed (4 times, 5 min each with PBST; standard rinsing step, unless noted otherwise) and incubated in a blocking solution containing 5% normal goat serum (NGS; Jackson Labs, West Grove, PA) containing 0.1% BSA in PBST, for 1 h. Tissue was then incubated in rabbit anti-GR (1:500, Thermo PA1–511A; Table 1) in blocking solution for 2 h at room temperature followed by 18 h at 4°C. The next day, tissue was rinsed, then incubated in goat anti-rabbit conjugated to Alexa 594 (1:300, Life A-11012) in blocking solution for 2 h and rinsed again. Tissue was then incubated in rabbit anti-GFP conjugated to Alexa 488 (1:1000, Life A-21311; Table 1) in blocking solution for 18 h at 4°C. Finally, tissue was rinsed, mounted on Superfrost slides (Fisher Scientific), coversliped with gelvatol [39], and stored at 4°C until microscopy. Because the antiserum raised against GFP was directly conjugated to the fluorophore (Alexa 488), and it was used after the detection of G R no cross reactivity is expected, though the primary antisera were raised in the same species. Moreover, the expected cellular distribution of these antigens and the staining pattern (GR in the nucleus and GFP throughout the cell) further demonstrates a lack of cross reactivity. This GR antiserum has been used extensively for detection of GR in mouse neural tissue [40, 41]; in our laboratory, preadsorption with recombinant GR resulted in no specific staining (Supplemental Fig. 1A). No specific staining was observed when the GFP antisera was applied to wild-type mouse neural tissue (Supplemental Fig. 1B).

**IHC for GFP and c-Fos—**One series of 30 μm sections, encompassing the entire arcuate nucleus, was processed for each animal. The following steps were performed at room temperature with gentile agitation, unless noted otherwise. Tissue was rinsed in PB, 12 times for 15 minutes, and incubated overnight at 4°C. The next day, tissue was rinsed 6 times in PB and then 6 times in PBS, for 5 min each (the following rinsing steps were performed in PBS). Antigen retrieval was performed by incubating tissue in boiling Citra Buffer for 10 min, twice. Tissue was rinsed and incubated in 0.3% hydrogen peroxide in PBS for 10 minutes. Next, tissue was rinsed and then incubated in blocking solution, containing PBS with 0.4% Triton X-100 and 4% NGS, for 1 hr. Tissue was then incubated in rabbit anti-c-

Fos (1:15,000, MilliporeSigma, ABE457; Table 1) in blocking solution for 18 h at 4°C. The next day tissue was rinsed and then incubated with biotinylated goat anti-rabbit antisera in blocking solution (1:500, Vector Laboratories, BA1000, Burlingame, CA) for 1 h. Next, tissue was rinsed and then signal was amplified with Vectastain Elite ABC Kit (1:500, Vector Laboratories, PK-6100), in PBS for 1 h. Tissue was rinsed and then incubated with biotinylated tyramine (1:250, Perkin Elmer Inc, SAT70001EA, Shelton, CT) in PBS containing 0.003% hydrogen peroxide for 10 minutes. Tissue was rinsed and incubated with streptavidin conjugated to Alexa 647 (1:100, Life S32357) in PBS. Tissue was rinsed and incubated in blocking solution for 1 h and incubated in rabbit anti-GFP conjugated to Alexa 488 (1:1000, Life A-21311) in blocking solution for 18 h at 4°C. Finally, tissue was rinsed, mounted on Superfrost slides, coversliped with gelvatol [39], and stored at 4°C until microscopy. Omission of the c-Fos antisera resulted in no specific staining in the arcuate or any other region we examined (Supplemental Fig. 1C). As described in the previous section, the anti-GFP antisera was conjugated directly to the fluorophore (Alexa 488) which obviates the need for a secondary antiserum which could have cross-reacted with the other primary antibody. Additionally, the c-Fos antiserum was used at a sufficiently low concentration that omission of the tyramide step prevented detection of the antigen (Supplemental Fig. 1D).

## Imaging

Imaging was performed with a Nikon Ti2-E inverted microscope with DS-Qi2 monochrome CMOS camera controlled with NIS-elements. At least two hemi-sections from each of three arcuate regions were selected for analysis (i.e. rostral, middle, and caudal). Within each region of the arcuate, the total number of GFP cells, and the percentage of GFP cells that contained GR (Experiment 4) or the percentage of GFP cells that contained c-Fos (Experiment 5), were determined in each hemi-section. The mean ± SEM number of GFP cells per hemisection or mean percentage of GFP cells that contained either GR (Experiment 4) or c-Fos (Experiment 5) is reported. Comparisons were then made between treatment groups (within region) with animal as the experimental unit. All cell counting was done by an observer blinded to treatment group using ImageJ software [42].

## Ultrasensitive Mouse LH Enzyme-Linked Immunosorbent Assay

Whole blood (3 μL) samples for measurement of LH were immediately diluted (1:20) into 57 μL of assay buffer, mixed, and placed on ice until storage at −20°C [33]. LH was measured in singleton by in-house enzyme-linked immunosorbent assay at the University of Virginia Ligand Assay Core, based on a method and reagents published in Steyn *et al.* [43]. The limit of quantitation (functional sensitivity) is defined as the lowest concentration that demonstrates accuracy within 20% of expected values and intraassay coefficient of variation (%CV) <20% and was determined by serial dilutions of a defined sample pool. Functional sensitivity was 0.320 ng/mL. Intra- and interassay %CVs were 2.3% and 7.0%, respectively.

## Corticosterone Enzyme-Link Immunosorbent Assay

Blood was centrifuged at 5000 rpm for 15 min and serum was isolated and stored at −20°C until assayed. Serum (4 μL) was diluted 1:100–1:400 and assayed using DetectX Corticosterone EIA kit (K014; Arbor Assays, Ann Arbor, MI), per Luo *et al.* [4]. Intra-assay and interassay %CVs were 1.8% and 4.4%, respectively. Assay sensitivity was 18.6 pg/mL.

### Data Analysis

**LH pulse analysis.—**Detection of an LH pulse was based on three criteria, proposed by Goodman and Karsch [44]: 1) the pulse peak must be within 3 consecutive samples (i.e. 18 min) from the preceding nadir, 2) the amplitude must be greater than the sensitivity of the assay, and 3) the amplitude must also be 2 standard deviations above the variability of the assay. Average values for pulse frequency, mean LH, and pulse amplitude were calculated across the pre and post periods. Pulse frequency was defined as the number of pulses in each 90-min period. Mean LH was calculated by averaging all LH values in each sampling period. Pulse amplitude was calculated as the difference from the pulse peak to the preceding nadir, and an average pulse amplitude value for each animal during the pre and post sampling period was determined. Note, 2 of 8 animals did not demonstrate detectable pulses following corticosterone and were excluded from the assessment of pulse amplitude.

Two-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test was used to determine significant differences across time (pre *vs.* post, AM *vs.* PM) or treatments or groups (oil *vs.* estradiol, cholesterol *vs.* corticosterone), and to identify significant interactions between variables: LH characteristics, mean corticosterone, $^{\Delta\Delta}$Ct relative quantity, and colocalization of c-Fos/Kiss1 values. One-way ANOVA followed by Tukey's honestly significant difference test was used to determine significant differences in estradiol response measures: uterine weight, weight gain, or pulse frequency. Values for c-Fos/Kiss1 were square-root transformed prior to analysis to conform with the assumptions for ANOVA, as the non-transformed values and were not normally distributed and the standard deviations not homogeneous. Student's t-test was used to determine differences in mean corticosterone, LH response to GnRH, or colocalization of GR/Kiss1. Values are expressed as mean ± SEM. All statistical analyses were performed using JMP 10.0.0 (SAS Institute, Cary, NC) and statistical significance was defined as $p<0.05$.

## Results

### Experiment 1: Does chronic corticosterone reduce pulsatile LH secretion?

Representative LH pulse profiles collected from OVX mice prior to (−1.5–0 h, pre) and 48 h following (46.5–48 h, post) implantation with cholesterol (A, chol) or corticosterone (B, cort), are shown in Figure 2. During the pre-implant period in both groups, mean LH averaged 4.5 ng/ml and LH pulse frequency averaged 4 pulses/90 minutes (Fig. 2C–D). Regardless of treatment, values for mean LH and LH pulse frequency did not significantly differ across pre and post periods. Although LH pulse amplitude did not significantly differ between groups in the pre period, amplitude was significantly increased in the post period in both groups (Fig. 2E, pre *vs.* post, $p<0.05$), likely due to increased time from OVX. Circulating levels of corticosterone were significantly elevated in animals treated with corticosterone compared to controls (Fig. 2F, 23 ± 3 ng/ml vs. 632 ± 38 ng/ml, chol vs. cort, $p<0.05$).

## Experiment 2: Does estradiol enable chronic corticosterone to inhibit pulsatile LH secretion?

**OVX+E treatment.—**Mice were OVX and implanted subcutaneously with either an implant containing 0 ng estradiol (oil control) or an implant containing 20, 40, or 100 ng estradiol. Approximately 10 d after OVX and estradiol replacement, uterine weight, weight gain following OVX, and LH pulse frequency were examined to document and determine the effectiveness of each estradiol treatment. In the absence of estradiol replacement, average uterine weight was 18.9 ± 1.0 mg, which did not significantly differ from mice implanted with 20 ng estradiol or 40 ng estradiol (Fig. 3A, $p>0.05$). In contrast, 100 ng estradiol significantly increased uterine weight compared to values in oil control animals (Fig. 3A, 35 ± 1.3 mg, 0 *vs.* 100 ng estradiol, $p<0.05$), and values did not significantly differ from uterine weight values obtained from sham OVX controls sampled in diestrus (Fig. 3A, 37.3 ± 2.5 mg, $p>0.05$). Although all animals gained weight during the 10 d period from OVX, the body weight change in animals treated with 100 ng estradiol was not different from the increase in body weight in sham OVX controls, indicating treatment with 100 ng estradiol was sufficient to prevent the weight gain that resulted from OVX (Fig. 3B, 2.4 ± 0.2 g *vs.* 0.8 ± 0.1 g, 0 ng *vs.* 100 ng E2, $p<0.05$). LH pulse frequency in response to 20 or 40 ng estradiol did not differ from the frequency in control animals (Fig. 3C, $p>0.05$); yet, replacement with 100 ng estradiol significantly slowed LH pulse frequency (Fig. 3C, 0 *vs.* 100 ng E2, $p<0.05$). Although not significant, treatment with 100 ng estradiol lowered mean LH and elicited an increase in pulse amplitude (0 ng *vs.* 100 ng E2, $p<0.1$; Supplemental Table 2). Interestingly, treatment with 100 ng estradiol also restored an aspect of HPA axis activity that dissipates when estradiol is removed, as indicated by elevated evening corticosterone that was not significantly different from diestrus control females (Fig. 3D).

Figure 4 shows representative LH pulse profiles during the two periods of frequent tail-tip blood sampling collected from OVX+E mice prior to and 48 h following implantation with cholesterol (Fig. 4 A–B) or corticosterone (Fig. 4 C–F). In animals treated with cholesterol, mean LH averaged 3.2 ng/ml, pulse frequency averaged 2.7 pulses/90 minutes, and pulse amplitude averaged 2.8 ng/ml in the pre-implant period; values for each LH characteristic did not significantly differ during the pre period between corticosterone- and cholesterol-treated controls (Fig. 4 G–I, chol pre *vs.* cort pre, $p>0.05$). During the post-implant period in animals treated with cholesterol, mean LH values were significantly elevated while frequency and amplitude were both unchanged compared to pre values. In contrast, corticosterone significantly reduced mean LH by 51.7% and decreased LH pulse frequency by 45.8% across the two sampling windows (Fig. 4G–H, cort, pre *vs.* post, $p<0.05$). Notably, the individual LH responses during treatment with corticosterone were variable: in two animals, no pulses were identified (Fig. 4C): in four animals, low amplitude pulses were identified (0.4–1.7 ng/ml, Fig. 4D); and in two animals, high amplitude pulses were identified (5.1–6.6 ng/ml, Fig. 4E–F). Again, corticosterone levels were significantly elevated by the corticosterone treatment (Fig. 4J, chol *vs.* cort, $p<0.05$).

### Experiment 3: Does estradiol enable chronic corticosterone-induced suppression of pituitary gene expression or gonadotrope responsiveness to GnRH?

We tested the hypothesis that a suppression in gonadotrope cell activity contributes to decreased LH during chronic corticosterone. Representative profiles of LH in tail-tip blood, collected prior to and following an injection of GnRH, from OVX+E mice either implanted with cholesterol or corticosterone are shown (Fig. 5A–B). Prior to injection, LH levels averaged 3.7 ng/ml in the control group and 1.8 ng/ml in the corticosterone group (Fig. 5C, pre). In response to GnRH, peak LH levels reached an average value of 7.9 ng/ml in mice treated with cholesterol compared to 5.6 ng/ml in mice treated with corticosterone (Fig. 5C, $p>0.05$); the amplitude of the LH response to GnRH did not significantly differ between treatments (4.1 ± 0.6 vs. 3.8 ± 0.9 ng/ml, chol *vs*. cort, $p>0.05$).

Pituitaries were collected from animals treated with chronic corticosterone or cholesterol in the absence or presence of estradiol. Although significant effects of corticosterone or estradiol treatment were identified, no significant interactions between these two factors were observed. For example, *Lhp* was suppressed by estradiol in animals treated with or without corticosterone and increased by corticosterone regardless of estradiol treatment (Fig. 5D, $p<0.05$). *Gnrhr* also showed a significant increase in response to corticosterone regardless of estradiol treatment (Fig. 5E). *Gr* expression was suppressed by estradiol in both groups (Fig. 5F).

### Experiment 4: Is GR expressed in KNDy neurons?

To determine if corticosterone can act directly on KNDy cells, we performed immunohistochemistry for GR using brain tissue from Kiss1CreGFP females treated with or without estradiol. The majority of GFP-labeled Kiss1 neurons throughout the arcuate hypothalamus, in either OVX or OVX+E mice, contained clear nuclear labeling for GR (Fig. 6). Estradiol treatment did not significantly alter the percentage of Kiss1 neurons that contained GR, except for a modest increase in the rostral arcuate (Table 2).

### Experiment 5: Does chronic corticosterone inhibit or KNDy gene expression or KNDy neuron activity?

Brains were collected from OVX or OVX+E mice following treatment with chronic corticosterone or cholesterol and gene expression for KNDy peptides and receptors were analyzed in micropunches of the arcuate hypothalamus (Fig. 7). Both *Kiss1* and *Tac2* were downregulated by estradiol ($p<0.05$), confirming the effectiveness of the estradiol treatment; however, neither *Kiss1* nor *Tac2* were further suppressed by corticosterone in OVX+E mice. In contrast, corticosterone decreased *pDyn*, the gene encoding dynorphin, regardless of estradiol treatment. (Fig. 7, $p<0.05$). Neither the mRNA expression of receptors for neurokinin B or dynorphin, *Tac3r* and *Oprk*, respectively, nor *Gr* or *E ra* were altered by corticosterone or estradiol (Fig. 7, $p>0.05$).

Using Kiss1hrGFP females, we investigated whether corticosterone-induced inhibition of pulsatile LH was associated with changes in Kiss1 neuronal activity. Figure 8 shows representative photomicrographs of nuclear c-Fos within GFP-labeled Kiss 1 cells in a Kiss1hrGFP OVX+E female treated with either cholesterol (Fig. 8A–C) or corticosterone

(Fig. 8D–F). A robust population of Kiss1 cells were identified in the rostral, middle, and caudal regions of the arcuate nucleus using this mouse model, in which GFP expression is under the control of the Kiss1 promoter. Although treatment with estradiol did not alter the total number of Kiss1hrGFP cells detected (Fig. 8G–I) according to our methods, we did observe a 70% suppression in GFP intensity/cell in OVX+E females compared to OVX animals across all three regions of the arcuate hypothalamus. This may indicate estradiol-dependent regulation of the Kiss1 population in Kiss1hrGFP mice. Using this mouse model, we determined that the number of Kiss1hrGFP cells detected was not changed by chronic corticosterone in either OVX or OVX+E females (Fig. 8G–I); under these conditions, GFP intensity/cell was not altered by chronic corticosterone. Approximately 17–20% GFP-labeled Kiss1 cells contained c-Fos in OVX mice treated with cholesterol; in OVX mice, corticosterone did not significantly change co-expression in any region (Fig. 8J–L). In contrast, corticosterone induced a robust suppression in the percent of Kiss1 cells expressing c-Fos in OVX+E females compared to controls ($p<0.05$, 72–78% suppression); the suppression was present in all three regions of the arcuate nucleus.

## Discussion/Conclusion

The goal of the present study was to test the hypothesis that a chronic elevation in glucocorticoids can disrupt pulsatile LH secretion in female mice. This investigation was based on our prior finding that a chronic elevation of corticosterone impairs ovarian cyclicity in mice by preventing progression through the follicular phase [4]. We demonstrate that chronic corticosterone has no effect on LH secretion in OVX mice; however, in the presence of a physiologic level of estradiol, corticosterone is able to reduce LH secretion by slowing LH pulse frequency. The suppression in LH is not associated with reduced *Lhβ* or *Gnrhr* expression or impaired pituitary responsiveness to GnRH, suggesting corticosterone is acting upstream of the gonadotrope cell. As for a potential neuronal cell type mediating the suppression of pulsatile LH secretion, this study shows that a majority of arcuate Kiss1 neurons in the female mouse contain GR and that corticosterone decreases neuronal activation of this population of Kiss1 cells, as measured by c-Fos, in females treated with estradiol. Taken together, these data demonstrate that estradiol is necessary for corticosterone to impair the frequency of LH pulses in female mice, potentially via a direct action upon arcuate kisspeptin neurons.

A pertinent question raised by these findings is whether chronic corticosterone is acting directly or indirectly on arcuate kisspeptin cells to inhibit LH. Arcuate kisspeptin cells contain ERα [28], and the current study reveals that ~90% of arcuate Kiss1 neurons contain GR. Together, these observations identify the KNDy cell as a potential site whereby estradiol could enable corticosterone to impair pulsatile LH secretion. Our results in the mouse are consistent with studies in sheep. Considering that nearly all arcuate dynorphin cells coexpress kisspeptin in ewes [20], and that 80% of dynorphin cells are colocalized with GR [29], we can infer that a majority of arcuate kisspeptin neurons in the sheep contain GR. Interestingly, the female rat may demonstrate a species difference in both GR colocalization and sensitivity to corticosterone-induced LH suppression. Evidence that less than 3% of arcuate kisspeptin neurons express GR in diestrus female rats [45] offers a potential explanation for why OVX+E female rats did not demonstrate a disruption in LH pulsatile

secretion following either acute or chronic corticosterone [46]. Takumi et al. did observe that a majority of kisspeptin-containing cells within the anteroventral periventricular nucleus and periventricular nucleus continuum were found to express GR in female rats administered colchicine. Although interesting to consider a potential species difference, a direct comparison cannot be made as the gonad-status of the animals and GR antibody employed were also different. These findings do clearly show that corticosterone has the potential to act on KNDy cells, at least in the mouse, and provide the foundation for future studies utilizing neuronal specific GR knockouts to elucidate whether chronic corticosterone is acting directly on Kiss1 neurons or via afferent projections to this cell population.

Another question raised by these findings is the cellular mechanism whereby estradiol enables the suppression of pulse frequency by chronic corticosterone. A role for estradiol was demonstrated by previous work in female sheep which showed that elevated glucocorticoids decreased LH pulse frequency, also in an ovarian steroid-dependent manner, via a reduction in the frequency of GnRH pulses [6, 16]. We extend this observation by demonstrating that chronic corticosterone markedly impairs Kiss1 neuronal activity throughout the arcuate nucleus in an estradiol-dependent manner. Based on our current understanding that these cells function as the GnRH pulse generator, we hypothesized that both estradiol and corticosterone could induce changes directly within this population of cells to influence pulse frequency. We assessed levels of GR and determined that estradiol does not upregulate *Gr* expression nor increase GR colocalization in kisspeptin cells. In lieu of a change in GR expression, we explored transcriptional regulation by estradiol and corticosterone. Estradiol caused a robust reduction in *Tac2* and *Kiss1*, lowering levels by 40% and 70%, respectively, in OVX+E females; however, levels were not further reduced by corticosterone treatment.

Dynorphin is another interesting candidate that could mediate corticosterone-induced suppression of KNDy activity. Indeed, blocking dynorphin action with a kappa-opioid receptor antagonist elicits an increase in LH pulse frequency [47, 48] which is associated with increased multi-unit activity in the mediobasal hypothalamus [48]. We evaluated dynorphin expression in animals treated with chronic corticosterone and estradiol, based on observations that ER and GR can alter transcriptional activity via crosstalk at promoter response elements [49, 50], including at the dynorphin promoter leading to increased dynorphin expression [51]. However, the current study showed a decrease in dynorphin mRNA expression in response to corticosterone, suggesting upregulation of this inhibitory peptide is not likely to underlie the suppression in LH here. Interestingly, a few pieces of evidence support an alternate mechanism, whereby estradiol enhances the inhibitory action of dynorphin on the GnRH pulse generator. Electrophysiological recordings of KNDy cells in male mice showed that *in vivo* treatment with estradiol enhanced the inhibitory effect of dynorphin on KNDy neuron firing [52]. Furthermore, work in the female rat demonstrates that central delivery of a kappa-opioid receptor antagonist has no effect on the pulse generator in OVX animals, but prevents the negative feedback effect of estradiol on LH pulses in OVX+E females [53]. Future work is needed to test the possibility that estradiol enables chronic corticosterone to inhibit KNDy cells via a dynorphin-dependent pathway.

A couple of comments are warranted regarding the estradiol and corticosterone treatments employed in the current study. First, the estradiol replacement dose utilized here recapitulates a host of normal physiologic responses. For example, this dose restores peak serum corticosterone and prevents increased weight gain induced by OVX. Based on uterine weight, which is a simple and effective correlate of circulating estradiol [54], this estradiol dose mimics a diestrus level. Importantly for our studies, while this level of estradiol slows pulse frequency, the frequency and amplitude of LH pulsatile secretion is robust, and the detection of pulses is reliable as compared to published reports of LH profiles in diestrus mice that may be slower and lower in amplitude [55]. Therefore, we utilized this OVX+E model, which normalized estradiol levels across animals and removed other factors influenced by the ovarian cycle, to directly test the effect of estradiol on the LH response during a chronic elevation of corticosterone. Although the magnitude of the increment of corticosterone is physiologic [4, 35], the duration of this elevation exceeds that of an acute stress-induced increase, which would be lowered over time due to negative feedback. Whether an acute rise in corticosterone can also inhibit LH via a suppression in frequency in the female mouse remains to be tested. However, findings in OVX sheep that an acute increase in cortisol inhibits LH pulse amplitude via a pituitary mechanism without altering frequency [7] points to a potential role of glucocorticoid duration underlying LH suppression, in addition to a role of estradiol. Interestingly, we have shown that acute psychosocial restraint stress dramatically reduces LH pulse frequency in OVX mice, demonstrating that estradiol is not necessary for the suppression in frequency in response to this stress type [33]. Therefore, acute stressors may act via pathways independent of corticosterone or corticosterone may not require estradiol to inhibit LH pulses when administered acutely.

In summary, we utilized a female mouse model that allows for reliable detection of LH pulses to demonstrate that estradiol is necessary for chronic corticosterone to impair the frequency of LH pulses. We show that arcuate kisspeptin cells contain GR and demonstrate reduced kisspeptin neuronal activation in response to chronic corticosterone via an estradiol-dependent mechanism. Future studies are warranted to investigate acute elevations in glucocorticoids and whether a direct action by this stress hormone upon arcuate kisspeptin neurons mediates altered gonadotropin secretion commonly associated with stress [56, 57].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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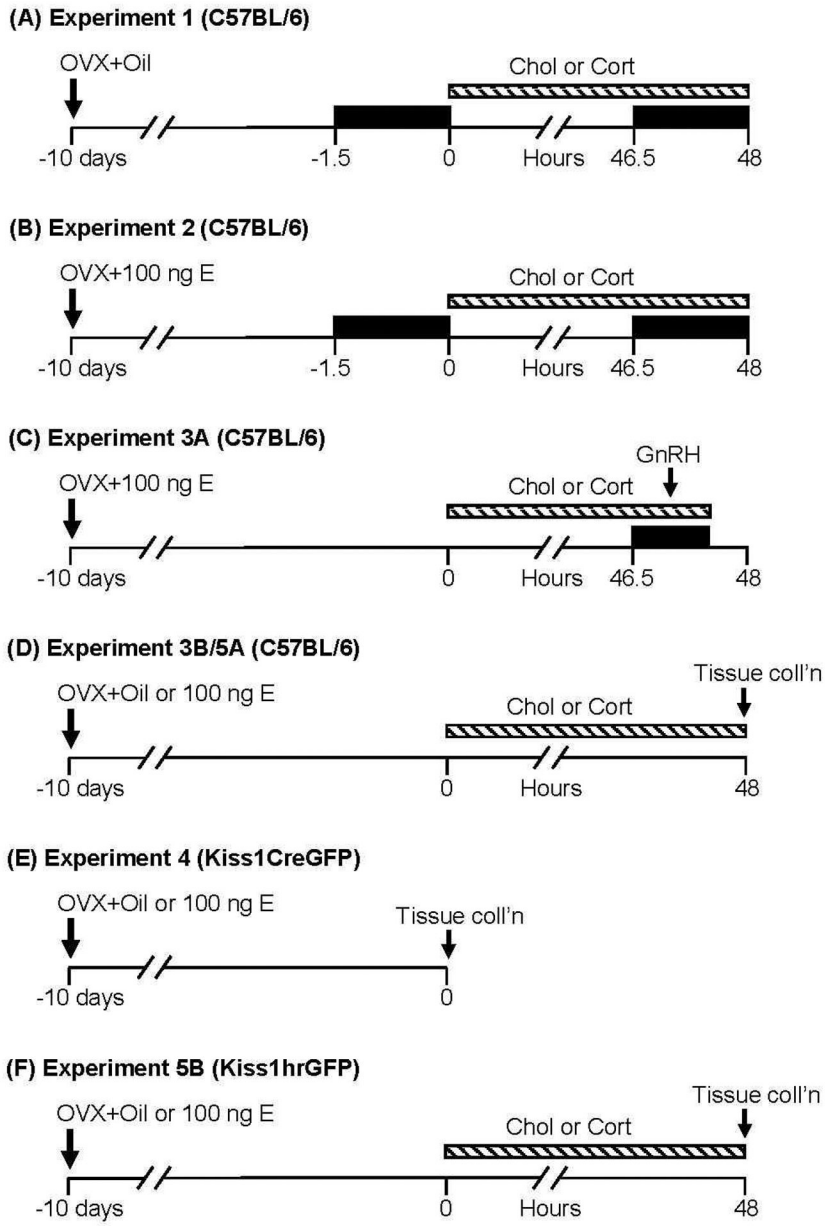


**Fig. 1. Animal strains and experimental designs.**

Time is depicted as hours relative to the implantation of corticosterone (Cort) or cholesterol (Chol), indicated by the dashed horizontal bar. Time of frequent sampling for LH analysis is identified by the black boxes. As indicated by the arrow, OVX and implantation with oil or estradiol (E) implant occurred 10 d prior to experimentation. GnRH (800 ng/kg, ip) was administered ~47 h after corticosterone or cholesterol in Experiment 3A. Time of tissue collection is indicated for Experiment 3B/5A (fresh frozen) and Experiment 4/5B (fixed).

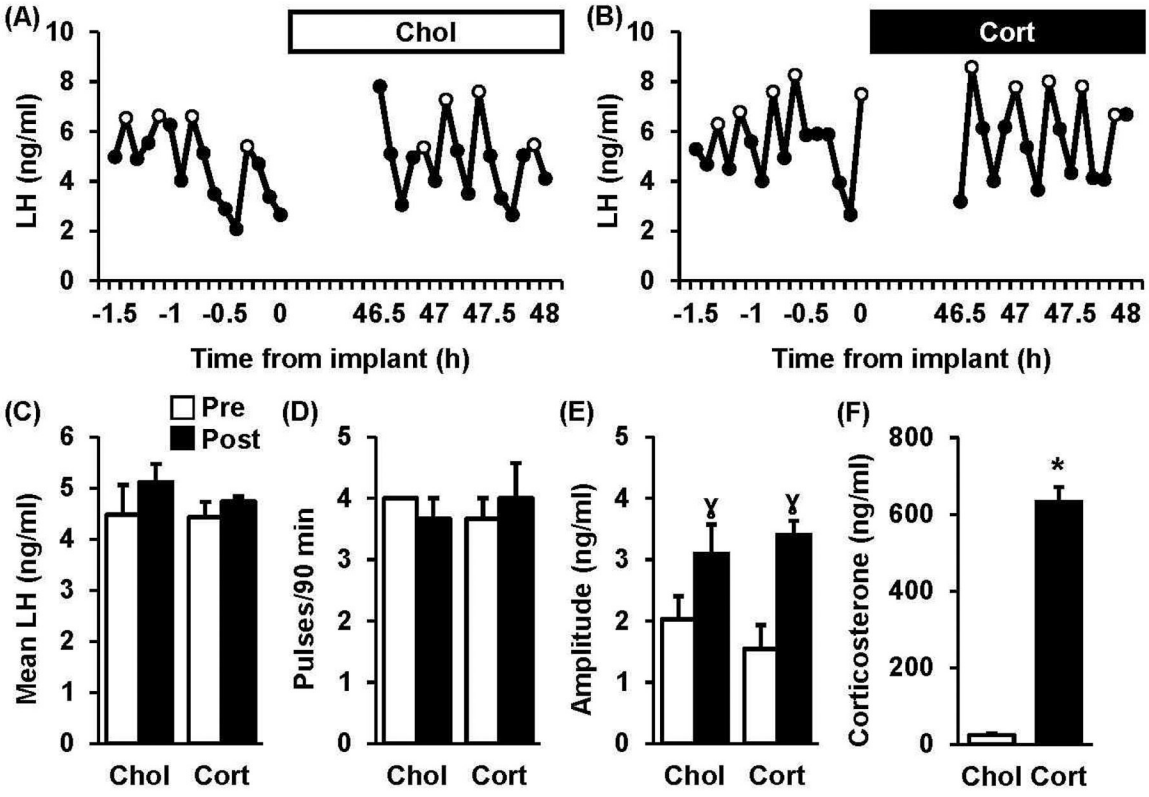


**Fig. 2. Chronic Corticosterone does not disrupt LH pulses in OVX mice.**

Pattern of pulsatile LH secretion in a representative OVX mouse treated with either cholesterol (A, Chol) or corticosterone (B, Cort) via a subcutaneous implant as depicted by the bar at the top of each panel. LH pulses are identified by open circles. Values (mean ± SEM) for mean LH (C), pulse frequency (D), and pulse amplitude (E) were calculated across time (Pre, white bars *vs.* Post, black bars) in each treatment group and analyzed (n=3 mice/group). Note, no significant time × treatment interactions were identified. γ, effect of time ($p<0.05$). (F) Serum corticosterone concentrations (mean ± SEM) in mice prior to the serial sampling which occurred 2 d following implantation (n=4–5 per group). *, effect of treatment ($p<0.05$).

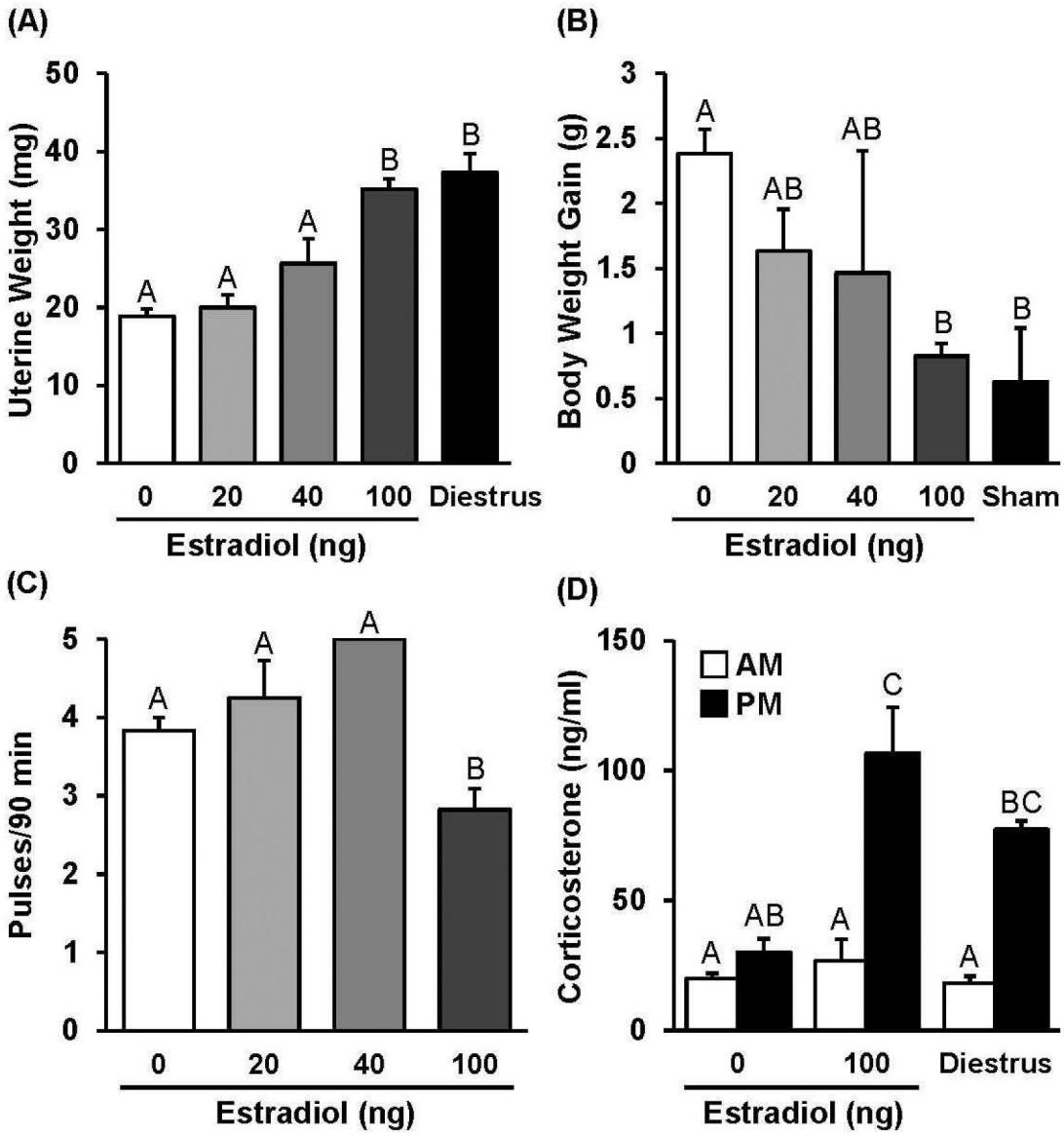


**Fig. 3. Assessment of OVX+E model.**

Mean (± SEM) uterine weight (A) or weight gain (B) in mice OVX and replaced with an implant containing 0, 20, 40, or 100 ng of estradiol, compared to a diestrus control group or a control group that were sham OVX and replaced with an oil implant. (C) Mean (± SEM) LH pulse frequency measured in OVX mice replaced with an implant containing 0, 20, 40, or 100 ng of estradiol. (D) Mean (± SEM) serum corticosterone concentrations measured at 0700 h (AM) and 1700 h (PM) following OVX plus 0 ng estradiol (OVX) or 100 ng estradiol (OVX+E) and compared to diestrus controls. Unique letters signify significant differences between values ($p<0.05$).

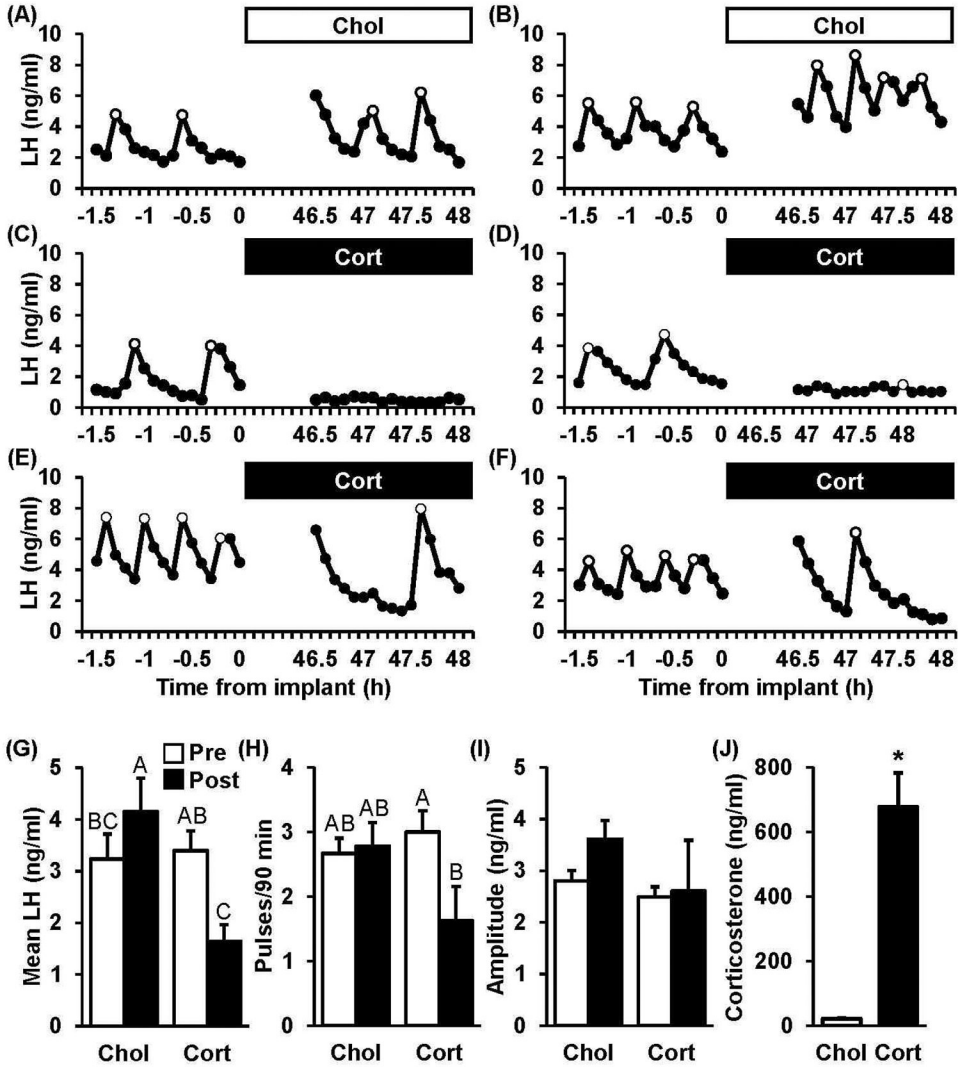


**Fig. 4. Chronic Corticosterone suppresses LH pulse frequency only in OVX+E mice.**

Patterns of pulsatile LH secretion in representative OVX+E mice, either implanted with cholesterol (A-B, Chol) or corticosterone (C-F, Cort). LH pulses are identified by open circles. Values (mean ± SEM) for mean LH(G), pulse frequency (H), and pulse amplitude (I) were compared across time (Pre, white bars *vs.* Post, black bars) in each treatment group (n=8–9 mice/group). Unique letters signify significant differences between values ($p<0.05$). (J) Serum corticosterone concentrations (mean ± SEM) in mice prior to the serial sampling which occurred 2 d following implantation (n=13/group). *, effect of treatment ($p<0.05$).

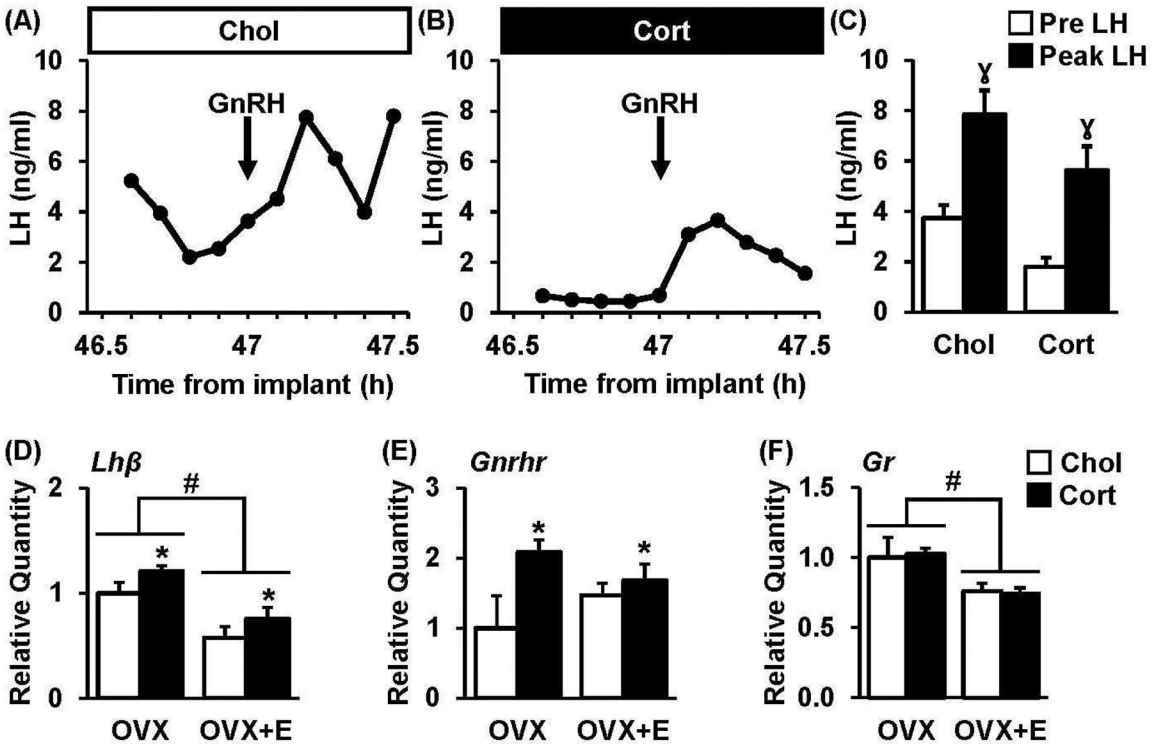


**Fig. 5. Chronic corticosterone does not impair pituitary responsiveness.**

Representative LH responses to GnRH in OVX+E mice treated with cholesterol (A, Chol) or corticosterone (B, Cort) and 800 ng/kg of GnRH (ip) at time 0. (C) Mean (± SEM) LH at time 0 (Pre LH) and in response to GnRH (Peak LH). γ, $p<0.05$, effect of time. Analysis of (D) *Lhβ*, (E) *Gnrhr*, and (F) *Gr* expression in individual pituitary glands collected from OVX or OVX+E mice treated with cholesterol (white bars) or corticosterone (black bars). Note, no significant estradiol × treatment interactions were identified. #, effect of estradiol; *, effect of corticosterone, $p<0.05$.

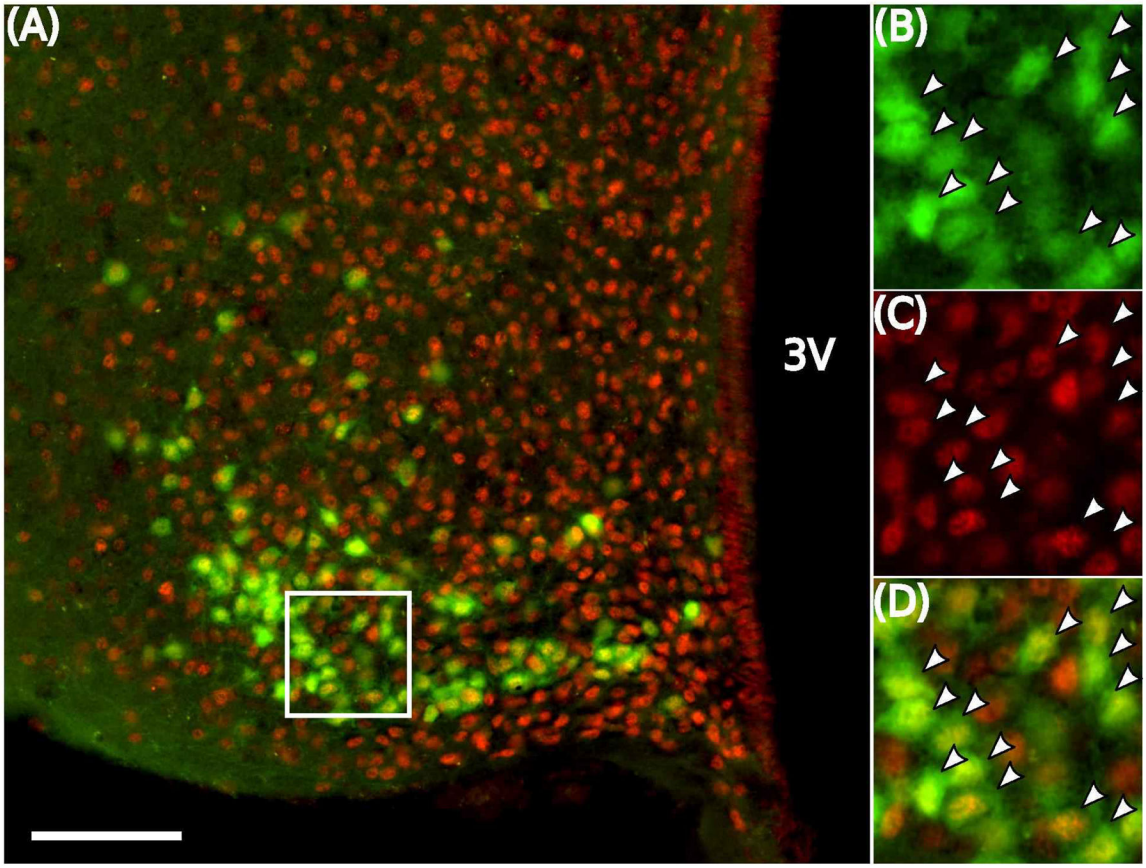


**Fig. 6. GR is expressed in Kiss1 cells of the mediobasal hypothalamus.**

Representative photomicrograph of dual-labeled Kiss1CreGFP and GR cells in the middle arcuate (A). White box indicates location of zoomed panels depicting staining for GFP (B, green), GR (C, red), and the merged image (D). White arrowheads indicate dual-labeled Kiss1CreGFP-GR cells. Scale bar equals 40 μm.

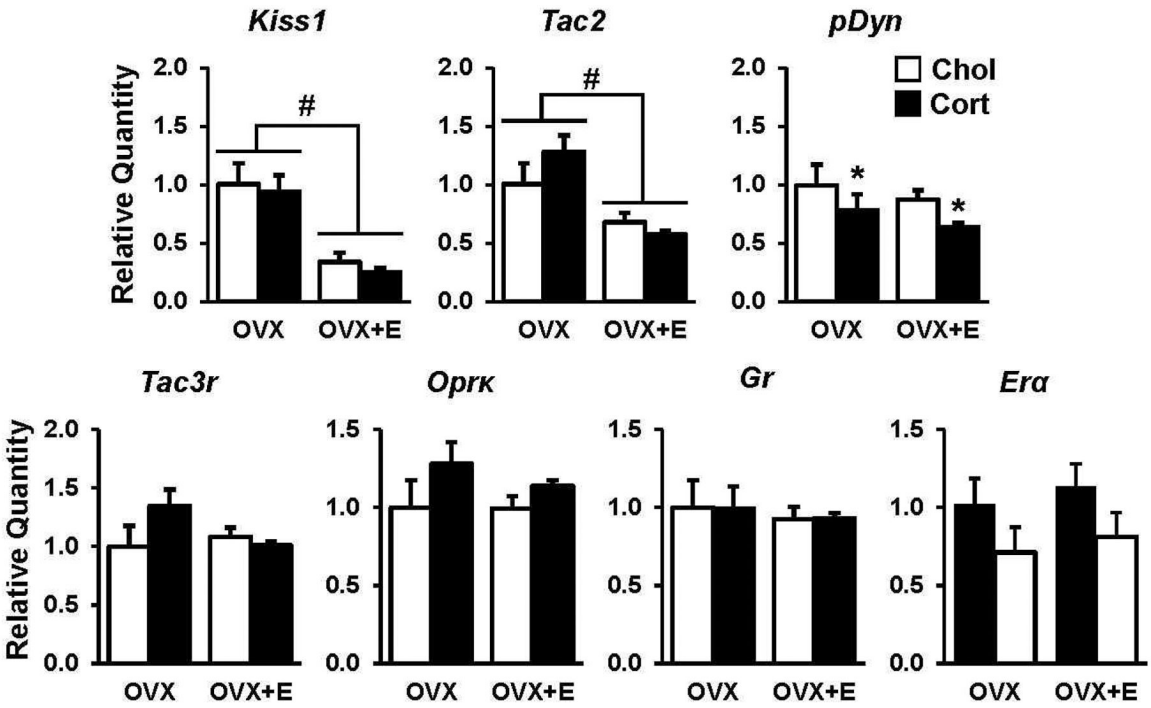


**Fig. 7. Chronic corticosterone does not suppress arcuate KNDy-associated gene expression.**

qPCR analysis of *Kiss1, Tac2, pDyn, Tac3r, OprK, Gr, and Erα* gene expression in arcuate micropunches collected from OVX or OVX+E mice and treated with cholesterol (white bars, Chol) or corticosterone (black bars, Cort). Note, no significant estradiol × treatment interactions were identified. #, effect of estradiol; *, effect of corticosterone, $p<0.05$. All data represented as mean ± SEM.

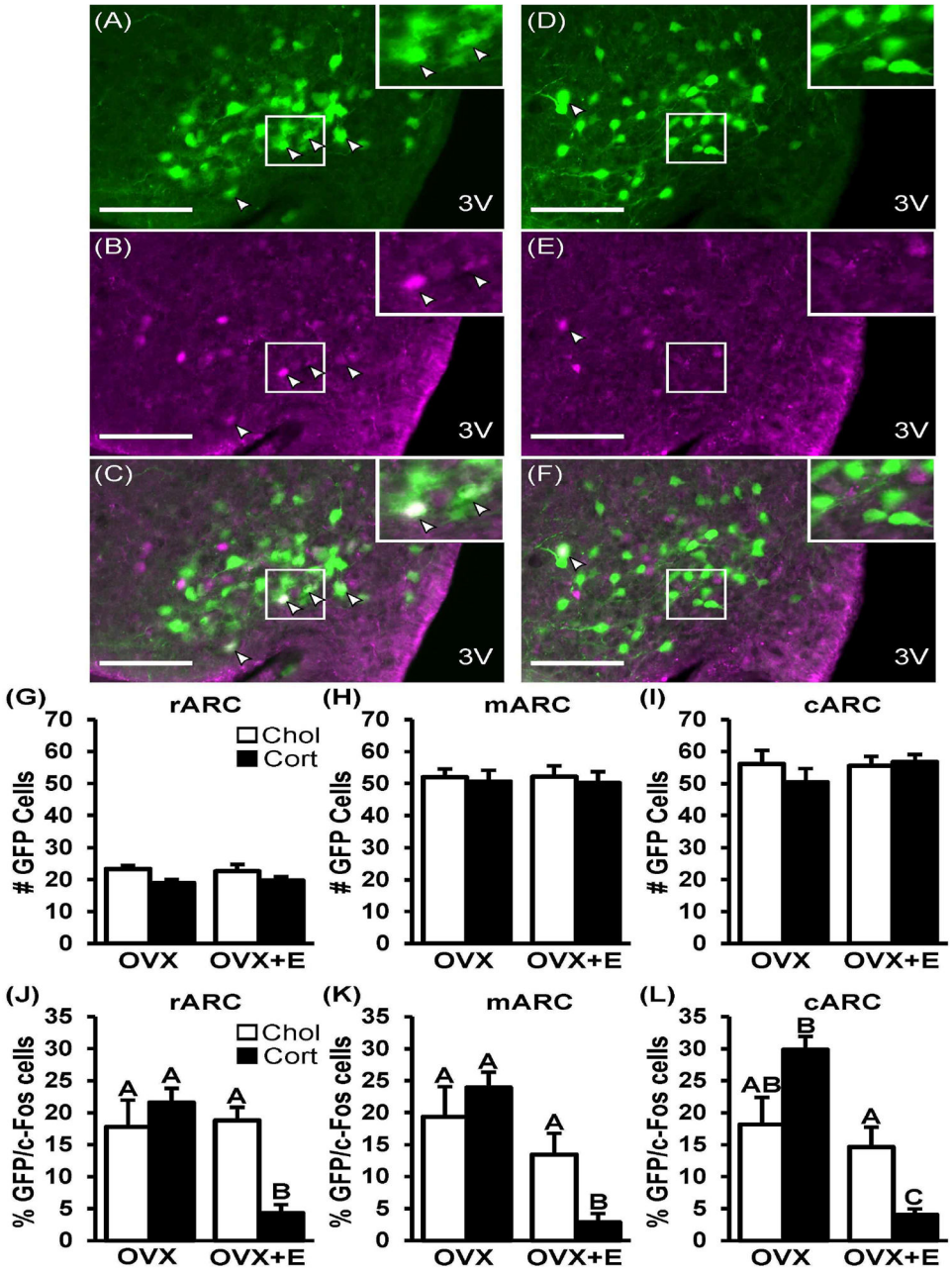


**Fig. 8. Corticosterone-induced suppression of Kiss1 neuronal activation requires estradiol.**

Representative photomicrographs depicting staining for Kiss1hrGFP (A and D, green), c-Fos (B and E, purple), and dual-labeled Kiss1/c-Fos cells (C and F) in the middle arcuate of OVX+E mice treated with cholesterol (A-C) or corticosterone (D-F). White box indicates location of zoomed panels. White arrowheads indicate dual labeled Kiss1/c-Fos cells. Scale bar equals 40 μm. Number of Kissl cells (G-I) and percent of Kiss1 cells with c-Fos (J-L) per hemi-section of OVX or OVX+E mice treated with cholesterol (white bars, Chol) or corticosterone (black bars, Cort). Regions of the arcuate hypothalamus are referred to as:

rostral (r), middle (m), and caudal (c). Back-transformed mean (± SEM) values are displayed. Unique letters signify significant differences between values ($p<0.05$).

**Table 1:**

Primary antibodies

| Peptide/protein target | Antigen Sequence (if known) | Name of Antibody | Manufacturer, Catalog # and/or name of individual providing the antibody | Species raised in; monoclonal or polyclonal | Dilution used | RRID |
|---|---|---|---|---|---|---|
| Glucocorticoid Receptor | Synthetic sequence from human GR: D(346) Q K P I F N V I P P I P V G S E N W N R C(367) | Glucocorticoid Receptor Polyclonal Antibody | Thermo Fisher Scientific Cat# PA1–511A | Rabbit; polyclonal | 1:500 | AB_2236340 |
| Green Fluorescent Protein | GFP from jellyfish (Aequorea Victoria). | GFP Polyclonal Antibody, Alexa Fluor 488 | Thermo Fisher Scientific Cat# A-21311 | Rabbit; polyclonal | 1:1000 | AB_221477 |
| c-Fos | N-terminus c-Fos | Anti-c-Fos Antibody | MilliporeSigma, Cat# ABE457 | Rabbit; polyclonal | 1:15,000 | AB_2631318 |

**Table 2:**

IHC for GFP and GR

| | OVX | OVX+E |
|---|---|---|
| # GFP Cells/hemi-section | | |
| rARC | 26.4 ± 8.0 | 24.2 ± 9.2 |
| mARC | 69.4 ± 9.5 | 54.9 ± 8.0 |
| cARC | 54.1 ± 9.4 | 74.2± 14.8 |
| % GFP Cells with GR | | |
| rARC | 86.0 ± 1.3$^{A}$ | 97.4 ± 1.3$^{B}$ |
| mARC | 93.3 ± 0.7 | 92.7 ± 3.1 |
| cARC | 96.2 ± 2.2 | 89.3 ± 4.0 |

Values are mean ± SEM.

Regions of the arcuate hypothalamus are referred to as: rostral (r), middle (m), and caudal (c).

Values with unique superscripts are significantly different between OVX and OVX+E ($p<0.05$).