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Differential Regulation of KiSS-1 mRNA Expression by Sex Steroids in the Brain of the Male Mouse

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Kisspeptins are products of the *Kiss1* gene, which bind to GPR54, a G protein-coupled receptor. Kisspeptins and GPR54 have been implicated in the neuroendocrine regulation of GnRH secretion. To test the hypothesis that testosterone regulates *Kiss1* gene expression, we compared the expression of KiSS-1 mRNA among groups of intact, castrated, and castrated/testosterone (T)-treated male mice. In the arcuate nucleus (Arc), castration resulted in a significant increase in KiSS-1 mRNA, which was completely reversed with T replacement, whereas in the anteroventral periventricular nucleus, the results were the opposite, *i.e.* castration decreased and T increased KiSS-1 mRNA expression. In the Arc, the effects of T on KiSS-1 mRNA were completely mimicked by estrogen but only partially mimicked by dihydrotestosterone, a nonaromatizable androgen, suggesting that both estrogen receptor (ER) and androgen receptor (AR) play a role in T-mediated regulation of KiSS-1. Studies of the effects of T on KiSS-1 expression in mice with either a deletion of the ERα or a hypomorphic allele to the AR revealed that the effects of T are mediated by both ERα and AR pathways, which was confirmed by the presence of either ERα or AR coexpression in most KiSS-1 neurons in the Arc. These observations suggest that KiSS-1 neurons in the Arc, whose transcriptional activity is inhibited by T, are targets for the negative feedback regulation of GnRH secretion, whereas KiSS-1 neurons in the anteroventral periventricular nucleus, whose activity is stimulated by T, may mediate other T-dependent processes. (*Endocrinology* 146: 2976–2984, 2005)

KISSPEPTINS ARE PRODUCTS of the *Kiss1* gene[1] that bind to the G protein-coupled receptor (GPR)54 (1–3). KiSS-1 and GPR54 are widely distributed in the forebrain (4–6), but their physiological function has only recently begun to emerge. In both humans and mice, mutations in GPR54 result in the failure of normal pubertal progression, which becomes manifest as hypogonadotropic hypogonadism (7–9). Moreover, central and peripheral administration of kisspeptin stimulates GnRH and gonadotropin secretion in the rodent and primate (6, 10–13), suggesting that kisspeptins and GPR54 play a role in the neuroendocrine regulation of gonadotropin secretion.

In the male, the negative feedback effects of testosterone (T) regulate GnRH and, in turn, gonadotropin secretion from the pituitary (14). However, the precise neural targets for the inhibitory action of T on GnRH secretion remain unclear. Attempts to identify steroid receptors, in particular estrogen receptor (ER) α and androgen receptor (AR), in GnRH neurons have generally been inconclusive (15, 16), and it is widely held that other steroid-sensitive neurons act as intermediaries to relay sex steroid signals to GnRH neurons (17). Steroid receptors are expressed throughout the forebrain, notably within the arcuate nucleus (Arc) and the anteroventral periventricular nucleus (AVPV) (18–20), which are known to send projections to regions of the brain in which GnRH neurons reside (21–23). However, the phenotypic identity of the steroid-sensitive neurons that couple directly to GnRH neurons has yet to be fully elucidated (24).

KiSS-1 mRNA is expressed in various areas of the mouse forebrain, including the Arc and AVPV (6), but whether KiSS-1 neurons are direct targets for regulation by T is unknown. Here we report the results of several experiments designed to investigate the effects of T on KiSS-1 neurons. First, we evaluated the effects of T on the expression of KiSS-1 mRNA in individual neurons of the mouse forebrain. Second, having observed that T differentially regulates KiSS-1 expression in various regions of the forebrain, we used steroid treatments and steroid receptor mutants to identify which steroid receptor mediates those effects. Finally, to determine whether T can act directly on KiSS-1 neurons, we sought to identify neurons that express both KiSS-1 mRNA and either ERα or AR mRNA.

Materials and Methods

Animals

Adult male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Male ERα null (ERKO) mice and wild-type littermates (WT) were purchased from Taconic (Germantown, NY). Male mice

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Abbreviations: AR, Androgen receptor; Arc, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BnST, bed nucleus of the stria terminalis; DHT, dihydrotestosterone; DIG, digoxigenin; E, estrogen; ER, estrogen receptor; ERKO, ERα null; GPR, G protein-coupled receptor; PeN, periventricular nucleus; SBR, signal to background ratio; T, testosterone; WT, wild type.

[1] The Mouse Genome Informatics database at the Jackson Laboratory (Bar Harbor, ME; http://www.informatics.jax.org) states that the correct nomenclature for the KiSS-1 gene is "*Kiss1*" and KiSS-1 protein is "KiSS-1." For consistency to previously published work, we have referred to the mRNA as "KiSS-1 mRNA."

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possessing a hypomorphic allele of the AR ($Ar^{invflox(ex1)-neo}$) and WT littermates (Ar^{+}) were generated as previously described (25). $Ar^{invflox(ex1)-neo}$ mice develop testes but have elevated circulating levels of gonadotropins and T, indicating that they have impaired feedback control of gonadotropin secretion at the level of the brain-pituitary axis. Animals were individually housed and were maintained on a 12-h light, 12-h dark cycle (lights on at 0600 h). Animals 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 in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Castration and steroid treatments

Gonads were removed from adult mice while anesthetized under isoflurane inhalation anesthesia (Abbott Laboratory, North Chicago, IL) delivered by a vaporizer (Veterinary Anesthesia Systems, Bend, OR). Vasculature to the gonad was sutured to prevent internal bleeding and wound clips were used to close the incision. Immediately after castration, steroid capsules were implanted sc via a small incision at the base of the neck; wound clips were used to close the incisions. All animals that were left intact underwent sham surgery.

T (4-androsten-17β-ol-3one), dihydrotestosterone (DHT; 5α-androstan-17β-ol-3one), and estrogen (E; β-estradiol) were all purchased from Sigma (St. Louis, MO). For T implants, SILASTIC brand tubing (inner diameter = 1.47 mm; outer diameter = 1.95 mm; Dow Corning Corp., Midland, MI) was cut to 15 mm, one end sealed with silicone glue and allowed to cure overnight. T crystals were packed into the tube to a length of 10 mm, and then the remaining length of the tube was occluded with silicone glue. Implants were left to cure overnight. The 10-mm length of tubing exposed to T crystals was based on previous studies (26, 27) and was designed to achieve normal physiological levels. Capsules containing DHT were made as described above. This dose was chosen based on previous research, which established that it would produce significant androgenic actions at target tissues, *e.g.* spermatogenesis in gonadotropin-deficient mice (27, 28). The dose of crystalline E was chosen based on a previous study that established its efficacy in significantly elevating serum E levels (29), and the capsules were constructed by packing SILASTIC brand tubing with 4 mm of an E-cholesterone mix (1:4). The day before surgery, implants were washed with two changes of 100% ethanol (10 min each) and then placed in physiological saline overnight. All untreated animals received empty (sham) capsules.

Experimental design

Experiment 1. The purpose of the experiment was to examine the effects of castration and T replacement on KiSS-1 mRNA in the forebrain of male mice. Mice were divided into three groups (n = 6 per group): intact, castrated, and castrated plus T replacement. Seven days after treatment, mice were weighed, anesthetized with isoflurane, and killed by decapitation. Trunk blood was collected for T 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 the effects of T on KiSS-1 expression in the forebrain (found in the previous experiment) could be mimicked by either (or both) E or a nonaromatizable androgen, DHT. Mice were divided into four groups (n = 5–7 per group): intact; castrated; castrated plus DHT; and castrated plus E. Tissue collection and preparation for DHT and E RIAs and KiSS-1 mRNA *in situ* hybridization occurred as described in experiment 1. To further confirm DHT treatment, seminal vesicles were dissected and weighed.

Experiment 3. The purpose of this experiment was to determine whether T can regulate expression of KiSS-1 mRNA in male mice lacking a functional ERα (ERKOs). Twelve male ERKO mice and 12 WT littermates were castrated and half of each group received T replacement. Tissue collection and preparation for T RIA and KiSS-1 mRNA *in situ* hybridization occurred as described in experiment 1.

Experiment 4. The purpose of this experiment was to determine whether T can regulate expression of KiSS-1 mRNA in male mice lacking a fully functional AR ($Ar^{invflox(ex1)-neo}$). Ten male $Ar^{invflox(ex1)-neo}$ mice and eight Ar^{+} littermates were castrated and half of each group received T replacement. Tissue collection and preparation for T RIA and KiSS-1 mRNA *in situ* hybridization occurred as described in experiment 1.

Experiment 5. The purpose of this experiment was to determine whether KiSS-1 neurons in the Arc coexpress ERα and AR. To accomplish this objective, we performed double-label *in situ* hybridization on a set of coronal sections of brains taken from castrated male mice in experiment 1 (n = 4). Castrated mice were used to adequately visualize KiSS-1 mRNA in the Arc via digoxigenin (DIG)-labeled riboprobes.

RIAs

Serum levels of T and E were measured at Northwestern University (Evanston, IL). T was measured with a double antibody kit (MP Biomedicals, Orangeburg, NY). The assay sensitivity was 0.02 ng/ml and the intraassay coefficient of variation was 15%. E was measured with a double antibody kit (Diagnostics Products Corp., Los Angeles, CA). The assay sensitivity was 2.0 pg/ml and the intraassay coefficient of variation was 6%. DHT was measured at the Department of Medicine, University of Washington with a kit (Diagnostic Systems Laboratory, Webster, TX). The assay sensitivity was 1.7 nmol/liter and the intraassay coefficient of variation was 10%. Where sufficient blood volume was available, serum LH levels were determined (experiments 1 and 3). Reagents for the LH assay were from the National Institute of Diabetes and Digestive and Kidney Diseases, the antiserum 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%.

Radiolabeled KiSS-1 cRNA probes

Antisense and sense mouse KiSS-1 probes were generated as previously described (6). Briefly, antisense mouse KiSS-1 probes were transcribed from linearized pAMP1 plasmid containing the mouse KiSS-1 insert with T7 polymerase (New England Biologicals, Beverly, MA). Radiolabeled probes were synthesized *in vitro* by inclusion of the following ingredients in a volume of 20 μl: 250 μCi ^{33}P-UTP (PerkinElmer Life Sciences, Boston, MA); 1 μg linearized DNA; 0.5 mM each ATP, CTP, and GTP; 40 U polymerase; 1 μl RNase inhibitor; and 4 μl 5 × transcription buffer (New England Biologicals). Residual DNA was digested with 4 U DNase (Ambion, Austin, TX) and the DNase reaction was terminated by addition of 2 μl of 0.5 M EDTA (pH 8.0). The riboprobes were separated from unincorporated nucleotides with NucAway spin columns (Ambion) and quantified in a scintillation counter. The KiSS-1-specific sequence spanned bases 76–486 of the mouse cDNA sequence (GenBank accession no. AF_472576). Abolishing all specific signal with excess unlabeled antisense probe and no signal with radiolabeled sense probe was previously determined (6).

In situ hybridization

Radioactive *in situ* hybridization was performed as previously described (30). Briefly, slides were removed from −80 C, rapidly thawed, fixed in 4% paraformaldehyde, acetylated in triethanolamine buffer, delipidated in chloroform, and dehydrated in graded ethanols. Radiolabeled, antisense riboprobe was denatured, diluted in hybridization solution at a concentration of 0.03 pmol/ml along with tRNA (2 mg/ml), and applied to slides (100 μl/slide). Slides were covered with glass coverslips and incubated in a humidified chamber at 55 C for 16 h. After hybridization, slides were treated with RNase (32 μg/ml), washed, and dehydrated as previously reported (30). Slides were then dipped in NTB-3 liquid emulsion (Eastman Kodak Co., Rochester, NY). Slides were developed approximately 3 d later and coverslips were then applied.

KiSS-1 mRNA quantification and analysis

All KiSS-1 mRNA-containing sections were analyzed unilaterally. Slides from all of the animals were assigned a random three-letter code,

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alphabetized, and read under dark-field illumination with custom-designed software designed to count the total number of cells and the number of silver grains (corresponding to radiolabeled KiSS-1 mRNA) over each cell (31). Cells were counted as KiSS-1 mRNA positive when the number of silver grains in a cluster exceeded that of background. Thus, cell counts represent the number of cells that achieved a detectability threshold, and the grains per KiSS-1 cell reflects a semiquantitative index of mRNA content in those cell that achieve the detectability threshold.

Double-label in situ hybridization for KiSS-1 mRNA/ERα mRNA and KiSS-1/AR mRNA

The cDNA template for the ERα riboprobe was generated by PCR as previously described for GPR54 (13) 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 GAACCGC-CCA 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 described for the radiolabeled KiSS-1 cRNA riboprobe.

Antisense and sense mouse AR probes were transcribed from the cDNA template as described for the radiolabeled KiSS-1 cRNA riboprobe. The AR-specific sequence spanned bases 710-1120 of the mouse cDNA sequence (GenBank accession no. NM_013476). No specific labeling was detected with radiolabeled AR sense cRNA probe (data not shown).

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. After synthesis, the DIG-labeled riboprobe was treated with DNase and purified as described above.

Slides were processed for *in situ* hybridization as above with modifications. Radiolabeled antisense ERα (0.03 pmol/ml) or AR (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. Sections were incubated in blocking buffer and then in Tris buffer containing anti-digoxigenin fragments conjugated to alkaline phosphatase (Roche), diluted 1:300, overnight at room temperature. KiSS-1 mRNA-positive cells were visualized using the Vector Red substrate kit (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 approximately 3 d later and coverslips were applied.

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 AR mRNA) over each cell (31). Signal to background ratios (SBRs) for individual cells were calculated; an individual 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 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 was assessed by least significant difference tests. All analyses were performed with Statview 5.0.1 for Macintosh (Apple, Cupertino, CA).

Results

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

KiSS-1 mRNA was present in the Arc, AVPV, periventricular nucleus (PeN), and anterodorsal preoptic area of all animals. Few cells were found in the medial amygdala and bed nucleus of the stria terminalis (BnST). Expression of KiSS-1 was most robust in the Arc and AVPV (Fig. 1), and the data from these areas and the PeN are presented below. There was no effect of treatments on KiSS-1 expression in the anterodorsal preoptic area, medial amygdala, or BnST (data not shown).

Experiment 1: effects of castration and T replacement on KiSS-1 mRNA in the forebrain

In the Arc, castration increased in the number of cells expressing KiSS-1 mRNA by 65% ($P < 0.01$) and increased the per-cell content of KiSS-1 mRNA (as reflected by grains per KiSS-1 cell) by 2-fold ($P < 0.0001$), compared with intact controls. Treatment with T completely reversed the effects of castration on both cell number and grains per KiSS-1 cell (both $P < 0.0001$; castration *vs.* castration + T; Fig. 2). In the AVPV, the results were the opposite of those found in the Arc. Castration decreased the number of identifiable KiSS-1 mRNA-containing cells by 61% ($P < 0.05$), and T treatment returned the number of KiSS-1 expressing cells to that of intact mice (Fig. 2). No difference was seen for grains per KiSS-1 cell. Results in the PeN were similar to those in the AVPV, *i.e.* castration decreased KiSS-1 cell number by 72% ($P < 0.05$) and T restored KiSS-1 cell number to that of intact controls. Grains per KiSS-1 cell in the PeN decreased with castration (41%, $P < 0.05$) and was restored by T replacement (Fig. 2).

Experiment 2: the effects of castration and E or DHT replacement on KiSS-1 mRNA in the forebrain of male mice

In the Arc, as had been observed in experiment 1, the number of detectable KiSS-1-expressing cells was increased by castration (3.5-fold, $P < 0.0001$, Fig. 3). This effect was partially reversed by DHT treatment to castrated animals, with the number of KiSS-1 cells in the Arc being reduced by

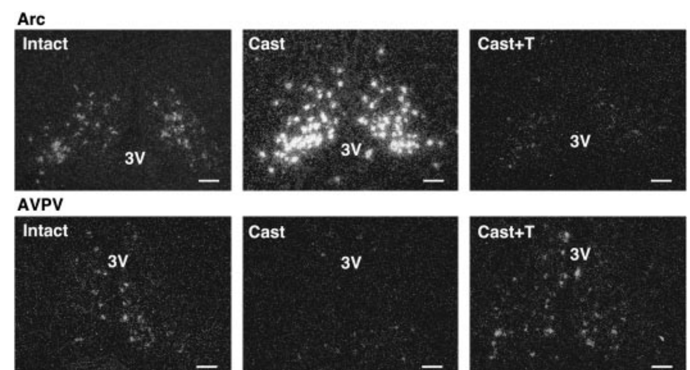

FIG. 1. Representative dark-field photomicrographs showing KiSS-1 mRNA-expressing cells (as reflected by the presence of *white clusters of silver grains*) in the Arc and AVPV from intact, castrated (Cast), and castrated with T-replaced (Cast + T) mice. 3V, Third ventricle. *Scale bars*, 100 μm.

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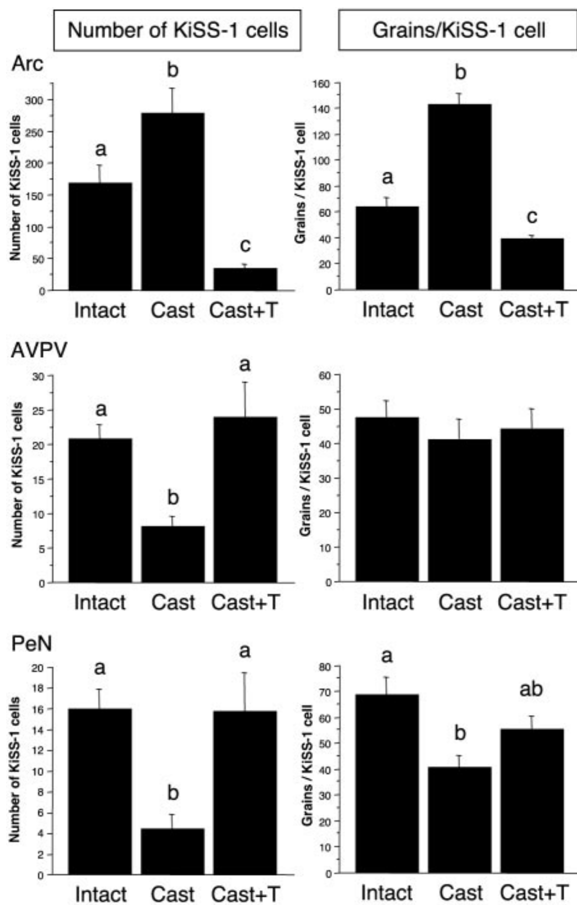

FIG. 2. Effects of castration (Cast) and T replacement on KiSS-1 mRNA in the Arc, AVPV, and PeN. One week after treatments the number of KiSS-1 mRNA-positive cells and grains per KiSS-1 cell in the Arc was significantly greater in Cast animals than intact controls, and T replacement completely reversed the effects of Cast. Values without common notation (*a*, *b*, *c*) differ significantly ($P < 0.01$). In the AVPV and PeN, the results were the opposite of those in the Arc. Cast reduced the number of KiSS-1 mRNA-positive cells, compared with intact controls, and T replacement restored KiSS-1 cell number. Values without common notation (*a*, *b*) differ significantly ($P < 0.05$). Values are presented as the mean ± SEM.

35%, compared with the castrated/sham-treated group ($P < 0.05$); however, KiSS-1 cell number was still significantly higher in the castrated DHT-treated animals than in intact controls (2.3-fold, $P < 0.01$). E treatment in castrated animals completely reversed the effects of castration ($P < 0.0001$, castration *vs.* castration + E, Fig. 3). A similar result was observed for grains per KiSS-1 cell in the Arc, castration resulted in a 2.6-fold increase ($P < 0.0001$), and E treatment reduced grains per KiSS-1 cell by 61% from castration alone (Table 1).

In the AVPV, as observed in experiment 1, castration significantly reduced the number of identifiable KiSS-1 cells (by 90%, $P < 0.05$, Fig. 3). Treatment with DHT had no discernible effect on KiSS-1 cell number; however, E treatment in castrated animals fully restored the number of identifiable KiSS-1 cells to that seen in intact animals ($P < 0.05$, compared with castrated/sham-treated animals, Fig. 3). There was no difference in grains per KiSS-1 cell in the AVPV (Table 1).

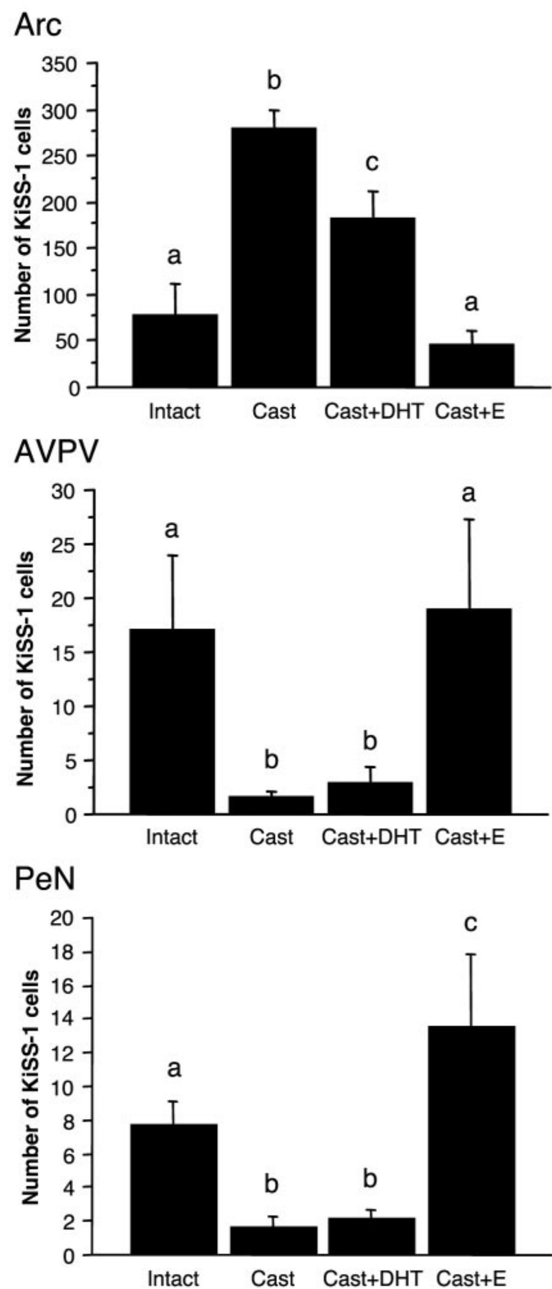

FIG. 3. Effects of castration (Cast) and DHT or E treatment on KiSS-1 mRNA in the Arc, AVPV, and PeN. The number of identifiable KiSS-1 mRNA-positive cells in the Arc was significantly greater in Cast animals than intact controls. DHT treatment decreased KiSS-1 cell number, but E treatment completely reversed the effects of Cast. Values without common notation (*a*, *b*, *c*) differ significantly ($P < 0.05$). In the AVPV and PeN, Cast reduced the number of KiSS-1 mRNA-positive cells, compared with intact controls, and E replacement increased KiSS-1 cell number. Values without common notation (*a*, *b*, c) differ significantly ($P < 0.05$). All values are presented as the mean ± SEM.

Results in the PeN were similar to the AVPV. In the PeN, castration significantly reduced the number of identifiable KiSS-1 cells (by 78%, $P < 0.05$), and treatment with E increased KiSS-1 cell number to values that were significantly greater than that seen in the intact animal (Fig. 3).

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TABLE 1. KiSS-1 mRNA expression (grains per KiSS-1 cell) in the Arc, AVPV, and PeN from intact, castrated, castrated plus DHT, and castrated plus estrogen-treated mice (experiment 2)

KiSS-1 mRNA (grains/KiSS-1 cell)	Intact (n = 6)	Cast (n = 6)	Cast + DHT (n = 7)	Cast + E (n = 5)
Arc	36 ± 3^a	94 ± 11^b	73 ± 7^b	37 ± 5^a
AVPV	38 ± 3	24 ± 3	31 ± 5	48 ± 12
PeN	41 ± 6	29 ± 4	25 ± 5	52 ± 8

Data are presented as the mean ± SEM for intact, castrated (Cast), castrated plus dihydrotestosterone-treated (Cast + DHT), and castrated plus estrogen-treated (Cast + E) mice. Values without common notations (a, b) differ significantly ($P < 0.05$).

DHT had no discernible effect on KiSS-1 cell number. There was no difference in grains per KiSS-1 cell in the PeN (Table 1).

Experiment 3: the effects of castration and T replacement on KiSS-1 mRNA in the forebrain of male ERKO mice

T retained its ability to regulate the expression of KiSS-1 in castrated ERKO mice, reducing KiSS-1 expression in the Arc and increasing its expression in the AVPV. In the Arc, the number of KiSS-1-positive cells and grains per KiSS-1 cell in the Arc varied with treatment ($P < 0.0001$ for both, two-way ANOVAs), and there was a significant interaction between treatment and genotype for cell number ($P < 0.01$). In WT animals, T treatment reduced the number of identifiable KiSS-1 neurons and grains per KiSS-1 cell by 76 and 78%, respectively (compared with castrated/sham-treated controls; $P < 0.001$ for both). In ERKOs, T treatment reduced KiSS-1 cell number by 42% and grains per KiSS-1 cell by 68%, compared with sham-treated castrates ($P < 0.01$ and $P < 0.001$, respectively; Fig. 4 and Table 2).

In the AVPV, the number of identifiable KiSS-1 cells and grains per KiSS-1 cell varied with both treatment (both $P < 0.0001$) and genotype ($P < 0.01$ and $P < 0.001$, respectively), and there was a significant interaction between treatment and genotype for mRNA content ($P < 0.001$). In the AVPV of WT mice, T treatment increased KiSS-1 cell number by 2-fold ($P < 0.01$) and grains per KiSS-1 cell by 73% ($P < 0.01$), compared with sham-treated castrated controls. In the AVPV of ERKO mice, T treatment increased KiSS-1 cell number by 2.5-fold, compared with sham-treated, castrated controls ($P < 0.001$, Fig. 4). Grains per KiSS-1 cell in the AVPV of ERKO mice was increased 3-fold after castration and T treatment ($P < 0.001$; Table 2).

A significant effect of treatment on KiSS-1 cell number was also present in the PeN ($P < 0.01$), but there was no effect of genotype and no interaction (two-way ANOVA, Fig. 4, although it appeared that T increased KiSS-1 cell number only in WT mice). A similar result was also seen for grains per KiSS-1 cell (Table 2).

Experiment 4: the effects of castration and T replacement on KiSS-1 mRNA in the forebrain of male $Ar^{invflox(ex1)-neo}$ mice

T retained its ability to regulate the expression of KiSS-1 mRNA in castrated $Ar^{invflox(ex1)-neo}$ mice, reducing KiSS-1 expression in the Arc and increasing its expression in the AVPV. In the Arc, the number of KiSS-1-positive cells and grains per KiSS-1 cell varied with treatment (both $P <$

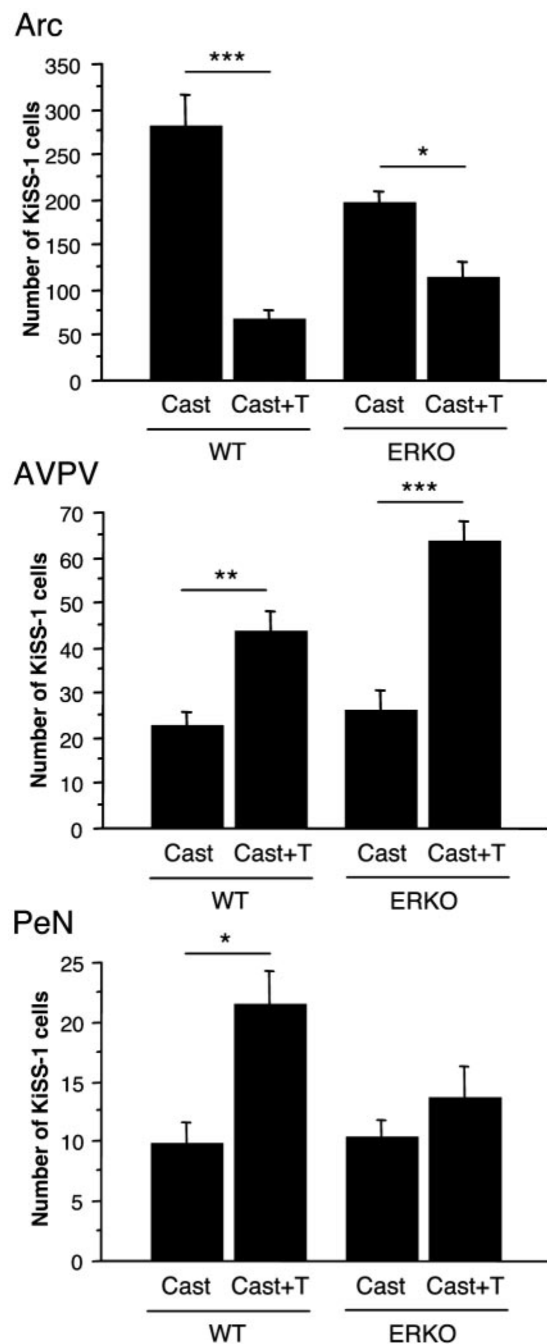

FIG. 4. Effect of T replacement on KiSS-1 mRNA in the Arc, AVPV, and PeN in castrated (Cast) ERKO and WT controls. T treatment reduced the number of KiSS-1 mRNA-positive cells in the Arc, compared with Cast alone, in both ERKO and WT mice. In the AVPV, T increased the number of KiSS-1 cells in both ERKO and WT mice. In the PeN, T increased the number of KiSS-1 cells in WT mice only. Data are presented as the mean ± SEM. *, $P < 0.05$;**, $P < 0.01$; ***, $P < 0.001$.

0.0001), with no interaction between treatment and genotype (two-way ANOVAs). In castrated Ar^+ mice, T treatment reduced the number of identifiable KiSS-1 cells and grains per KiSS-1 cell by 80 and 73%, respectively (both $P < 0.001$), and in castrated $Ar^{invflox(ex1)-neo}$ mice, T treatment significantly reduced KiSS-1 cell number and grains per KiSS-1 cell by 56

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TABLE 2. KiSS-1 mRNA expression (grains per KiSS-1 cell) in the Arc, AVPV, and PeN from intact, castrated, and castrated with testosterone-treated WT and ERKO mice (experiment 3)

KiSS-1 mRNA (grains/KiSS-1 cell)	WT		ERKO	
	Cast (n = 6)	Cast + T (n = 6)	Cast (n = 6)	Cast + T (n = 6)
Arc	134 ± 5	43 ± 4[a]	126 ± 7	28 ± 2[a]
AVPV	26 ± 1	45 ± 6[b]	26 ± 2	80 ± 4[a]
PeN	39 ± 7	73 ± 7[b]	25 ± 5	53 ± 5[b]

Data are presented as the mean ± SEM for castrated (Cast) and castrated plus testosterone-treated (Cast + T), wild-type (WT), and estrogen receptor α null (ERKO) mice. T treatment reduced grains per KiSS-1 cell in the Arc, compared with Cast alone, in both WT and ERKO mice. In the AVPV and PeN, T increased grains per KiSS-1 cell in both WT and ERKO mice.

[a] $P < 0.001$, [b] $P < 0.01$, compared with Cast alone.

and 58%, respectively (both $P < 0.001$), compared with sham-treated castrates (Fig. 5 and Table 3).

In the AVPV, a significant effect was only seen with treatment ($P < 0.01$, two-way ANOVAs). In the AVPV of Ar^+, T treatment significantly increased both KiSS-1 cell number and grains per KiSS-1 cell by approximately 7.2-fold and 93%, respectively ($P < 0.05$ and $P < 0.01$, respectively). In the AVPV of $Ar^{invflox(ex1)\text{-}neo}$ mice, T treatment significantly increased both KiSS-1 cell counts and grains per KiSS-1 cell by 6.7-fold and 65%, respectively, compared with sham-treated, castrated controls (both $P < 0.01$; Fig. 5 and Table 3).

In the PeN, the number of KiSS-1 cells differed only with treatment ($P < 0.05$, two-way ANOVA), but there was no effect of genotype and no interaction (Fig. 5). A significant effect of treatment on KiSS-1 mRNA content was also present in the PeN ($P < 0.01$), and there was also an effect of genotype ($P < 0.05$, two-way ANOVA, Table 3).

Experiment 5: double-label in situ hybridization for KiSS-1 mRNA/ERα mRNA and KiSS-1 mRNA/AR mRNA

Cells expressing ERα mRNA were observed in areas in which they have previously been reported, including the preoptic area, AVPV, Arc, ventromedial hypothalamus, medial amygdala, and BnST. The vast majority of identifiable KiSS-1 mRNA-positive neurons in the Arc had clusters of silver grains (representing ERα mRNA) overlying them (Fig. 6A). Quantitative analysis of SBRs, with a criterion for double labeling of signal 3 times over background, showed that 87 ± 4% of all KiSS-1 mRNA-expressing cells in the Arc also expressed ERα mRNA.

Cells expressing AR mRNA were also observed where they have previously been shown to reside, including the preoptic area, Arc, VMH, medial amygdala, BnST, and lateral septum. The majority of identifiable KiSS-1-positive neurons in the Arc had clusters of silver grains (representing AR mRNA) overlying them (Fig. 6B). Quantitative analysis of SBRs, with a criterion for double labeling of signal three times over background, revealed that 64 ± 4% of all KiSS-1 mRNA-expressing cells in the Arc also expressed AR mRNA.

Body weight and serum hormone concentrations

Body weights were similar among treatment groups for all experiments with the exception of experiment 2. Here ani-

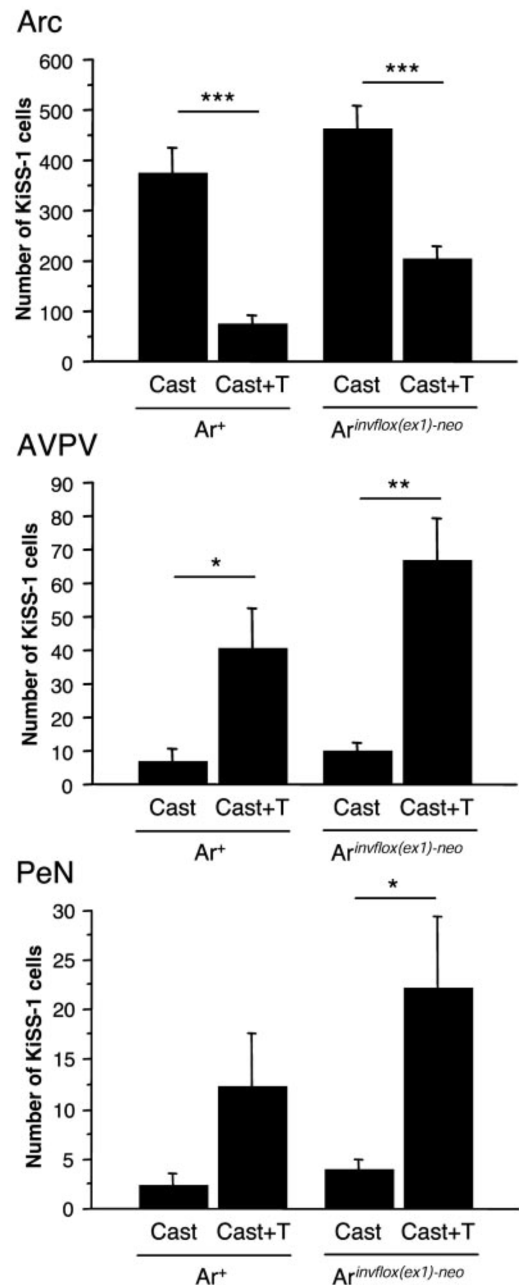

FIG. 5. Effect of T replacement on KiSS-1 mRNA in the Arc, AVPV, and PeN in castrated mice possessing a hypomorphic allele to the AR ($Ar^{invflox(ex1)\text{-}neo}$) and WT controls ($Ar^+$). T treatment reduced the number of KiSS-1 mRNA-positive cells in the Arc, compared with Cast alone, in both $Ar^{invflox(ex1)\text{-}neo}$ and Ar^+ mice. In the AVPV, T increased the number of KiSS-1 cells in both $Ar^{invflox(ex1)\text{-}neo}$ and Ar^+ mice. In the PeN, T increased the number of KiSS-1 cells in $Ar^{invflox(ex1)\text{-}neo}$ mice only. Data are presented as the mean ± SEM. *, $P < 0.05$; **$P < 0.01$; ***, $P < 0.001$.

mals that received E treatment with castration (27 ± 1 g) were slightly heavier than mice that were castrated alone (24 ± 1 g, $P < 0.05$) or castrated and given DHT treatment (24 ± 1 g, $P < 0.01$). Serum T levels were, as expected, undetectable in castrated mice, and within the physiological range in intact and castrated animals treated with T (average 4.2 ± 2.3 and 11.1 ± 0.8 ng/ml, respectively). Serum levels of DHT were

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TABLE 3. KiSS-1 mRNA expression (grains per KiSS-1 cell) in the Arc, AVPV, and PeN from castrated and castrated with testosterone-replaced wild-type and $Ar^{invflox(ex1)-neo}$ mice (experiment 4)

KiSS-1 mRNA (grains/KiSS-1 cell)	Ar^+		$Ar^{invflox(ex1)-neo}$	
	Cast (n = 6)	Cast + T (n = 6)	Cast (n = 6)	Cast + T (n = 6)
Arc	154 ± 19	41 ± 1[a]	156 ± 11	66 ± 5[a]
AVPV	27 ± 3	52 ± 4[b]	37 ± 2	61 ± 4[b]
PeN	30 ± 2	40 ± 3	37 ± 3	57 ± 6[c]

Data are presented as the mean ± SEM for castrated (Cast) and castrated plus testosterone-treated (Cast + T), wild-type (Ar^+), and mice possessing a hypomorphic allele to the androgen receptor ($Ar^{invflox(ex1)-neo}$). T treatment reduced grains per KiSS-1 cell in the Arc, compared with Cast alone, in both Ar^+ and $Ar^{invflox(ex1)-neo}$ mice. In the AVPV and PeN, T increased grains/KiSS-1 cells in both Ar^+ and $Ar^{invflox(ex1)-neo}$ mice.

[a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$, compared with Cast alone.

increased with DHT treatment (intact, 1.0 ± 0.5 ng/ml; Cast + DHT, 2.8 ± 0.7 ng/ml), and E levels were detectable only after treatment (87.9 ± 10.0 pg/ml). To further test the physiological relevance of DHT treatment, seminal vesicle weights were recorded in experiment 2. Castration decreased seminal vesicle weight, compared with intact controls (182 ± 13 *vs.* 47 ± 4 g; $P < 0.0001$), and this was restored with DHT treatment (203 ± 8 g). Serum LH levels were as expected in experiment 1 (intact, 0.22 ± 0.01 ng/ml; Cast, 3.80 ± 1.40 ng/ml; and undetectable in Cast + T). In experiment 3, LH levels in WT and ERKO mice were similar to those in experiment 1 (WT-Cast, 4.99 ± 0.96 ng/ml; undetectable in WT-Cast + T; ERKO-Cast, 3.60 ± 0.90 ng/ml; ERKO-Cast + T, 0.26 ± 0.06 ng/ml).

Discussion

This investigation has demonstrated that T regulates the expression of KiSS-1 mRNA in the forebrain of the mouse, confirming the observation by Navarro *et al.* (32) that the total KiSS-1 mRNA content of the male rat hypothalamus is inhibited by circulating T . Based on a cellular analysis of KiSS-1 mRNA in forebrain nuclei, we found that T does indeed inhibit the expression of KiSS-1 in the Arc in which the majority of KiSS-1 neurons reside. However, in other regions that contain KiSS-1 neurons (the AVPV, PeN), T stimulates the expression of KiSS-1 mRNA. Furthermore, our results suggest that both AR and ER are involved in the regulation of KiSS-1 mRNA via receptors expressed within KiSS-1 neurons. Thus, KiSS-1 neurons appear to be direct targets for T, making them plausible candidates for mediating both the positive and negative effects of T on a variety of neuroendocrine processes.

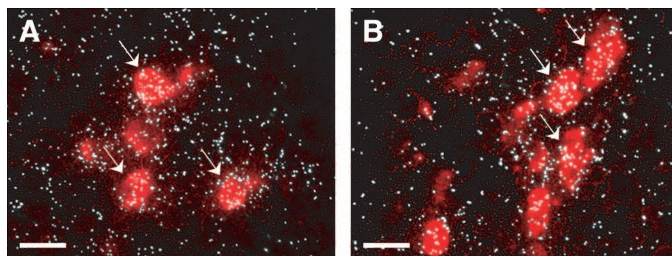

FIG. 6. Representative photomicrographs showing coexpression of KiSS-1 mRNA with ERα (A) and AR (B) in the Arcs. KiSS-1 mRNA-expressing cells are fluorescent with Vector red substrate, and *clusters of silver grains* reflect the presence of ERα (A) or AR (B) mRNA. The *arrows* indicate KiSS-1 neurons that coexpress either ERα or AR. Approximately 87 ± 4% of KiSS-1 neuron coexpress ERα mRNA and 64 ± 4% coexpress AR (n = 4 for each). *Scale bars*, 20 μm.

One possible role for KiSS-1 in the Arc is to mediate the negative feedback effects of T on GnRH secretion. Four lines of evidence suggest that KiSS-1 neurons in the Arc interact with GnRH neurons. First, centrally and peripherally administered kisspeptins stimulate gonadotropin secretion, and this effect is GnRH dependent (6, 10–13). Second, there are well-described anatomical connections between the Arc, in which there is an abundance of KiSS-1 neurons, and the medial preoptic area, in which many GnRH neurons reside (21, 22). Third, kisspeptin-containing fibers are found in areas possessing GnRH neurons (33). Finally, virtually all GnRH neurons express the kisspeptin receptor, GPR54 (13), which would imply that the effects of kisspeptin are mediated directly on GnRH neurons. Therefore, KiSS-1 neurons in the Arc are poised to play a critical role in the negative feedback regulation of GnRH neurons by T.

If KiSS-1 neurons in the Arc mediate the negative feedback effects of T on GnRH secretion, the steroid receptors that regulate KiSS-1 neurons should also in turn regulate GnRH/LH release. Indeed, both E and DHT, which is not aromatized to E, have been shown to inhibit LH secretion in the male (27, 34). The suppression of LH by T in ERKO mice suggests that at least some of the inhibitory effects of T on LH are mediated by AR. However, the fact that E (which does not activate AR) completely inhibits LH (and DHT does not) indicates that the primary mechanism of T's action is coupled to the ER after aromatization to E (27, 34). The effects of T, E, and DHT on KiSS-1 expression in the Arc appear to mirror their effects on LH secretion. We found that the expression of KiSS-1 mRNA in the Arc was increased after castration and was suppressed in castrated animals by T, E, or DHT, implying that both ER and AR are involved in the regulation of KiSS-1 expression. A role for both AR and ERα is also indicated by the observations that both AR and ERα are expressed in the majority of KiSS-1 neurons in the Arc and that T regulates KiSS-1 expression in the Arc of animals lacking functional AR or ERα. Because each of these receptors is expressed by a majority of KiSS-1-expressing cells, it seems reasonable to conclude that some KiSS-1 cells express both AR and ERα mRNA. Although it is conceivable that ERβ also mediates some of the effects of T in the Arc, we consider this unlikely because there is little ERβ expressed in the Arc (19, 20, 35). Furthermore, male mice lacking ERβ show no significant reproductive abnormalities (36), and treatment of castrated rats with a selective ERβ ligand has no effect on LH concentration or hypothalamic KiSS-1 mRNA expression (32). Taken together, these observations suggest that both LH secretion and KiSS-1 mRNA expression in the Arc are regulated by AR and ERα, but not ERβ, bolstering the concept that KiSS-1 neurons mediate the negative feedback effects of T on GnRH/LH secretion.

KiSS-1 neurons in the AVPV and PeN are different from those in the Arc. Here castration and T replacement altered KiSS-1 expression in the exact opposite fashion to that found

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in the Arc. In the AVPV, castration reduced both KiSS-1 cell counts and the cellular content of KiSS-1 mRNA, and T replacement completely restored these values back to those of intact controls. The inverse effect of T on KiSS-1 expression in the AVPV and Arc suggests that different receptor mechanisms are involved in those two areas. Unlike the Arc, the effect of T on KiSS-1 mRNA in the AVPV (and PeN) appears to be mediated exclusively by the ER because after castration E treatment fully restores KiSS-1 expression in these regions, whereas DHT had no discernible effect. However, another ER receptor besides ERα must be involved because T still induced KiSS-1 gene expression in the AVPV of ERKO mice. The other receptor is most likely ERβ, which could also play a role in reversing the effect of T on KiSS-1 expression from inhibitory in the Arc (in which there is little ERβ) to stimulatory in the AVPV. Indeed, ERβ (protein and mRNA) is expressed in the AVPV (19, 20). Confirming the existence of ERβ in KiSS-1 neurons of the AVPV and developing an understanding the mechanisms for differential regulation of KiSS-1 expression across regions of the forebrain represent important topics for future research.

What is the physiological significance of the ability of T to stimulate KiSS-1 expression in the AVPV and PeN? The AVPV has been implicated in the control of GnRH secretion, particularly in the generation of the preovulatory LH surge in the female rodent (37, 38). Thus, it seems conceivable that KiSS-1 neurons in the AVPV may be involved in estrogen-dependent, positive feedback regulation of GnRH secretion in the female, but their physiological significance in the AVPV of the male remains uncertain. It is notable that the AVPV is highly sexually differentiated (38) and that there are far fewer ($< 10\%$) KiSS-1 neurons in the AVPV and PeN of the male than the female (Smith, J. T., M. J. Cunningham, E. F. Rissman, D. K Clifton, and R. A. Steiner, unpublished observations). It may be that some remnant of the GnRH-positive feedback circuitry (that exists in the AVPV of the female) persists in males and that any function in the male is vestigial. It is also imaginable that KiSS-1 cells in the AVPV (and PeN) of the male serve other physiologically relevant functions, such as sexual behavior, which is enhanced by T. The AVPV has been implicated in the regulation of sexual behavior (38). The AVPV receives sensory inputs from the BnST and medial amygdala and sends projections to the preoptic area (23), all of which are recognized to be involved in sexual behavior in the male (39). The AVPV also contains a rich population of dopaminergic neurons (40), and there is recent evidence to suggest that some KiSS-1 neurons coexpress dopamine (33), which has been implicated in male sex behavior (41). Thus, the activation effects of T on KiSS-1 expression in the AVPV may be an irrelevant vestige or serve a role in mediating T's effect on sexual behavior, the latter of which is readily testable.

It became apparent in this study that mice lacking ERα had more KiSS-1-expressing cells in the AVPV, compared with WT controls. This is likely due to the masculinization effects of E on sexually dimorphic brain systems early in development (for review see Ref. 39). Interestingly, normal female mice do appear to possess more KiSS-1-expressing cells in the AVPV (Smith, J. T., M. J. Cunningham, E. F. Rissman, D. K Clifton, and R. A. Steiner, unpublished observations). It is possible that ERKO male mice possess a population of KiSS-1 cells that WT male mice do not.

In summary, we have shown that KiSS-1 mRNA is differentially regulated by T in distinct regions of the mouse forebrain, with T down-regulating KiSS-1 expression in the Arc and up-regulating KiSS-1 in the AVPV and PeN. We conclude that KiSS-1 neurons in the Arc may be involved in the T-mediated negative feedback control of gonadotropin secretion, whereas KiSS-1 neurons in the AVPV and PeN may be involved in other T-dependent physiological processes in the male mouse.

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