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

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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 ERβ, we evaluated the effects of E2 in OVX mice that lacked functional ERα or ERβ. In OVX mice that lacked functional ERα, KiSS-1 mRNA did not respond to E2 in either the Arc or AVPV, suggesting that ERα is essential for mediating the inhibitory and stimulatory effects of E2. In contrast, KiSS-1 mRNA in OVX mice that lacked functional ERβ 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 ERα, 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 ERα) (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). 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 ERα or ERβ, we evaluated the effects of E2 on KiSS-1 expression in mice bearing genetically targeted deletions of these receptors (ERαKO and ERβKO, respectively). Finally, to determine whether E2 acts directly on KiSS-1 neurons, we performed double-label *in situ* hy-

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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; ERαKO, mice that lack functional ERα; ERβKO, mice that lack functional ERβ; 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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bridization for KiSS-1 mRNA coupled with ERα or ERβ mRNA.

Materials and Methods

Animals

Adult female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female ERα null mice were produced by breeding pairs, each carrying a single copy of the disrupted ER gene, as previously described (27). Female ERβ 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 × 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-μm 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 μm apart).

Experiment 2. The purpose of this experiment was to determine whether E2 can regulate expression of KiSS-1 mRNA in ERαKO female mice. Seven female ERαKO 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 ERβKO female mice. Twelve female ERβKO 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 ERα and ERβ. 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 (^{33}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 μl/slide). After hybridization, slides were treated with RNase (32 μg/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/ERα mRNA and KiSS-1 mRNA/ERβ mRNA

The cDNA template for the ERα 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 CTCAGATCG; sense: CAGAGATGCA ATTAACCCTC ACTAAAGGGA GAACCGCCCA TGATCTATTC TG). The ERα-specific sequence spanned bases 1163–1990 of the mouse cDNA sequence (GenBank accession no. NM_007956). Antisense and sense mouse ERα probes were transcribed from the cDNA template as previously described for the radiolabeled KiSS-1 cRNA riboprobe (19).

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

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 ERα (0.03 pmol/ml) or ERβ (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 ERα or ERβ 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 ± SEM.

Statistical analysis

All data are expressed as mean ± 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 ERαKO mice

E2 had no effect on the expression of KiSS-1 mRNA in OVX ERαKO 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 ERβKO mice

The effect of E2 on the expression of KiSS-1 mRNA in OVX ERβKO 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

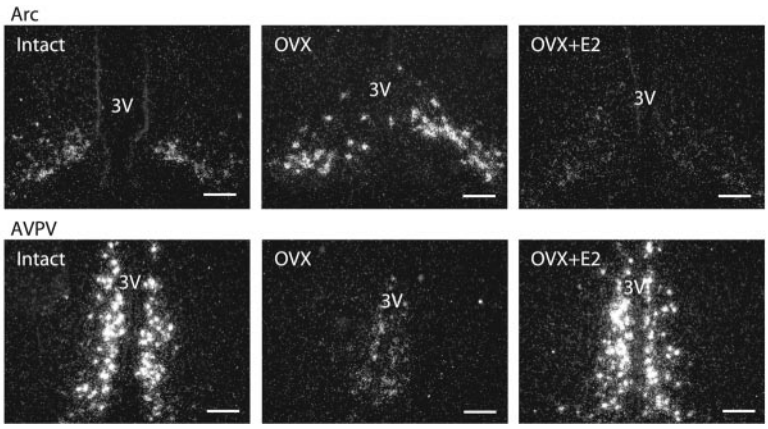

FIG. 1. Dark-field photomicrographs showing KiSS-1 mRNA-expressing cells (as reflected by the presence of *white clusters of silver grains*) in representative sections of the Arc and AVPV from ovary-intact, OVX, OVX + E2 mice. 3V, Third ventricle. *Scale bars*, 100 μm.

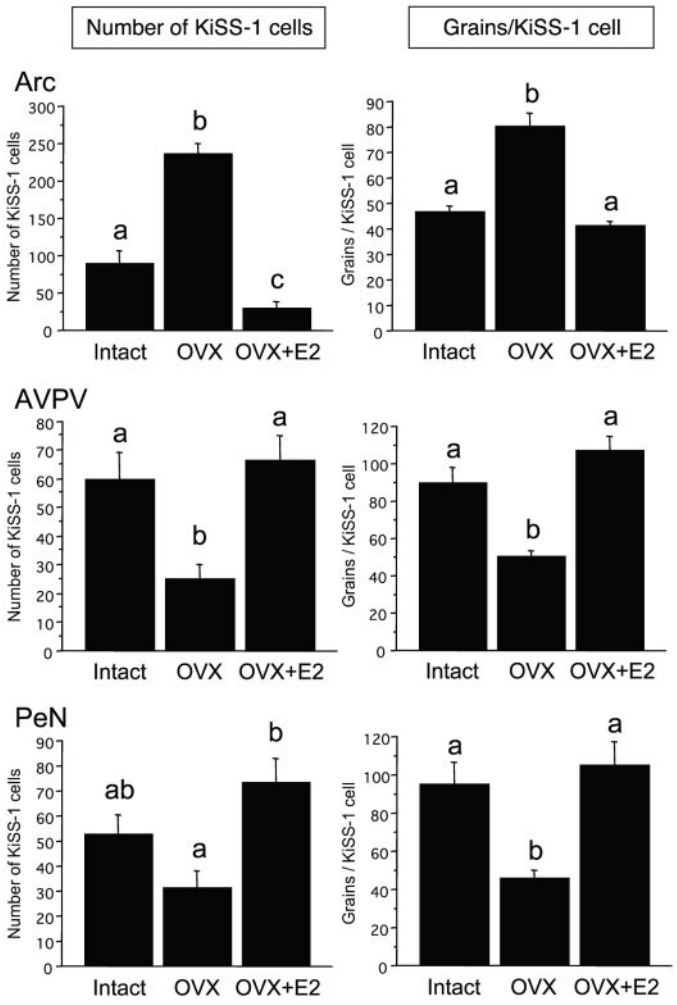

FIG. 2. The number of identifiable KiSS-1 mRNA-positive cells and grains per KiSS-1 cell after OVX and E2 replacement in the Arc, AVPV, and PeN. Values without *common notations* (a, b, c) differ significantly ($P < 0.01$). Values are presented as the mean ± SEM.

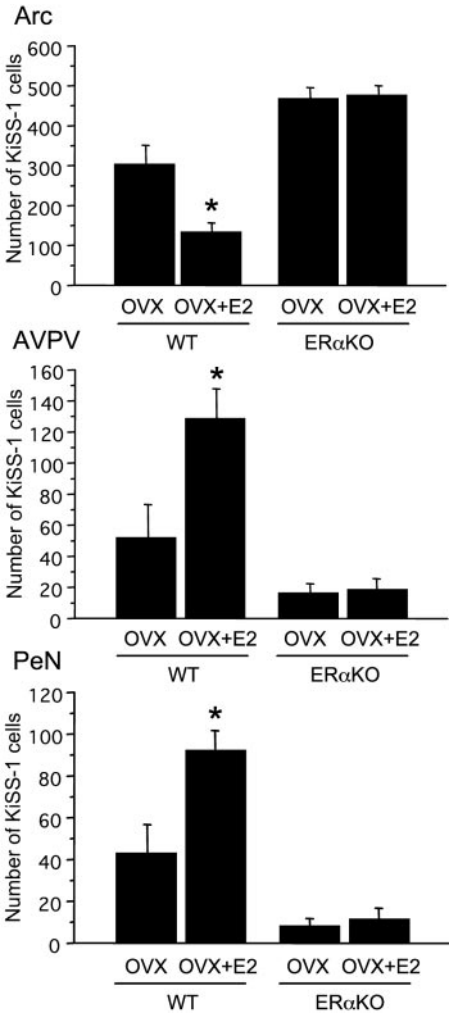

FIG. 3. Effect of E2 replacement on the number of KiSS-1-expressing cells in the Arc, AVPV, and PeN in OVX ERαKO and WT controls. Data are presented as the mean ± SEM. *, $P < 0.01$.

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

Experiment 4: KiSS-1 mRNA coexpression with ERα and ERβ mRNAs

Cells expressing ERα 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 ERα mRNA) overlying them (Fig. 4A). Quantitative analysis, with a criterion for double labeling of signal 3 times over background, indicated that 99.8 ± 0.1% of all KiSS-1 mRNA-expressing cells in the Arc, 98.7 ± 0.8% of all KiSS-1 mRNA-expressing cells in the AVPV, and 97.9 ± 1.8% of all KiSS-1 mRNA-expressing cells in the PeN also expressed ERα mRNA.

Cells expressing ERβ mRNA were observed in the preoptic area, AVPV, PeN, Arc, ventromedial hypothalamus, medial amygdala, BnST, and the paraventricular nucleus. Numerous identifiable KiSS-1 mRNA-positive neurons also possessed clusters of silver grains (representing ERβ mRNA) overlying them (Fig. 4B). Quantitative analysis, with a criterion for double labeling of signal 3 times over background, indicated that that 25.0 ± 2.6% of all KiSS-1 mRNA-expressing cells in the Arc, 30.5 ± 5.1% of all KiSS-1 mRNA-expressing cells in the AVPV, and 43.3 ± 10.8% of all KiSS-1 mRNA-expressing cells in the PeN also expressed ERβ mRNA.

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

KiSS-1 mRNA (grains/cell)	WT		ERαKO	
	OVX (n = 6)	OVX+E2 (n = 6)	OVX (n = 6)	OVX+E2 (n = 6)
Arc	128 ± 21	58 ± 6[a]	189 ± 5	170 ± 4
AVPV	101 ± 29	167 ± 4	30 ± 2	31 ± 2
PeN	114 ± 35	185 ± 3	35 ± 4	23 ± 5

Data are presented as the mean ± SEM for OVX and OVX plus estradiol-treated (OVX+E2), WT, and ERαKO 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 ERβKO mice (experiment 3)

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

Data are presented as the mean ± SEM for OVX and OVX plus estradiol-treated (OVX+E2), WT, and ERβKO 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 ERαKO or ERβKO 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 ERα 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 ERα 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 ERα, 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 ERα.

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

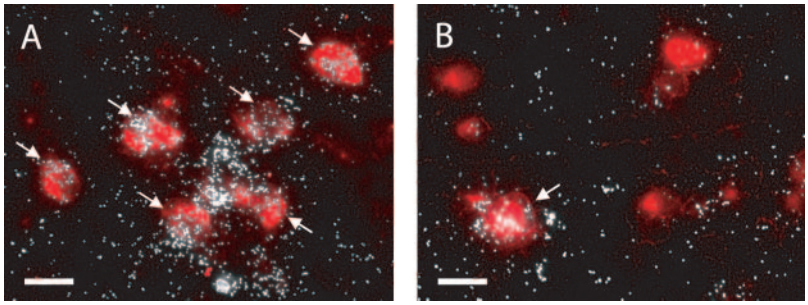

FIG. 4. Representative photomicrographs showing coexpression of KiSS-1 mRNA with ERα (A) and ERβ (B). KiSS-1 mRNA-expressing cells are fluorescent with Vector Red substrate, and *clusters of silver grains* reflect the presence of ERα (A) or ERβ (B) mRNA. The *arrows* indicate KiSS-1 neurons that coexpress either ERα or ERβ. *Scale bars*, 20 μm.

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 (ERα) 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 (ERα and ERβ) between these regions of the brain. These two ER isoforms have been shown to produce opposing molecular actions (45, 46). ERα and ERβ are colocalized in many areas of the forebrain and can form heterodimers (47). In the presence of ERα/ERβ heterodimers, ERβ appears to be the dominant regulator and can oppose ERα-mediated gene transcription (45). In the AVPV, there appears to be a greater ratio of ERβ to ERα expression than that in the Arc (8). However, our data show that in the absence of ERα, 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 ERβ, E2 fully retains its capacity to regulate KiSS-1. Because all KiSS-1 cells in the Arc, AVPV, and PeN express ERα and only a minority coexpress ERβ, it would appear that the ERβ 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 ERα 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 ERβ 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 ERβKO than in WT mice. It is tempting to speculate that under normal circumstances the formation of ERβ/ERα heterodimers in KiSS-1 neurons in the Arc attenuates E2-dependant transcriptional regulation of the *Kiss1* gene (45). Thus, in the absence of ERβ, the inhibitory effect of E2 on transcription of KiSS-1 is enhanced.

OVX mice lacking ERα 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 ERα 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 ERα 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 ERα (12). Whether KiSS-1 neurons are organized in a similar fashion and whether their development is altered in the absence of ERα 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 ERα. 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 ERα 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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References

1. **Moore CR, Price D** 1932 Gonadal hormone functions and the reciprocal influence between gonads and hypophysis, with its bearing on sex hormone antagonism. Am J Anat 50:13–71
2. **Caligaris L, Astrada JJ, Taleisnik S** 1971 Release of luteinizing hormone induced by estrogen injection into ovariectomized rats. Endocrinology 88: 810–815
3. **Hrabovszky E, Shughrue PJ, Merchenthaler I, Hajszan T, Carpenter CD, Liposits Z, Petersen SL** 2000 Detection of estrogen receptor-β messenger ribonucleic acid and 125I-estrogen binding sites in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology 141:3506–3509
4. **Dorling AA, Todman MG, Korach KS, Herbison AE** 2003 Critical role for estrogen receptor α in negative feedback regulation of gonadotropin-releasing hormone mRNA expression in the female mouse. Neuroendocrinology 78: 204–209
5. **Herbison AE** 1998 Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocr Rev 19:302–330
6. **Petersen SL, Ottem EN, Carpenter CD** 2003 Direct and indirect regulation of gonadotropin-releasing hormone neurons by estradiol. Biol Reprod 69:1771–1778
7. **Simerly RB, Chang C, Muramatsu M, Swanson LW** 1990 Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an *in situ* hybridization study. J Comp Neurol 294:76–95
8. **Shughrue PJ, Lane MV, Merchenthaler I** 1997 Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. J Comp Neurol 388:507–525
9. **Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS, Alves SE** 2003 Immunolocalization of estrogen receptor β in the mouse brain: comparison with estrogen receptor α. Endocrinology 144:2055–2067
10. **Canteras NS, Simerly RB, Swanson LW** 1994 Organization of projections from the ventromedial nucleus of the hypothalamus: a *Phaseolus vulgaris*-leukoagglutinin study in the rat. J Comp Neurol 348:41–79
11. **Simonian SX, Spratt DP, Herbison AE** 1999 Identification and characterization of estrogen receptor α-containing neurons projecting to the vicinity of the gonadotropin-releasing hormone perikarya in the rostral preoptic area of the rat. J Comp Neurol 411:346–358

12. **Simerly RB** 2002 Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. Annu Rev Neurosci 25:507–536
13. **Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SN, Vassart G, Parmentier M** 2001 The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem 276:34631–34636
14. **Stafford LJ, Xia C, Ma W, Cai Y, Liu M** 2002 Identification and characterization of mouse metastasis-suppressor KiSS1 and its G-protein-coupled receptor. Cancer Res 62:5399–5404
15. **Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M** 2001 Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411:613–617
16. **de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E** 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci USA 100:10972–10976
17. **Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley Jr WF, Aparicio SA, Colledge WH** 2003 The GPR54 gene as a regulator of puberty. N Engl J Med 349:1614–1627
18. **Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma FJ, Gustafson EL** 2003 The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem Biophys Res Commun 312:1357–1363
19. **Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA** 2004 A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145:4073–4077
20. **Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA** 2005 Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80:264–272
21. **Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM** 2005 Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc Natl Acad Sci USA 102:2129–2134
22. **Matsui H, Takatsu Y, Kumano S, Matsumoto H, Ohtaki T** 2004 Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. Biochem Biophys Res Commun 320:383–388
23. **Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2005 Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology 146:156–163
24. **Navarro VM, Castellano JM, Fernandez-Fernandez R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2004 Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 145:4565–4574
25. **Freeman ME** The neuroendocrine control of the ovarian cycle of the rat. In: Knobil E, Neill JD, eds. Physiology of reproduction. 3rd ed. New York: Raven Press, in press
26. **Simerly RB** 1998 Organization and regulation of sexually dimorphic neuroendocrine pathways. Behav Brain Res 92:195–203
27. **Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O** 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166
28. **Pak TR, Lynch GR, Ziegler DM, Lunden JB, Tsai PS** 2003 Disruption of pubertal onset by exogenous testosterone and estrogen in two species of rodents. Am J Physiol Endocrinol Metab 284:E206–E212
29. **Cunningham MJ, Scarlett JM, Steiner RA** 2002 Cloning and distribution of galanin-like peptide mRNA in the hypothalamus and pituitary of the macaque. Endocrinology 143:755–763
30. **Chowen JA, Steiner RA, Clifton DK** 1991 Semiquantitative analysis of cellular somatostatin mRNA levels by *in situ* hybridization histochemistry. Methods Neurosci 5:137–158
31. **Soper BD, Weick RF** 1980 Hypothalamic and extrahypothalamic mediation of pulsatile discharges of luteinizing hormone in the ovariectomized rat. Endocrinology 106:348–355
32. **Parhar IS, Ogawa S, Sakuma Y** 2004 Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. Endocrinology 145:3613–3618
33. **Wersinger SR, Haisenleder DJ, Lubahn DB, Rissman EF** 1999 Steroid feedback on gonadotropin release and pituitary gonadotropin subunit mRNA in mice lacking a functional estrogen receptor α. Endocrine 11:137–143
34. **Gu GB, Simerly RB** 1997 Projections of the sexually dimorphic anteroventral periventricular nucleus in the female rat. J Comp Neurol 384:142–164
35. **Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA** 2005 Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 146:2976–2974
36. **Everett JW, Sawyer CH** 1950 A 24-hour periodicity in the "LH-release apparatus" of female rats, disclosed by barbiturate sedation. Endocrinology 47: 198–218
37. **Legan SJ, Karsch FJ** 1975 A daily signal for the LH surge in the rat. Endocrinology 96:57–62
38. **Raisman G, Brown-Grant K** 1977 The 'suprachiasmatic syndrome': endocrine and behavioural abnormalities following lesions of the suprachiasmatic nuclei in the female rat. Proc R Soc Lond B Biol Sci 198:297–314
39. **Gray GD, Soderstein P, Tallentire D, Davidson JM** 1978 Effects of lesions in various structures of the suprachiasmatic-preoptic region on LH regulation and sexual behavior in female rats. Neuroendocrinology 25:174–191
40. **Coen CW, MacKinnon PC** 1980 Lesions of the suprachiasmatic nuclei and the serotonin-dependent phasic release of luteinizing hormone in the rat: effects on drinking rhythmicity and on the consequences of preoptic area stimulation. J Endocrinol 84:231–236
41. **Simerly RB, Swanson LW** 1987 The distribution of neurotransmitter-specific cells and fibers in the anteroventral periventricular nucleus: implications for the control of gonadotropin secretion in the rat. Brain Res 400:11–34
42. **Watson Jr RE, Langub Jr MC, Engle MG, Maley BE** 1995 Estrogen-receptive neurons in the anteroventral periventricular nucleus are synaptic targets of the suprachiasmatic nucleus and peri-suprachiasmatic region. Brain Res 689:254–264
43. **Peterfi Z, Churchill L, Hajdu I, Obal Jr F, Krueger JM, Parducz A** 2004 Fos-immunoreactivity in the hypothalamus: dependency on the diurnal rhythm, sleep, gender, and estrogen. Neuroscience 124:695–707
44. **Le WW, Berghorn KA, Rassnick S, Hoffman GE** 1999 Periventricular preoptic area neurons coactivated with luteinizing hormone (LH)-releasing hormone (LHRH) neurons at the time of the LH surge are LHRH afferents. Endocrinology 140:510–519
45. **Matthews J, Gustafsson JA** 2003 Estrogen signaling: a subtle balance between ERα and ERβ. Mol Interv 3:281–292
46. **Temple JL, Fugger HN, Li X, Shetty SJ, Gustafsson J, Rissman EF** 2001 Estrogen receptor β regulates sexually dimorphic neural responses to estradiol. Endocrinology 142:510–513
47. **Shughrue PJ, Scrimo PJ, Merchenthaler I** 1998 Evidence for the colocalization of estrogen receptor-β mRNA and estrogen receptor-α immunoreactivity in neurons of the rat forebrain. Endocrinology 139:5267–5270
48. **Klinge CM, Jernigan SC, Smith SL, Tyulmenkov VV, Kulakosky PC** 2001 Estrogen response element sequence impacts the conformation and transcriptional activity of estrogen receptor α. Mol Cell Endocrinol 174:151–166

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