

Regulation of *Kiss1* Gene Expression in the Brain of the Female Mouse

Jeremy T. Smith, Matthew J. Cunningham, Emilie F. Rissman, Donald K Clifton, and Robert A. Steiner

Departments of Physiology and Biophysics (J.T.S., M.J.C., R.A.S.) and Obstetrics and Gynecology (D.K.C., R.A.S.), University of Washington, Seattle Washington 98195-7290; and Department of Biochemistry and Molecular Genetics (E.F.R.), University of Virginia, Charlottesville, Virginia 22908

The *Kiss1* gene encodes a family of neuropeptides called kisspeptins, which activate the receptor G protein-coupled receptor-54 and play a role in the neuroendocrine regulation of GnRH secretion. We examined whether estradiol (E2) regulates KiSS-1 in the forebrain of the female mouse by comparing KiSS-1 mRNA expression among groups of ovary-intact (diestrus), ovariectomized (OVX), and OVX plus E2-treated mice. In the arcuate nucleus (Arc), KiSS-1 expression increased after ovariectomy and decreased with E2 treatment. Conversely, in the anteroventral periventricular nucleus (AVPV), KiSS-1 expression was reduced after ovariectomy and increased with E2 treatment. To determine whether the effects of E2 on KiSS-1 are mediated through estrogen receptor (ER) α or ER β , we evaluated the effects of E2 in OVX mice that lacked functional ER α or ER β . In OVX mice that lacked func-

tional ER α , KiSS-1 mRNA did not respond to E2 in either the Arc or AVPV, suggesting that ER α is essential for mediating the inhibitory and stimulatory effects of E2. In contrast, KiSS-1 mRNA in OVX mice that lacked functional ER β responded to E2 exactly as wild-type animals. Double-label *in situ* hybridization revealed that virtually all KiSS-1-expressing neurons in the Arc and AVPV coexpress ER α , suggesting that the effects of E2 are mediated directly through KiSS-1 neurons. We conclude that KiSS-1 neurons in the Arc, which are inhibited by E2, may play a role in the negative feedback regulation of GnRH secretion, whereas KiSS-1 neurons in the AVPV, which are stimulated by E2, may participate in the positive feedback regulation of GnRH secretion. (*Endocrinology* 146: 3686–3692, 2005)

SUCCESSFUL REPRODUCTION DEPENDS on tightly orchestrated communication between the brain-pituitary axis and the gonads. In the female, the tonic and cyclic release of LH and FSH occurs as a function of the negative and positive feedback effects of estradiol (E2) acting on the brain and pituitary (1, 2); however, the cellular and molecular basis for the differential effects of E2, negative and positive, on GnRH secretion are unknown. Although GnRH neurons appear to express estrogen receptor (ER) β (but not ER α) (3), it is generally thought that other steroid-sensitive neurons mediate the predominant physiological effects of sex steroids on GnRH secretion (4–6). Steroid receptors are expressed throughout the forebrain, particularly within the arcuate nucleus (Arc) and the anteroventral periventricular nucleus (AVPV) (7–9), and these areas are known to send projections to the preoptic area in close proximity to GnRH neurons (6, 10–12). Nevertheless, the phenotypic identities of steroid-sensitive neurons in the Arc and AVPV that couple directly to GnRH neurons have yet to be revealed.

The *Kiss1* gene codes for neuropeptides called kisspeptins, which bind to a G protein-coupled receptor, GPR54 (13–15).

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Abbreviations: Arc, Arcuate nucleus; AVPV, anteroventral periventricular nucleus; BnST, bed nucleus of the stria terminalis; DIG, digoxigenin; E2, estradiol; ER, estrogen receptor; ER α KO, mice that lack functional ER α ; ER β KO, mice that lack functional ER β ; GPR, G protein-coupled receptor; OVX, ovariectomized; PeN, periventricular nucleus; SBR, signal to background ratio; SCN, suprachiasmatic nucleus; WT, wild type.

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Kisspeptins and GPR54 have recently been implicated in the neuroendocrine regulation of GnRH secretion, based on several lines of evidence. First, mutations and targeted deletions in GPR54 in humans and mice result in sexual infantilism and hypogonadotropic hypogonadism, caused by GnRH deficiency (16–18). Second, KiSS-1 mRNA is expressed in the forebrain of the rat, mouse, and monkey (19–21), and administration of kisspeptin evokes GnRH-dependent gonadotropin secretion in these species (19, 21–23). Finally, sex steroids inhibit hypothalamic expression of KiSS-1 mRNA in the rat and monkey (20, 21, 24). Thus, it would appear that KiSS-1 neurons provide a link between the physiological action of sex steroids and the regulation of GnRH secretion; however, these initial studies of KiSS-1 regulation lack precise anatomical resolution. This limitation is compounded by the fact that KiSS-1 is expressed discretely in several hypothalamic nuclei, including the Arc and AVPV, which are each thought to play separate (and unique) roles in the regulation of GnRH and gonadotropin secretion (25, 26).

The objectives of our studies were 2-fold: to dissect the anatomical regulation of KiSS-1 mRNA expression by E2 in the hypothalamus and identify the molecular pathway through which E2 exerts its action. To study the regulation of *Kiss1* gene expression as a function of area, we examined the effects of E2 on KiSS-1 mRNA expression in individual nuclei of the female mouse. To determine whether the effects of E2 were mediated by either ER α or ER β , we evaluated the effects of E2 on KiSS-1 expression in mice bearing genetically targeted deletions of these receptors (ER α KO and ER β KO, respectively). Finally, to determine whether E2 acts directly on KiSS-1 neurons, we performed double-label *in situ* hy-

bridization for *KiSS-1* mRNA coupled with ER α or ER β mRNA.

Materials and Methods

Animals

Adult female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female ER α null mice were produced by breeding pairs, each carrying a single copy of the disrupted ER gene, as previously described (27). Female ER β null mice were purchased from Taconic (Germantown, NY). Animals were individually housed, maintained on a 12-h light, 12-h dark cycle (lights on at 0600 h) and had access to standard rodent chow and water *ad libitum*. All procedures were approved by the Animal Care Committee of the School of Medicine of the University of Washington as well as the Animal Use and Care Committee at the University of Virginia, in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Ovariectomy (OVX) and steroid replacement

Gonads were removed from adult mice under isoflurane inhalation anesthesia (Abbott Laboratory, North Chicago, IL) delivered by a vaporizer (Veterinary Anesthesia Systems, Bend, OR). Vasculature to the ovary was sutured and wound clips were used to close the incision. Immediately after OVX, E2-filled capsules were implanted sc via a small incision at the base of the neck; wound clips were used to close the incision.

For E2 implants, SILASTIC brand tubing (inner diameter = 1.47 mm; outer diameter = 1.95 mm; Dow Corning, Midland, MI) was cut to 9 mm; one end sealed with silicone cement and allowed to cure overnight. The dose of crystalline E2 (Sigma, St. Louis, MO) was based on a previous study (28) and involved packing silastic tubing with 4 mm of an E2/cholesterol mix (1:4). The day before surgery, implants were washed with 2 \times 10 min changes of 100% ethanol and then placed in physiological saline overnight. All untreated animals received empty (sham) capsules.

Experimental design

Experiment 1. The purpose of this experiment was to examine the effects of OVX and E2 replacement on hypothalamic *KiSS-1* mRNA. Female mice were divided into three groups ($n = 6-8$ per group): ovary intact (diestrus), castrated, and castrated plus E2 replacement. For intact mice, vaginal cytology was examined for two consecutive cycles. At approximately 0900 h either on diestrus or 7 d after castration, mice were weighed, anesthetized with isoflurane, and killed by decapitation. Trunk blood was collected for E2 and LH RIA. Brains were removed for *KiSS-1* mRNA *in situ* hybridization, frozen on dry ice, and then stored at -80 C until sectioned. Five sets of 20- μ m sections in the coronal plane were cut on a cryostat (from the diagonal band of Broca to the mammillary bodies), thaw mounted onto SuperFrost Plus slides (VWR Scientific, West Chester, PA), and stored at -80 C. A single set was used for *in situ* hybridization (adjacent sections 100 μ m apart).

Experiment 2. The purpose of this experiment was to determine whether E2 can regulate expression of *KiSS-1* mRNA in ER α KO female mice. Seven female ER α KO mice and seven wild-type (WT) littermates were OVX and four from each group received E2 replacement. Tissue collection and preparation for *KiSS-1* mRNA *in situ* hybridization was carried out as described in experiment 1.

Experiment 3. The purpose of this experiment was to determine whether E2 can regulate expression of *KiSS-1* mRNA in ER β KO female mice. Twelve female ER β KO mice and 12 WT littermates were OVX and half from each group received E2 replacement. Tissue collection and preparation for *KiSS-1* mRNA *in situ* hybridization occurred as described in experiment 1.

Experiment 4. The purpose of this experiment was to determine whether *KiSS-1* neurons in the female forebrain coexpress ER α and ER β . We performed double-label *in situ* hybridization on a set of coronal sections from brains of ovary-intact and OVX female mice (used in experiment

1, $n = 3-4$). Intact (diestrus) mice were used to examine coexpression in the AVPV and periventricular nucleus (PeN), whereas OVX mice were used to determine coexpression in the Arc because this treatment allows for adequate visualization of *KiSS-1* mRNA in the Arc via digoxigenin (DIG)-labeled riboprobes.

Radioimmunoassays

Serum levels of LH and E2 were measured at Northwestern University (Evanston, IL). Reagents for the LH assay were from the NIH, the antiserum used was anti-r-LH-S11, and the standard was rLH-RP3. The assay sensitivity was 0.2 ng/ml, and the intraassay coefficient of variation was 4%. E2 was measured with a double-antibody kit (Diagnostics Production Corp., Los Angeles, CA). The assay sensitivity was 2.0 pg/ml, and the intraassay coefficient of variation was 6%.

Radiolabeled *KiSS-1* cRNA riboprobes

Antisense and sense mouse *KiSS-1* riboprobes were generated as previously described (19). The *KiSS-1*-specific sequence spanned bases 76–486 of the mouse cDNA sequence (GenBank accession no. AF472576). We previously demonstrated that there is no signal with excess unlabeled antisense probe or radiolabeled sense probe (19).

In situ hybridization

Radioactive *in situ* hybridization was performed as described (29). Radiolabeled (32 P), antisense *KiSS-1* riboprobe was denatured, diluted in hybridization buffer at a concentration of 0.03 pmol/ml along with tRNA (2 mg/ml), and applied to slides (100 μ l/slide). After hybridization, slides were treated with RNase (32 μ g/ml), washed, and dehydrated as previously reported (29). Slides were then dipped in NTB-3 liquid emulsion (Eastman Kodak Co., Rochester, NY) and stored at 4 C. Slides were developed 3 d later.

KiSS-1 mRNA quantification and analysis

All slides were assigned a random three-letter code, alphabetized, and read unilaterally under dark-field illumination with custom software designed to count the total number of cells and the number of silver grains (corresponding to radiolabeled *KiSS-1* mRNA) over each cell (30). Cells were counted as *KiSS-1* mRNA positive when the number of silver grains in a cluster exceeded that of background. Data are expressed as the total number of identifiable cells and grains/cell (a semiquantitative index of mRNA content/cell).

Double-label *in situ* hybridization for *KiSS-1* mRNA/ER α mRNA and *KiSS-1* mRNA/ER β mRNA

The cDNA template for the ER α riboprobe was generated by PCR as previously described for GPR54 (20), with primers that were designed to contain promoters for T7 RNA polymerase in the antisense direction and T3 RNA polymerase in the sense direction (antisense: CCAAGCCTTC TAATACGACT CACTATAGGG AGAGGGAGCT CTCA-GATCG; sense: CAGAGATGCA ATTAACCCTC ACTAAAGGGA GAACCGCCCA TGATCTATTG). The ER α -specific sequence spanned bases 1163–1990 of the mouse cDNA sequence (GenBank accession no. NM_007956). Antisense and sense mouse ER α probes were transcribed from the cDNA template as previously described for the radiolabeled *KiSS-1* cRNA riboprobe (19).

The cDNA template for the ER β riboprobe was generated by PCR with primers as above for ER α (antisense: CCAAGCCTTC TAATACGACT CACTATAGGG AGACATCAGC ACCTCCATCC AGC; sense: CAGAGATGCA ATTAACCCTC ACTAAAGGGA GAACTCTCCT TTAGCCACCC ACTGC). The ER β -specific sequence spanned bases 662–1424 of the mouse cDNA sequence (GenBank accession no. NM_207707). Antisense and sense mouse ER β probes were transcribed from the cDNA template as above for ER α .

The cDNA template for the *KiSS-1* riboprobe was prepared as above for single label *in situ* hybridization. DIG-labeled antisense cRNA was synthesized with T7 RNA polymerase and DIG labeling mix (Roche, Indianapolis, IN) according to the manufacturer's protocol.

Slides were processed for *in situ* hybridization as above with mod-

ifications. Radiolabeled antisense ER α (0.03 pmol/ml) or ER β (0.05 pmol/ml) and DIG-label KiSS-1 riboprobes (concentration determined empirically) were denatured, dissolved in the same hybridization buffer along with tRNA (1.9 mg/ml), and applied to slides. Slides were hybridized, treated with RNase, and washed as above. KiSS-1 mRNA-positive cells were visualized using anti-DIG fragments conjugated to alkaline phosphatase (diluted 1:300; Roche) and Vector Red substrate (SK-5100; Vector Laboratories, Burlingame, CA) under the manufacturer's directions. Slides were dipped in 70% ethanol, air dried, and then dipped in NTB-3 liquid emulsion (Eastman Kodak). Slides were developed 7–18 d later.

KiSS-1 mRNA-containing cells were identified under fluorescent illumination, and custom-designed software was used to count the silver grains (corresponding to radiolabeled ER α or ER β mRNA) over each cell (30). Signal to background ratios (SBRs) for individual cells were calculated; a cell was considered to be double labeled if it had a SBR of 3 or more. For each animal, the amount of double labeling was calculated as a percentage of the total number of KiSS-1 mRNA-expressing cells and then averaged across animals to produce a mean \pm SEM.

Statistical analysis

All data are expressed as mean \pm SEM for each group. Variation in KiSS-1 mRNA expression among treatment groups was assessed by one-way ANOVA. For experiments 3 and 4, variation among genotype and treatment groups was assessed by two-way ANOVA. Where the F test for the ANOVA reached statistical significance ($P < 0.05$), differences among means were assessed by least significant difference tests. All analyses were performed with Statview 5.0.1 for Macintosh (SAS Institute, Cary, NC).

Results

Distribution of KiSS-1 mRNA in the brain of the female mouse

Cells expressing KiSS-1 mRNA were readily identifiable in the Arc, AVPV, and PeN of all animals. A few cells were found in the anterodorsal preoptic area, medial amygdala, and bed nucleus of the stria terminalis (BnST). The magnitude of KiSS-1 expression (as reflected by grain counts) was most robust in the Arc and AVPV (Fig. 1), and data from these areas and the PeN are presented in the descriptions that follow.

Experiment 1: effects of OVX and E2 replacement on KiSS-1 mRNA in the forebrain

In the Arc, OVX increased the number of cells expressing KiSS-1 mRNA by 2.6-fold ($P < 0.0001$) and increased the cellular content of KiSS-1 mRNA (as reflected by grains per cell) by 1.7-fold ($P < 0.0001$), compared with intact controls.

Treatment with E2 completely reversed the effects of OVX on both cell number and per cell content of KiSS-1 mRNA (both $P < 0.0001$; OVX *vs.* OVX + E2; Fig. 2). In the AVPV, the results were the opposite of those found in the Arc. OVX decreased the number of identifiable KiSS-1 mRNA-containing cells by 58% ($P < 0.01$), and E2 treatment restored this number to that of intact mice ($P < 0.01$; OVX *vs.* OVX + E2; Fig. 2). A similar effect was seen for mRNA content/cell ($P < 0.001$; intact *vs.* OVX; $P < 0.0001$; OVX *vs.* OVX + E2; Fig. 2). Results in the PeN were similar to those in the AVPV; however, in the PeN cell number differed significantly only between OVX and OVX + E2 ($P < 0.01$). KiSS-1 mRNA content/cell in the PeN decreased with OVX (52%, $P < 0.01$) and was restored by E2 treatment (Fig. 2).

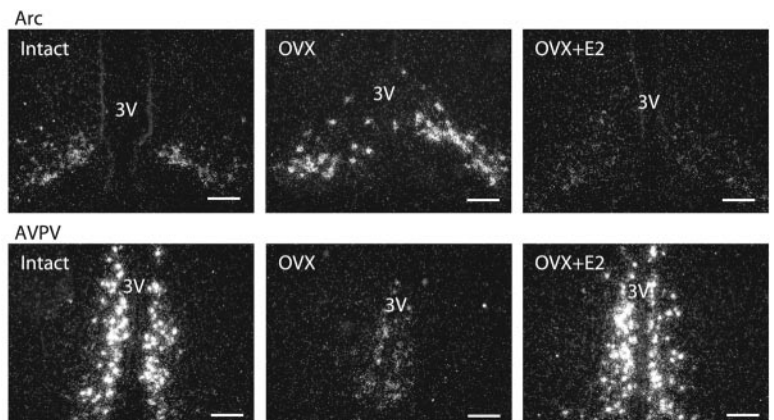
Experiment 2: the effects of OVX and E2 replacement on KiSS-1 mRNA in the forebrain of female ER α KO mice

E2 had no effect on the expression of KiSS-1 mRNA in OVX ER α KO mice in any anatomical area (Fig. 3 and Table 1). The effects of E2 in the WT animals confirmed those found in experiment 1. In the Arc of WT animals, E2 treatment reduced the number of identifiable KiSS-1 neurons and the per-cell content of KiSS-1 mRNA by 56 and 55%, respectively (compared with OVX/sham-treated controls; $P < 0.01$ for both). In the AVPV of WT mice, compared with OVX controls, E2 treatment significantly increased the number of identifiable KiSS-1 cells by 2.4-fold ($P < 0.01$) and increased the KiSS-1 mRNA grains per cell by 1.6-fold, although that increase was not statistically significant. A similar effect of E2 was seen in the PeN of WT mice. Here, E2 treatment significantly increased KiSS-1 cell number by 2.2-fold ($P < 0.01$) and also led to a 1.6-fold increase in KiSS-1 mRNA grains/cell that was not statistically significant.

Experiment 3: the effects of OVX and E2 replacement on KiSS-1 mRNA in the forebrain of female ER β KO mice

The effect of E2 on the expression of KiSS-1 mRNA in OVX ER β KO mice was indistinguishable from its effect in OVX WT animals, with E2 reducing the expression of KiSS-1 in the Arc and increasing its expression in the AVPV and PeN (Table 2). This was confirmed by two-way ANOVA, which detected a significant effect of treatment ($P < 0.001$), but not a significant effect of genotype nor a significant interaction

FIG. 1. Dark-field photomicrographs showing KiSS-1 mRNA-expressing cells (as reflected by the presence of white clusters of silver grains) in representative sections of the Arc and AVPV from ovary-intact, OVX, OVX + E2 mice. 3V, Third ventricle. Scale bars, 100 μ m.



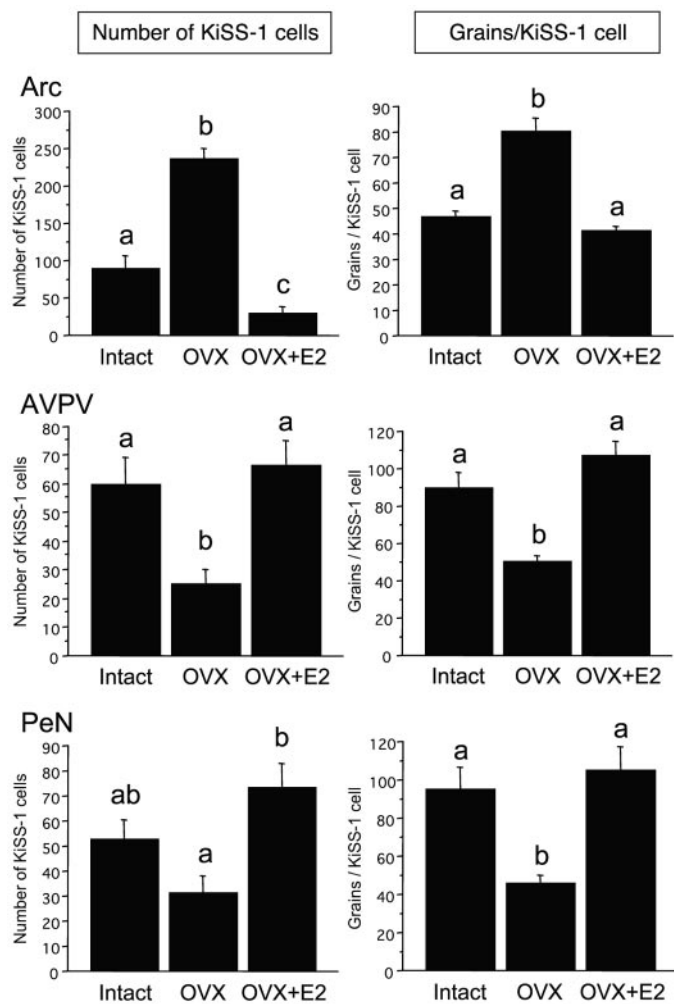


FIG. 2. The number of identifiable KiSS-1 mRNA-positive cells and grains per KiSS-1 cell after OVX and E2 replacement in the Arc, AVPV, and PeN. Values without *common notations* (a, b, c) differ significantly ($P < 0.01$). Values are presented as the mean \pm SEM.

between treatment and genotype for both the number of cells and grains per cell in each area.

Experiment 4: *KiSS-1* mRNA coexpression with *ER α* and *ER β* mRNAs

Cells expressing *ER α* mRNA were observed in areas in which they have previously been reported, including the preoptic area, AVPV, PeN, Arc, ventromedial hypothalamus, medial amygdala, and BnST. The vast majority of identifiable KiSS-1 mRNA-positive neurons in the Arc, AVPV, and PeN had clusters of silver grains (representing *ER α* mRNA) overlying them (Fig. 4A). Quantitative analysis, with a criterion for double labeling of signal 3 times over background, indicated that $99.8 \pm 0.1\%$ of all KiSS-1 mRNA-expressing cells in the Arc, $98.7 \pm 0.8\%$ of all KiSS-1 mRNA-expressing cells in the AVPV, and $97.9 \pm 1.8\%$ of all KiSS-1 mRNA-expressing cells in the PeN also expressed *ER α* mRNA.

Cells expressing *ER β* mRNA were observed in the preoptic area, AVPV, PeN, Arc, ventromedial hypothalamus, medial amygdala, BnST, and the paraventricular nucleus.

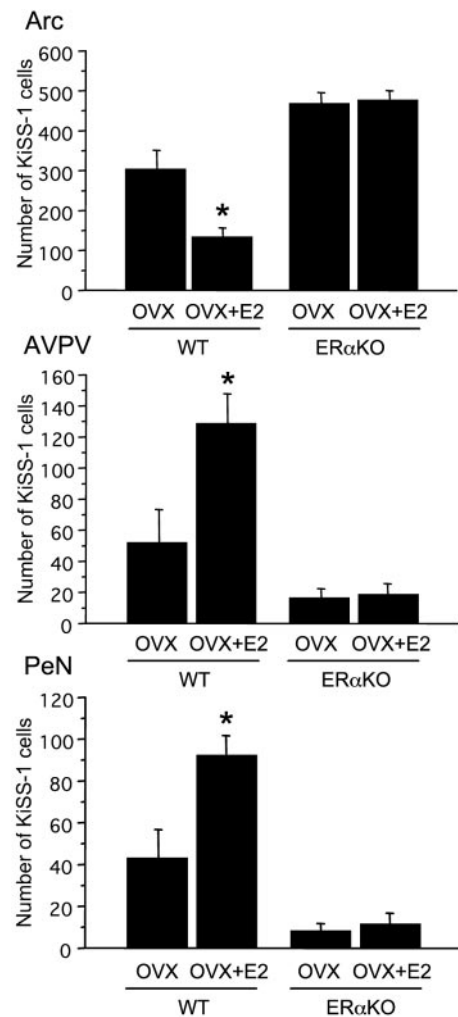


FIG. 3. Effect of E2 replacement on the number of KiSS-1-expressing cells in the Arc, AVPV, and PeN in OVX ER α KO and WT controls. Data are presented as the mean \pm SEM. *, $P < 0.01$.

Numerous identifiable KiSS-1 mRNA-positive neurons also possessed clusters of silver grains (representing *ER β* mRNA) overlying them (Fig. 4B). Quantitative analysis, with a criterion for double labeling of signal 3 times over background, indicated that that $25.0 \pm 2.6\%$ of all KiSS-1 mRNA-expressing cells in the Arc, $30.5 \pm 5.1\%$ of all KiSS-1 mRNA-expressing cells in the AVPV, and $43.3 \pm 10.8\%$ of all KiSS-1 mRNA-expressing cells in the PeN also expressed *ER β* mRNA.

TABLE 1. KiSS-1 mRNA expression (grains/cell) in the Arc, AVPV, and PeN from OVX and OVX with E2-replaced WT and ER α KO mice (experiment 2)

KiSS-1 mRNA (grains/cell)	WT		ER α KO	
	OVX (n = 6)	OVX+E2 (n = 6)	OVX (n = 6)	OVX+E2 (n = 6)
Arc	128 \pm 21	58 \pm 6 ^a	189 \pm 5	170 \pm 4
AVPV	101 \pm 29	167 \pm 4	30 \pm 2	31 \pm 2
PeN	114 \pm 35	185 \pm 3	35 \pm 4	23 \pm 5

Data are presented as the mean \pm SEM for OVX and OVX plus estradiol-treated (OVX+E2), WT, and ER α KO mice.

^a $P < 0.001$, compared with OVX alone.

TABLE 2. KiSS-1 mRNA expression in the Arc, AVPV, and PeN from OVX and OVX with E2-replaced WT and ER β KO mice (experiment 3)

KiSS-1 mRNA	WT		ER β KO	
	OVX (n = 6)	OVX+E2 (n = 6)	OVX (n = 6)	OVX+E2 (n = 6)
Arcuate				
Number of cells	334 \pm 31	157 \pm 21 ^a	369 \pm 20	90 \pm 14 ^a
Grains/cell	192 \pm 9	60 \pm 4 ^a	191 \pm 9	47 \pm 3 ^a
AVPV				
Number of cells	41 \pm 10	110 \pm 11 ^a	27 \pm 5	104 \pm 5 ^a
Grains/cell	69 \pm 8	194 \pm 5 ^a	73 \pm 10	186 \pm 5 ^a
PeN				
Number of cells	25 \pm 2	53 \pm 5 ^a	36 \pm 3	68 \pm 11 ^a
Grains/cell	64 \pm 6	178 \pm 10 ^a	76 \pm 9	180 \pm 10 ^a

Data are presented as the mean \pm SEM for OVX and OVX plus estradiol-treated (OVX+E2), WT, and ER β KO mice.

^a $P < 0.001$, compared with OVX alone.

Body weight and serum hormone concentrations

Body weight gain during the 7-d treatment period was similar among intact and OVX groups in all experiments, whereas OVX + E2 treated mice gained slightly more weight ($P < 0.05$, data not shown). Serum E2 levels were, as expected, undetectable in OVX mice, within the physiological range in diestrus mice (average 8 ± 3 pg/ml) and elevated in OVX mice treated with E2 (average 188 ± 16 pg/ml). Serum levels of E2 did not appear to differ between WT and ER α KO or ER β KO mice (data not shown). Serum LH levels were measured for experiment 1 only and as expected were minimal in intact mice (0.5 ± 0.2 ng/ml), elevated with OVX (3.7 ± 0.5 ng/ml), and undetectable in OVX mice treated with E2.

Discussion

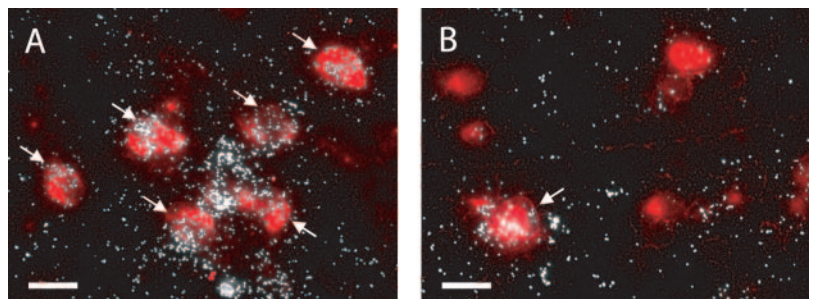
This study demonstrates that E2 differentially regulates KiSS-1 mRNA expression across different nuclei in the forebrain of the mouse. In the Arc, E2 inhibits the expression of KiSS-1 mRNA. These results are consonant with an earlier report on studies in the female rat in which it was demonstrated, based on gross dissections of the diencephalon, that treatment of OVX animals with E2 reduces total hypothalamic content of KiSS-1 mRNA (24). However, using techniques that permit quantitative analysis of specific nuclei in the forebrain, we discovered that E2 stimulates KiSS-1 mRNA in the AVPV and PeN. This observation demonstrates that E2 exerts anatomically specific and sometimes opposing effects on KiSS-1 expression in the brain. Both the stimulatory and inhibitory effects of E2 on KiSS-1 mRNA appear to be

mediated by ER α because KiSS-1 expression is unresponsive to E2 treatment in mice with genetically targeted deletions of this ER receptor isoform. Finally, the effect of E2 on KiSS-1 mRNA is likely to be direct on KiSS-1 neurons because virtually all KiSS-1 mRNA-expressing cells in the forebrain also coexpress ER α mRNA.

Steroid-sensitive projections from the Arc to the medial preoptic area have long been implicated for a role in the negative feedback control of GnRH secretion by E2 (7–12, 31). The identity of the cells that mediate this signal is unknown, but KiSS-1 neurons fulfill most of the major criteria. First, GnRH neurons are direct targets for kisspeptin because most GnRH neurons express the kisspeptin receptor (20, 32). Second, kisspeptin activates GnRH neurons (inducing Fos expression) and stimulates GnRH-dependent gonadotropin secretion (19, 20, 22, 23). Third, the present results demonstrate that all KiSS-1 neurons express ER α , which is thought to mediate the predominant effects of sex steroids on GnRH secretion (4, 33). Finally, low circulating levels of E2 (*e.g.* OVX) stimulate KiSS-1 mRNA expression in the Arc, whereas high levels of E2 inhibit the expression of KiSS-1 in the Arc. Together these observations suggest that afferents from KiSS-1 neurons in the Arc provide tonic stimulatory input to GnRH neurons, which increases in the absence and decreases in the presence of E2 and that E2 acts directly on KiSS-1 neurons to regulate the expression of the KiSS-1 mRNA through ER α .

In the AVPV and PeN, E2 induces the expression of KiSS-1 mRNA, which is precisely the opposite effect of E2 in the Arc. The AVPV is a sexually dimorphic nucleus and is recognized to play an important role in the preovulatory surge of GnRH and LH (26, 34). KiSS-1 mRNA expression in the AVPV is sexually differentiated, with the female AVPV harboring far more KiSS-1-positive cells than the male (35). Furthermore, the expression of KiSS-1 mRNA in the AVPV is elevated during the afternoon of proestrus in the female rat, during the time of the LH surge (Smith, J. T., S. M. Popa, D. K. Clifton, and R. A. Steiner, unpublished observations). If KiSS-1 neurons in the AVPV are necessary for the generation of the GnRH/LH surge, it would appear that increased KiSS-1 expression alone is insufficient for its generation because elevated levels of KiSS-1 mRNA in the AVPV were not accompanied by high levels of circulating LH (as shown in experiment 1). This is not unexpected because the generation of the GnRH/LH surge in the rodent is dependent on not only high levels of E2 (which induces KiSS-1 in the AVPV) but also a circadian signal that restricts the LH surge to the late afternoon (36, 37). Thus, if KiSS-1 neurons in the AVPV

FIG. 4. Representative photomicrographs showing co-expression of KiSS-1 mRNA with ER α (A) and ER β (B). KiSS-1 mRNA-expressing cells are fluorescent with Vector Red substrate, and clusters of silver grains reflect the presence of ER α (A) or ER β (B) mRNA. The arrows indicate KiSS-1 neurons that coexpress either ER α or ER β . Scale bars, 20 μ m.



are involved in generating the LH surge, it seems likely that they are coupled to circadian circuits that originate in the suprachiasmatic nucleus (SCN) (38–40). The SCN sends projections to estrogen-responsive neurons in the AVPV (41–43), and the AVPV in turn sends projections to GnRH neurons (11, 34, 44). Thus, it is plausible that estrogen-sensitive KiSS-1 neurons in the AVPV are the conduit between the circadian oscillator in the SCN and GnRH neurons in the medial preoptic area.

The molecular mechanisms by which E2 produces reverse effects on KiSS-1 mRNA expression in the Arc and AVPV are unknown. Whereas E2 apparently acts through the same receptor (ER α) to produce opposite results in the Arc and AVPV/PeN, it seems likely that something intrinsic to the two cell types causes the opposing regulatory effects of E2. We initially postulated that the opposing effect of E2 in these two regions might be attributed to differential expression of ER isoforms (ER α and ER β) between these regions of the brain. These two ER isoforms have been shown to produce opposing molecular actions (45, 46). ER α and ER β are colocalized in many areas of the forebrain and can form heterodimers (47). In the presence of ER α /ER β heterodimers, ER β appears to be the dominant regulator and can oppose ER α -mediated gene transcription (45). In the AVPV, there appears to be a greater ratio of ER β to ER α expression than that in the Arc (8). However, our data show that in the absence of ER α , both negative (in the Arc) and positive (in the AVPV) regulation of KiSS-1 mRNA by E2 is completely voided, whereas in the absence of ER β , E2 fully retains its capacity to regulate KiSS-1. Because all KiSS-1 cells in the Arc, AVPV, and PeN express ER α and only a minority coexpress ER β , it would appear that the ER β isoform does not play a significant role in mediating the effects of E2 on KiSS-1 mRNA expression. Thus, it is probable that regional differences in the regulation of KiSS-1 expression are the result of E2 acting through ER α alone, which may recruit coactivators of transcription in KiSS-1 neurons of the AVPV and corepressors of transcription in the Arc (48).

Our results demonstrate that the expression of ER β is not essential for the regulation of KiSS-1 mRNA by E2. In the Arc, the decline in KiSS-1 mRNA in response to E2 appeared greater in the ER β KO than in WT mice. It is tempting to speculate that under normal circumstances the formation of ER β /ER α heterodimers in KiSS-1 neurons in the Arc attenuates E2-dependant transcriptional regulation of the *Kiss1* gene (45). Thus, in the absence of ER β , the inhibitory effect of E2 on transcription of KiSS-1 is enhanced.

OVX mice lacking ER α appeared to express more KiSS-1 mRNA in the Arc and less in the AVPV, compared with OVX WT controls. One explanation for this phenomenon may be that ER α retains a small level of intrinsic activity in WT OVX animals. This could reflect low-level binding of estrogenic ligands that remain despite OVX. Alternatively, ER may be able to activate/repress KiSS-1 expression, even when unoccupied. However, a more likely possibility is that the congenital absence of ER α alters the development of KiSS-1-expressing neurons, leading to altered basal KiSS-1 expression in the adult. This argument seems tenable, considering that perinatal exposure to E2 has long been known to direct the formation of sexually dimorphic circuits within

the brain, particularly within the AVPV, and at least part of this effect is thought to be mediated by ER α (12). Whether KiSS-1 neurons are organized in a similar fashion and whether their development is altered in the absence of ER α is yet to be determined.

In summary, we have shown that E2 differentially regulates the expression of KiSS-1 mRNA in distinct forebrain nuclei and that these effects are mediated by ER α . We have also shown that the effect of E2 on KiSS-1 mRNA appears to be direct on these cells because nearly all KiSS-1 neurons (in all hypothalamic regions) coexpress ER α mRNA. We conclude that KiSS-1 neurons in the Arc are likely to be involved in the E2-mediated negative feedback control of gonadotropin secretion, whereas KiSS-1 neurons in the AVPV and PeN are involved in E2-mediated positive feedback control of gonadotropin secretion.

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Address all correspondence and requests for reprints to: Robert A. Steiner, Department of Physiology and Biophysics, Health Sciences Building, G-424, School of Medicine, University of Washington, Box 357290, Seattle, Washington 98195-7290. E-mail: steiner@u.washington.edu.

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