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Sexual Differentiation of *Kiss1* Gene Expression in the Brain of the Rat

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The *Kiss1* gene codes for kisspeptins, which have been implicated in the neuroendocrine regulation of reproduction. In the brain, *Kiss1* mRNA-expressing neurons are located in the arcuate (ARC) and anteroventral periventricular (AVPV) nuclei. *Kiss1* neurons in the AVPV appear to play a role in generating the preovulatory GnRH/LH surge, which occurs only in females and is organized perinatally by gonadal steroids. Because *Kiss1* is involved in the sexually dimorphic GnRH/LH surge, we hypothesized that *Kiss1* expression is sexually differentiated, with females having more *Kiss1* neurons than either males or neonatally androgenized females. To test this, male and female rats were neonatally treated with androgen or vehicle; then, as adults, they were left intact or gonadectomized and implanted with capsules containing sex steroids or nothing. *Kiss1* mRNA levels in the AVPV and ARC were determined by *in situ* hybridization. Normal females expressed significantly more *Kiss1* mRNA in the AVPV than normal males, even under identical adult hormonal conditions. This *Kiss1* sex difference was organized perinatally, as demonstrated by the observation that neonatally androgenized females displayed a male-like pattern of adulthood *Kiss1* expression in the AVPV. In contrast, there was neither a sex difference nor an influence of neonatal treatment on *Kiss1* expression in the ARC. Using double-labeling techniques, we determined that the sexually differentiated *Kiss1* neurons in the AVPV are distinct from the sexually differentiated population of tyrosine hydroxylase (dopaminergic) neurons in this region. Our findings suggest that sex differences in kisspeptin signaling from the AVPV subserve the cellular mechanisms controlling the sexually differentiated GnRH/LH surge. (*Endocrinology* 148: 1774–1783, 2007)

IN MAMMALS, OVULATION is triggered by a dramatic rise in circulating levels of LH, whose secretion is induced by GnRH. The preovulatory GnRH/LH surge mechanism is sexually differentiated and occurs only in females (1–3). The ability of females, but not males, to produce a GnRH/LH surge presumably reflects sexual differentiation of neural circuits in the forebrain, and this differentiation process is organized by gonadal steroids during the neonatal critical period (4–7). In the male, exposure to testosterone (T) or its metabolites during early postnatal life permanently alters the circuitry in the developing forebrain (8), averting its ability as an adult to generate a GnRH/LH surge in response to estradiol (E) (8). Because the brain of the female is normally unexposed to T during the neonatal period, it retains (or develops) the circuitry necessary to generate a GnRH/LH surge (2, 3). Males that are castrated during the neonatal critical period can produce a GnRH/LH surge as adults, just like normal females (3, 6); likewise, females that are exposed to T during the critical period lose their ability to generate GnRH/LH surges as adults (3, 6). However, despite recognition that the hormonal milieu of the neonatal rodent permanently alters the neural circuitry that generates the GnRH/LH surge mechanism in the adult, the identity of the cellular and molecular elements in the brain that subserve this process remains ambiguous.

The anteroventral periventricular nucleus (AVPV) is the anatomical nodal point for generating the preovulatory GnRH/LH surge (2, 9, 10). Lesions of the AVPV block the spontaneous and steroid-induced surge (11–14), and placement of E into the vicinity of the AVPV (but not other hypothalamic regions) elicits an LH surge (15). Estrogen receptor (ER) α is thought to mediate E's stimulatory effects on the surge mechanism (16), and many cells in the AVPV express ERα (17). The AVPV is also larger in females than males and contains sexually differentiated populations of neurons (18), including a large and anatomically discrete collection of dopaminergic neurons [positive for tyrosine hydroxylase (TH)], which are more abundant in females than males (2, 3, 19). The role, if any, of these dopaminergic neurons in the AVPV for the generation of the GnRH/LH surge is unknown, yet it is indisputable that their development is influenced by the neonatal sex steroid environment (19, 20).

The *Kiss1* gene codes for a family of peptides, known as kisspeptins, which act as endogenous ligands for the G protein-coupled receptor GPR54 (21–23). Kisspeptins and GPR54 are thought to play a critical role in the regulation of GnRH and gonadotropin secretion (24). Kisspeptins stimu-

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Abbreviations: ARC, Arcuate nucleus; AVPV, anteroventral periventricular nucleus; DIG, digoxigenin; E, estradiol; ER, estrogen receptor; GNX, gonadectomized; IHC, immunohistochemistry; ISH, *in situ* hybridization; OVX, ovariectomized; PeN, periventricular nucleus; RNAse, ribonuclease; SSC, sodium citrate, sodium chloride; T, testosterone; TH, tyrosine hydroxylase; TP, T propionate.

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late hypothalamic GnRH/LH release (25–29), most likely by acting directly on GnRH neurons, which express GPR54 (26). In mammals, the *Kiss1* gene is expressed in the forebrain, particularly in the hypothalamic arcuate nucleus (ARC) and the AVPV [including the periventricular nucleus (PeN)] (25, 26, 29–31). *Kiss1* neurons in the AVPV and ARC express ERα (30, 32), and gonadal steroids inhibit the expression of *Kiss1* in the ARC but stimulate its expression in the AVPV (26, 30–33). The ability of E to induce *Kiss1* gene expression in the AVPV/PeN led us to postulate that *Kiss1* neurons play a role in generating the preovulatory GnRH/LH surge (32). Several observations support this hypothesis. First, both the expression of *Kiss1* mRNA and induction of Fos protein in *Kiss1* neurons increase in the AVPV/PeN coincident with the LH surge (32), and second, blockade of kisspeptin's action by central administration of kisspeptin antiserum blocks the E-induced LH surge (34).

If *Kiss1* neurons in the AVPV/PeN participate in the generation of the sexually differentiated GnRH/LH surge, we would predict two things; first, that *Kiss1* expression in this region would be sexually differentiated, with higher levels of *Kiss1* mRNA in females than males; and second, that the sex steroid milieu during the neonatal critical period would determine the level of *Kiss1* expression in the AVPV of adults. Furthermore, if *Kiss1* expression in the AVPV is sexually differentiated, we might also predict that *Kiss1* neurons in the AVPV are the same cell population as the sexually differentiated TH-expressing neurons previously identified in this region. To test these hypotheses, we used *in situ* hybridization (ISH) to compare levels of *Kiss1* expression in the brain between adult male and female rats and examined the effects of altering the sex steroid environment of the neonate on the expression of *Kiss1* in the adult. We also analyzed the coexpression of *Kiss1* and TH in the AVPV by double-label ISH and double-label immunohistochemistry (IHC).

Materials and Methods

Animals

For experiments 1, 2, and 3B, Wistar male and female rats bred in the vivarium of the University of Córdoba were used. The animals were maintained under constant conditions of light (14 h light/d; lights on at 0700 h) and temperature (22 C). Rats were weaned at 21 d of age in groups of five animals per cage, with animals having free access to pelleted food and tap water. All experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals.

For experiment 3A, female Sprague Dawley rats were housed at the University of Maryland under 12 h light/d (lights on 0300 h), with food and water provided *ad libitum*. All protocols pertaining to these animals were approved by the University of Maryland Institutional Animal Care and Use Committee in accordance with National Institutes of Health policies.

Neonatal and adult hormone treatments and tissue collection

For Experiments 1, 2, and 3B, 10 groups (n = 5/group) of male and female rats were treated on the day of birth with single injections of either androgen [T propionate (TP); 1.25 mg/rat; Sigma, St. Louis, MO; female pups only] or vehicle (oil; male and female pups). At 60 d of age, adult males, females, and neonatally androgenized females were either left intact or gonadectomized (GNX) and simultaneously implanted with sc Silastic capsules containing either 17β-E (E; 20-mm length; inner diameter, 0.062 cm; exterior diameter, 0.125 cm) or nothing (empty capsules). An additional group of adult males was similarly GNX and given T-containing capsules (20 mm) to compare the effects of T and E. Table 1 lists the details of the 10 treatment groups. One week after gonadectomy and hormone replacement, animals were rapidly killed by decapitation. Brains were immediately removed, frozen on dry ice, and stored at −80 C until they were sectioned on a cryostat. During sectioning, five sets of coronal 20-μm sections, spanning the forebrain, were collected, thaw-mounted onto SuperFrost Plus slides (VWR Scientific, West Chester, PA), and stored at −80 C until use for ISH. Trunk blood samples for hormone measurements (RIA) were also collected at the time of decapitation.

For experiment 3A, adult female rats (n = 3) were ovariectomized (OVX) under fluorourethane anesthesia. One week later, each animal received a sc injection of 5 μg E in sesame oil at 0900 h followed 24 h later by a second sc injection of E (50 μg). At 1530–1630 h on the day after E administration, each animal was deeply anesthetized with pentobarbital (100 mg/kg) and then perfused transcardially first with saline containing 2% sodium nitrite, followed by 2.5% acrolein and 4% paraformaldehyde (11, 35, 36). Brains were collected and sunk in 30% aqueous sucrose. Serial sections containing the AVPV (28 μm) were cut on a freezing microtome and placed into antifreeze cryoprotectant until later used in the double IHC assay for TH and kisspeptin proteins.

Experiment 1: evaluation of sex differences in Kiss1 neurons in the AVPV and ARC

In experiment 1, we determined whether *Kiss1* mRNA is sexually dimorphic in the AVPV and ARC. The number of *Kiss1* mRNA-containing neurons and the level of *Kiss1* mRNA per cell were analyzed with single-label ISH, comparing levels in the AVPV and ARC from intact males and females (at diestrus).

TABLE 1. Experimental groups, treatments, and serum gonadotropin concentrations

Group	Sex	Neonatal treatment	Adult treatment	LH (ng/ml)	FSH (ng/ml)
1	Male	Vehicle	Intact	0.53 ± 0.13	8.26 ± 0.60
2	Male	Vehicle	GNX + sham	9.56 ± 1.66[a]	52.89 ± 4.58[a]
3	Male	Vehicle	GNX + E	0.60 ± 0.14	6.35 ± 1.39
4	Male	Vehicle	GNX + T	0.35 ± 0.10	5.49 ± 0.58
5	Female	Vehicle	Intact (diestrus)	0.68 ± 0.15	2.45 ± 0.40
6	Female	Vehicle	GNX + sham	3.58 ± 0.42[a]	53.88 ± 3.44[a]
7	Female	Vehicle	GNX + E	0.56 ± 0.14	8.72 ± 0.33
8	Female	Androgen	Intact	0.36 ± 0.06	3.53 ± 0.56
9	Female	Androgen	GNX + sham	1.35 ± 0.32[a]	28.41 ± 4.77[a]
10	Female	Androgen	GNX + E	0.28 ± 0.05	8.52 ± 0.93

Male and female rats were treated with either androgen (TP; female pups only) or vehicle (oil; male and female pups) on the day of birth. Later, at 60 d of age, adult rats were left intact or GNX and implanted (sc) with Silastic capsules containing E, T, or nothing (sham). Serum levels of LH and FSH were measured in adult rats at the time of kill (1 wk after gonadectomy and hormone implantation). Hormone data are presented as the mean ± SEM. Each group contained n = 5 animals.

[a] Hormone value is significantly different than that of other groups of the same sex receiving similar neonatal treatment ($P < 0.05$).

Experiment 2: evaluation of organizational and activational effects of gonadal steroids on sex differences in Kiss1 mRNA expression

Sex differences in *Kiss1* mRNA expression could reflect either sex differences in circulating hormone levels between intact adult males and diestrous females (so-called activational effects) or differences in the sex steroid milieu during the neonatal period (organizational effects). Experiment 2 addressed this issue by using single-label ISH to detect and compare levels of *Kiss1* mRNA in AVPV and ARC derived from adult females that had been neonatally treated with TP on the day of birth with levels measured in adult males and females that had been neonatally treated with vehicle. All animals were GNX in adulthood and given either an empty implant or an implant containing T or E, thereby allowing for controlled assessment of activational (*i.e.* adulthood) effects of steroids on *Kiss1* expression.

Experiment 3: evaluation of the colocalization of Kiss1 and TH neurons in the sexually dimorphic AVPV

TH neurons in the AVPV are known to be sexually differentiated, with females possessing greater numbers of TH cells in this region than males. In this experiment, we tested whether the sexually dimorphic *Kiss1* neurons in the AVPV/PeN (determined in experiments 1 and 2) comprise the same population of cells as the sexually dimorphic TH neurons, *i.e.* whether these two populations represent identical or two separate and distinct sexually differentiated systems within the same nucleus. In a preliminary study (experiment 3A), female rat tissue containing the AVPV/PeN of OVX + E females was processed for double-label IHC for TH and kisspeptin proteins (n = 3). In experiment 3B, a more quantitative analysis of the colabeling of *Kiss1* and *TH* messages was performed, controlling for possible effects of sex steroids on coexpression. For this experiment, brain sections from intact, OVX, and OVX and E-replaced females were processed by double-label ISH to label AVPV/PeN cells for both *Kiss1* mRNA and *TH* mRNA. In both experiments, AVPV/PeN sections were analyzed to determine the percentage of cells that express both kisspeptin and TH proteins (or *Kiss1* and *TH* mRNA).

RIAs of plasma hormone levels

A double-antibody method was used to measure serum levels of LH and FSH in 25–50-μl samples. The RIA kits were kindly supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rat LH-I-9 and FSH-I-9 were labeled with ^{125}I by the chloramine-T method; LH-RP-3 and FSH-RP2 were used as the reference standards. Intra- and interassay coefficients of variation were less than 8 and 10% for LH and 6 and 9% for FSH, respectively. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH.

Radiolabeled riboprobe preparation

Antisense mouse *Kiss1* probes were generated as previously described (25). The *Kiss1*-specific sequence spanned bases 76–486 of the mouse cDNA sequence (GenBank accession no. AF472576). The radiolabeled *Kiss1* riboprobe (made with ^{33}P) was generated against the mouse *Kiss1* mRNA; there is a 90% homology between mouse and rat in the cloned region, and we have previously demonstrated that this probe can be used successfully in rat tissue (26, 32). For the double-label ISH assay in experiment 3, radiolabeled *TH* riboprobe was generated by using a rat-specific sequence, as described previously (38, 39).

Single-label ISHs

Slide-mounted brain sections were processed for *Kiss1* ISH, as previously described (25, 26). Briefly, sections were fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2× sodium citrate, sodium chloride (SSC), delipidated in chloroform, dehydrated in graded ethanols, and then allowed to air-dry before the hybridization procedure. The volume of *Kiss1* riboprobe was calculated (0.03 pmol/ml) and combined with 1/20 volume yeast tRNA (Roche Biochemicals, Indianapolis, IN) in 0.1 M Tris/0.01 M EDTA (pH 8.0) to produce the probe mix. The probe mix was heat-denatured in boiling water for 3 min, then returned to ice for 5 min. The denatured probe mix was added to prewarmed hybridization buffer (60% deionized formamide, 5× hybridization salts, 0.1× Denhardt's buffer, 0.2% SDS), at a ratio of 1:4, and added to each slide (100 μl/slide). The sections were then coverslipped and placed in humidity chambers at 55 C for 16 h. After hybridization, coverslips were removed, and the slides were washed in 4× SSC at room temperature. Slides were then placed into ribonuclease (RNAse) [10 mg/ml RNAse (Roche Biochemicals) in 0.15 M sodium chloride, 10 mM Tris, 1 mM EDTA (pH 8.0)] for 30 min at 37 C, then in RNAse buffer, without RNAse, at 37 C for another 30 min. After a 3-min wash in 2× SSC at room temperature, slides were washed twice in 0.1× SSC at 62 C, then dehydrated in graded ethanols and air dried. The slides were then dipped in Kodak NTB emulsion (VWR, Inc.), air-dried, and stored at 4 C for 8–9 d. Slides were then developed, dehydrated in graded ethanols, cleared in Citrasol (VWR, Inc.), and coverslips were applied with Permaslip (Sigma).

Double-label ISH

Slides were treated similarly to single-label ISH with the following modifications. Digoxigenin (DIG)-labeled antisense *Kiss1* cRNA was synthesized with T7 RNA polymerase and DIG labeling mix (Roche) according to the protocol of the manufacturer. Radiolabeled antisense *TH* and DIG-labeled *Kiss1* riboprobes (concentration determined empirically) were denatured, dissolved in the same hybridization buffer along with 1/20 volume yeast tRNA, and applied to slides. Slides were then hybridized overnight as above. After the stringent washes on d 2, slides were instead incubated in 2× SSC with 0.05% Triton X-100 containing 2% normal sheep serum for 1 h at room temperature. The slides were then washed in buffer 1 [100 mM Tris-HCl (pH 7.5), 150 mM NaCl] and incubated overnight at room temperature with anti-DIG antibody fragments conjugated to alkaline phosphatase (Roche Biochemical; diluted 1:200 in buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100). The next day, slides were again washed with buffer 1 and incubated with Vector Red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA) for 4 h at room temperature, with changes in solution every 45–60 min. The slides were then dipped in 70% ethanol, air-dried, dipped in emulsion, stored at 4 C, and developed 11 d later.

Quantification and analysis of Kiss1 mRNA for ISHs

Slides were analyzed with an automated image processing system by a person unaware of the treatment group of each slide (109). The system consists of a Scion VG5 video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Power Macintosh G5 computer running custom grain-counting software. For single-label experiments, the software was used to count the number of cells and the number of silver grains over each cell (a semiquantitative index of mRNA content per cell) (25, 26, 30, 40). Cells were considered *Kiss1* positive when the number of silver grains in a cluster exceeded that of background by 3-fold. For double-label assays, DIG-containing cells (*Kiss1* cells) were identified under fluorescence microscopy, and the grain-counting software described above was used to quantify silver grains (representing *TH* mRNA) over each cell. Signal to background ratios for individual cells were calculated, and a cell was considered double-labeled if it had a signal to background ratio of 3 or greater and contained at least three grains (a typical TH cell in the rat AVPV contains between 50 and 100 grains). For each animal, the amount of double-labeling was calculated as a percentage of the total number of *Kiss1* mRNA-expressing cells and then averaged across animals to produce a mean ± SEM.

Double-label fluorescence IHC

This approach follows the approach of Shindler and Roth (41). The first reaction in the series is performed by using biotinylated tyramide amplification and streptavidin-fluorophore visualization, and the second uses a direct fluorophore-tagged secondary antibody (see 42, 43) This technique was used for antikisspeptin with amplified fluorescence, with the concentration of the primary kisspeptin antibody increased to 1:20,000–30,000 [antikisspeptin antibody no. 564; provided by Dr. Alain Caraty, University of Tours (44, 45)]. Briefly, the sections that contained the AVPV/PeN, from a one in six series, were removed from cryopro-

tectant antifreeze, rinsed in PBS, treated with a 1% NaBH4 solution (Sigma), rinsed, and then incubated with antikisspeptin antibody in PBS with 0.4% Triton X-100 for 48 h at 4 C. After rinsing, the tissue was incubated for 1 h at room temperature in biotinylated antirabbit IgG (heavy and light chains, Vector Laboratories) at a concentration of 1:5000 in PBS with 0.4% Triton X-100, rinsed, and incubated for 1 h in avidin-biotin complex solution (elite ABC kit, Vector Laboratories; 1.125 μl each/ml incubation mixture). After rinsing in PBS, the sections were placed into biotinylated tyramide and peroxidase for 20 min at room temperature according to Berghorn *et al.* (35). Biotinylated tyramide was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA) as a part of their tyramine amplification kits. After biotinylated tyramide incubation, the sections were rinsed and placed into streptavidin Cy2 (Invitrogen, Carlsbad, CA) for 3 h at 37 C temperature, rinsed, and placed into anti-TH (Chemicon International, Temecula, CA; mouse monoclonal, 1:30,000). Kisspeptin protein was visualized with red fluorescence, whereas TH protein was visualized with green fluorescence. Because the anti-TH was generated in mouse and the antikisspeptin in rabbit, no erroneous cross-reactivity of the secondary antibody fluorophore complex with the first primary antibody occurred. In addition, the optics did not show any bleed-through of the fluorescence from the Texas Red into the Cy2 channel or *vice versa* in these reactions.

Statistical analysis

All data are expressed as the mean ± SEM for each group. One-way ANOVA was used to assess variation among experimental groups in each experiment, and differences in means were assessed by least significant difference tests. Significance level was set at $P < 0.05$. All analyses were performed with Staview 5.0.1 for Macintosh (SAS Institute, Cary, NC).

Results

Serum hormone levels

Serum levels of gonadotropins of males and females that were GNX in adulthood were elevated relative to those of intact adults of the same sex, as would be expected with removal of steroidal feedback (Table 1). Treatment of GNX adults with hormone implants (either E or T) significantly reduced LH and FSH concentrations, indicating that the hormone capsules were functional and able to provide steroidal negative feedback (Table 1). The hormone levels of females treated neonatally with androgen were lower than those of vehicle-treated males and females of the corresponding adult treatment groups; however, these androgenized females still exhibited elevated gonadotropins after gonadectomy as well as low gonadotropins with hormone implants (Table 1), indicating that their neuroendocrine reproductive axis was functional and working properly.

Experiment 1: Kiss1 cells in the AVPV, but not the ARC, are sexually dimorphic

In the AVPV, the number of identifiable *Kiss1*-expressing neurons and the cellular content of *Kiss1* mRNA (as reflected by silver grains per cell) were both significantly higher in intact adult female rats (in diestrus) than intact adult males ($P < 0.01$ for both measures) (Fig. 1). Intact females possessed approximately 12-fold more *Kiss1* cells in this region than intact males, which typically possessed six or fewer *Kiss1* neurons in the sections that were examined. In contrast, in the ARC, neither the number of *Kiss1* cells nor the cellular content of *Kiss1* mRNA was significantly different between intact females and males, with the sections from both sexes containing approximately 40–50 *Kiss1* neurons (Fig. 1).

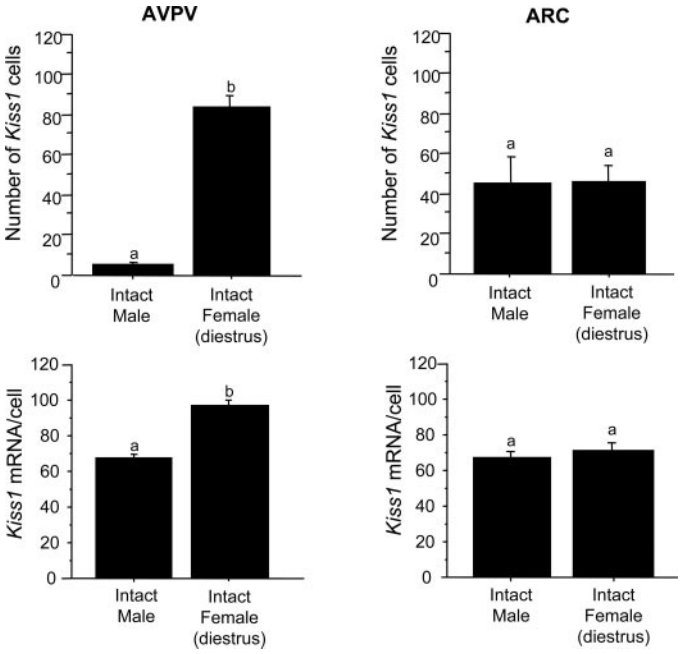

FIG. 1. The mean (+SEM) number of identifiable *Kiss1* mRNA-positive cells and grains per cell in the AVPV and ARC of intact adult male and female (diestrus) rats. Values with *different letters* differ significantly from each other ($P < 0.05$).

Experiment 2: sex differences in AVPV Kiss1 cells are organized early in development by perinatal hormones and are unaffected by the activational effects of adult hormones

To determine whether the sex difference in *Kiss1* expression in the AVPV was attributable to differences in circulating levels of sex steroids in the adult state or the hormonal milieu during neonatal development, we experimentally manipulated levels of sex steroids (E and T) in the neonatal period and adulthood. Levels of hormones in adulthood influenced levels of *Kiss1* in the AVPV of females but did not influence the sex difference in *Kiss1* expression in this same region; both castrated and castrated + E adult males (treated with vehicle at birth) possessed fewer *Kiss1* neurons in the AVPV than females that were OVX or OVX and treated with E as adults (also treated neonatally with vehicle) ($P < 0.05$ for all intersex comparisons; Figs. 2 and 3). For these neonatally vehicle-treated females, the presence of steroids in adulthood significantly increased the number of identifiable *Kiss1* neurons and *Kiss1* mRNA cell content in the AVPV ($P < 0.05$ OVX *vs.* OVX + E) (Figs. 2 and 3).

In contrast to sex steroid levels in adulthood, the sex steroid milieu during the neonatal period dramatically influenced the sex difference in *Kiss1* neurons in the adult AVPV. Female rats treated neonatally with androgen displayed fewer numbers of *Kiss1* cells in the AVPV as adults than normal females and did not differ significantly from neonatally vehicle-treated adult males on this index (Figs. 2 and 3). Both the number of *Kiss1* neurons and the cellular content of *Kiss1* mRNA were significantly lower in females given androgen at birth compared with females given vehicle alone at birth, regardless of the hormone status during adulthood ($P < 0.01$ for both measures; Figs. 2 and 3).

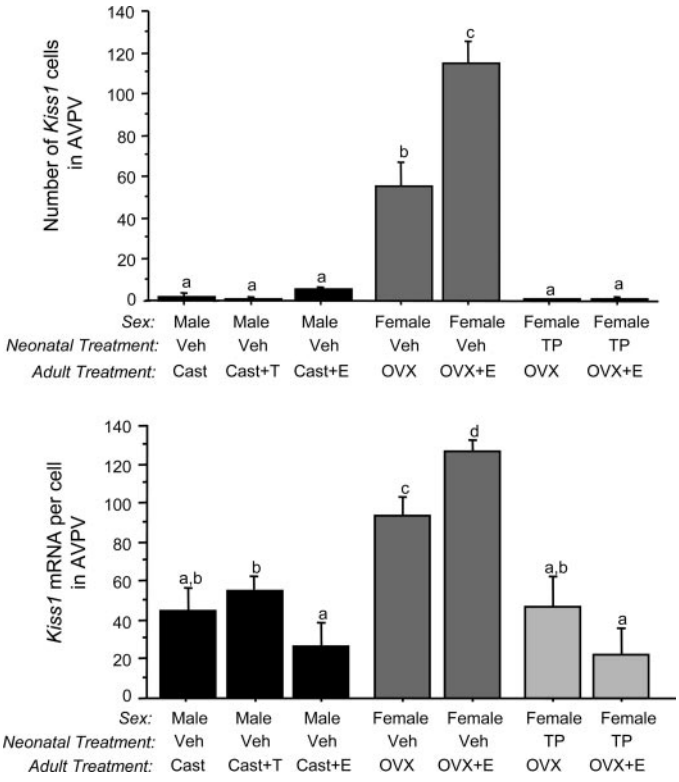

FIG. 2. Mean (+SEM) *Kiss1* mRNA expression in the AVPV of male and female rats that received vehicle (veh; oil) or androgen (TP) during neonatal development. In adulthood, animals were GNX and implanted with either a hormone-containing capsule (E or T) or an empty capsule. Values with *different letters* differ significantly from each other ($P < 0.05$). Cast, Castrated.

Kiss1 cells in the ARC are not sexually dimorphic, regardless of hormone levels in adulthood or development

The number of *Kiss1*-expressing neurons in the ARC did not differ between sexes, regardless of hormone levels present during development or adulthood. Thus, castrated males and OVX females both displayed similarly high numbers of *Kiss1* cells in the ARC, whereas GNX males and females given adulthood hormone implants had similarly low numbers of *Kiss1* neurons in this region (Figs. 4 and 5). Neonatal treatment of female pups with androgen did not significantly affect levels of *Kiss1* in the ARC; as adults, androgenized females showed high levels of *Kiss1* in the ARC after gonadectomy and low levels of *Kiss1* after hormone replacement, just as vehicle-treated males and nonandrogenized females (Figs. 4 and 5).

Experiment 3: the sexually dimorphic Kiss1 and TH systems in the AVPV are separate and distinct populations

In experiment 3A, we used double-label fluorescence IHC to examine the colocalization of TH and kisspeptin proteins in the AVPV/PeN of OVX + E female rats (the steroid condition similar to proestrus, when the LH surge occurs). Qualitatively, the vast majority of identifiable kisspeptin-positive cells in the AVPV/PeN did not overlap with the TH-containing neurons, with less than 5% of kisspeptin cells coexpressing TH protein (Fig. 6, A and B). The few kisspeptin-containing neurons that were colabeled showed only weak to moderate intensity of TH immunoreactivity (Fig. 6C).

In experiment 3B, we expanded on the initial findings above by performing a more detailed and quantitative analysis of the coexpression of *Kiss1* and *TH* mRNAs under several steroid hormone conditions. Using double-label ISH, we evaluated the coexpression of *TH* and *Kiss1* mRNAs in the AVPV/PeN of intact, OVX, and OVX + E females. Qualitatively, most TH neurons were found in the rostral and caudal AVPV, as well as the caudal PeN, whereas most *Kiss1* neurons tended to be located in the caudal AVPV and rostral, middle, and caudal PeN. Thus, although there was some overlap in the distribution of the two mRNAs, there were areas within the AVPV-PeN continuum where each mRNA was more abundantly expressed than the other (rostral and middle PeN for *Kiss1* and rostral AVPV and caudal PeN for *TH*). In AVPV/PeN areas where the two mRNAs were both present, the vast majority of large, identifiable *TH*-containing cells did not overlap with the *Kiss1* cells (Fig. 7A). Even so, there were a few *Kiss1* neurons that coexpressed low levels of *TH* mRNA, and this coexpression was influenced by the gonadal hormone status. Quantitative analyses indicted that in OVX + E females, fewer than 20% of *Kiss1* neurons coexpressed *TH* mRNA, whereas in intact diestrus females, approximately 30% of *Kiss1* cells showed coexpression (Fig. 7B). In OVX females, in which *TH* expression was up-regulated, there was a greater tendency for *TH* mRNA and *Kiss1* mRNA to colabel, with approximately 50% of *Kiss1* cells coexpressing *TH* mRNA (Fig. 7B). However, in all hormonal states, the amount of *TH* mRNA in each double-labeled *Kiss1* neuron (as determined semiquantitatively by the number of silver grains on each *Kiss1* cell) was low, typically less than 15 grains/cell (Fig. 7C). In comparison, the numerous large *TH* mRNA clusters throughout the AVPV and PeN that did not coexpress *Kiss1* mRNA typically contained between 40 and 100 grains per cell (or more) (for examples, see Fig. 7A).

Discussion

The brain is anatomically and physiologically differentiated between the sexes in all mammals (1, 3, 8). In rodents, this phenomenon develops during the perinatal critical period, which extends into the 1st week of postnatal life (3). Many differences between adult male and female phenotypes (*e.g.* morphology, reproductive physiology, and sexual behavior) reflect the organizational effects of the sex steroid environment that is present during the critical period. One important sexually differentiated trait is the ability of adult females, but not males, to display an E-induced, circadian-dependent GnRH/LH surge. The neuronal circuitry and molecular/cellular mechanisms that mediate this process are not fully characterized, although accumulating evidence suggests that E-sensitive neurons in the AVPV of the female are likely to serve as a relay station to integrate and transmit circadian and sex steroid signals that trigger GnRH secretion. In support of this contention, the AVPV is sexually differentiated, with females possessing more neurons overall than males, as well as greater numbers of TH-containing cells (2, 3, 18, 19) and GABA/glutamate cells (46). However, despite being aware (for decades) of sex differences in cell number

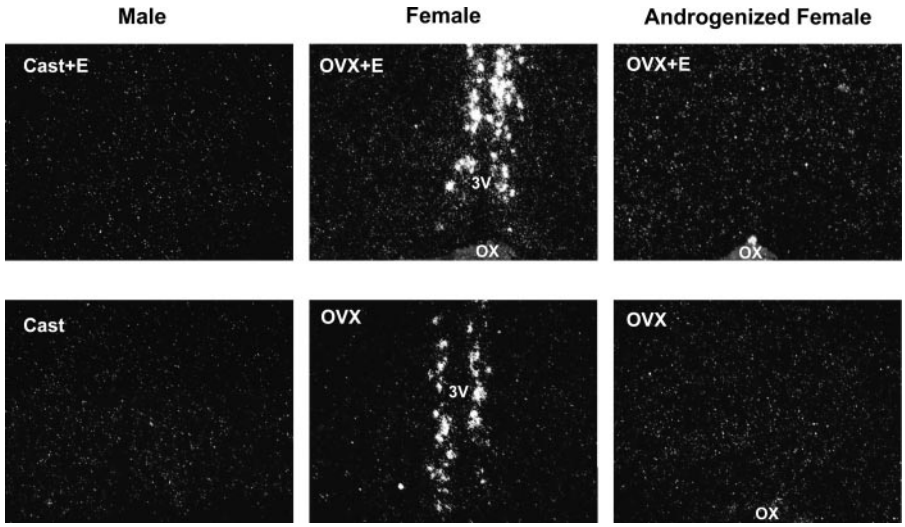

FIG. 3. Dark-field photomicrographs showing *Kiss1* mRNA-expressing cells (as reflected by the presence of *white clusters* of *silver grains*) in representative sections of the AVPV of male, female, and androgenized female rats. 3V, Third ventricle; Cast, castrated.

and type in the AVPV, we remain ignorant of the phenotypic identity of the E-sensitive neurons in the AVPV that control the GnRH/LH surge in females. In this report, we provide evidence that *Kiss1* neurons in the AVPV are sexually differentiated, with adult females possessing as much as 25 times more *Kiss1* cells than males and that the sex difference in *Kiss1* expression is organized by sex steroids early in perinatal development. We posit that this sexually differentiated population of *Kiss1* cells in the AVPV provides the molecular and cellular basis for the GnRH/LH surge that occurs in normal females and may explain its incompetence in normal males.

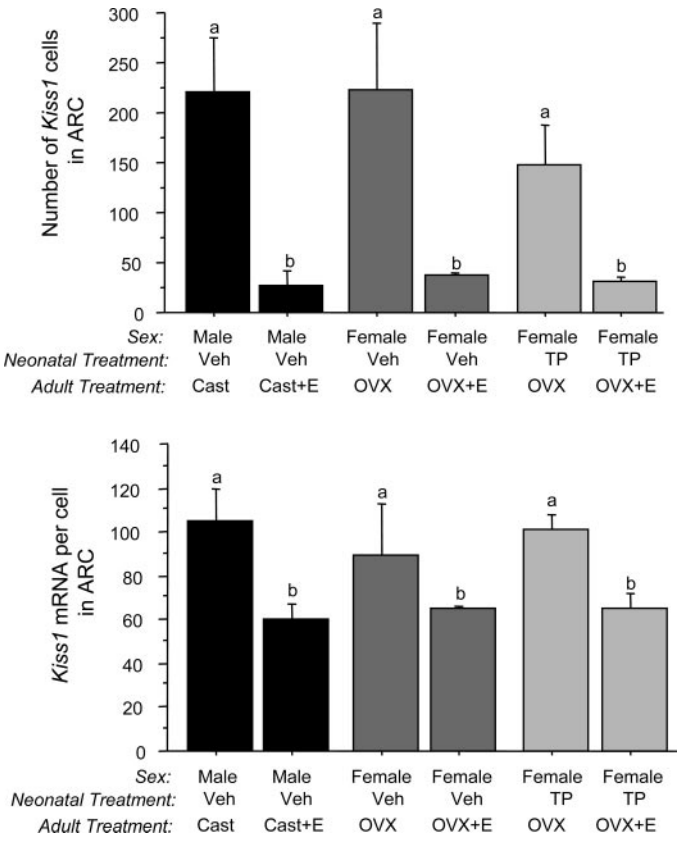

FIG. 4. Mean (+SEM) *Kiss1* mRNA expression in the ARC of male and female rats that received vehicle (veh; oil) or androgen (TP) during neonatal development. In adulthood, animals were GNX and implanted with either a hormone-containing capsule (E or T) or an empty capsule. Values with *different letters* differ significantly from each other ($P < 0.05$). Cast, Castrated.

We observed many more *Kiss1* cells (and *Kiss1* mRNA content per cell) in the AVPV of females than males (in fact, males had virtually no *Kiss1* cells in this region). Sex steroids dramatically up-regulate *Kiss1* expression in the AVPV, as reported here and demonstrated previously (24, 32). However, the sex difference in *Kiss1* neurons in the AVPV was not attributable to dissimilarities in the hormonal milieu between intact adult males and females because GNX males and females receiving similar hormone treatments in adulthood (*i.e.* either E-containing Silastic implants or empty implants) still displayed a large sex difference in *Kiss1* expression in the AVPV. In contrast, perinatal hormones had a dramatic effect on the sex difference in *Kiss1* neurons in the AVPV. Adult females that were treated with androgen on the day of birth showed vanishingly few *Kiss1* neurons (and *Kiss1* mRNA per cell) as adults, more similar to normal males than normal females. These data indicate that the *Kiss1* system in the AVPV is organized early in development under the influence of gonadal steroids, which produce robust gender differences in *Kiss1* expression in the AVPV later in adulthood. Clarkson and Herbison (44) recently reported similar findings of sex differences in kisspeptin protein levels (as determined by ICC) in the AVPV of intact mice, although it remains to be determined whether these mouse sex differences reflect differences in the hormonal environment of the adults or the organizational effects of perinatal exposure to sex steroids. Despite the apparent congruence of the observations on sex differences in both the mouse and rat, there are unresolved discrepancies in the reported anatomical distribution of *Kiss1* mRNA-containing cells and kisspeptin-containing neurons within (and among) species. *Kiss1* mRNA-containing neurons (as detected with ISH) do not appear in the dorsomedial nucleus in either the rat or mouse (25, 26, 30, 31), whereas kisspeptin-positive cell bodies are apparently detectable by ICC in this region (44). This discrepancy suggests either problems in the

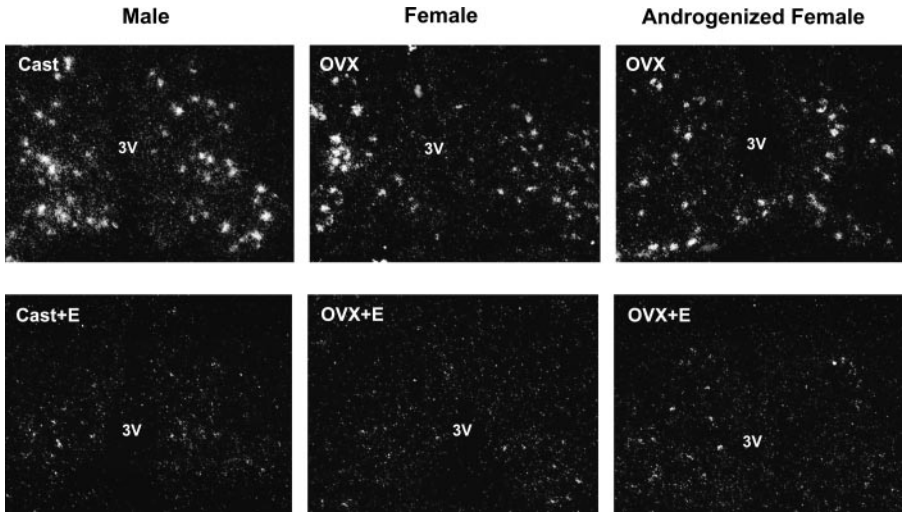

FIG. 5. Dark-field photomicrographs showing *Kiss1* mRNA-expressing cells (as reflected by the presence of *white clusters* of *silver grains*) in representative sections of the ARC of male, female, and androgenized female rats. 3V, Third ventricle; Cast, castrated.

sensitivity of the ISH technique (which we consider unlikely) or nonspecific binding of the kisspeptin antibody, possibly to other Arg-Phe amide peptides (*i.e.* RF-amides) in the dorsomedial nucleus that are similar to kisspeptin (47). Proper validation of the available kisspeptin antisera should help to resolve this inconsistency.

A circumstantial, but compelling, line of evidence suggests that the sexually differentiated population of *Kiss1* neurons in the AVPV drives the sexually differentiated E-induced GnRH/LH surge. First, kisspeptin is a potent secretagogue for GnRH, and most GnRH neurons express the kisspeptin receptor GPR54 (25–28, 48). Second, expression of *Kiss1* mRNA in the AVPV increases at the time of the GnRH/LH surge, coincident with increased coexpression of the transcription factor Fos in *Kiss1* neurons (32). Third, central infusion of antiserum to kisspeptin blocks the preovulatory LH surge in female rats (34). Fourth, lesions of the AVPV block spontaneous and steroid-induced preovulatory surges (12, 13, 49), indicating this anatomical site is indeed critical for producing the surge. Fifth, an unidentified population of ERα-containing neurons in the AVPV mediates the stimulatory effects of E on the preovulatory surge mechanism (17). Because virtually all *Kiss1* neurons in the AVPV express ERα (30, 31), we infer that these previously unidentified E-sensitive *Kiss1* neurons in the AVPV represent the critical group that mediates the effects of E on the surge. Finally, only females possess significant numbers of *Kiss1* cells in the AVPV (even after E or T treatment given to adult males). Because the ability to generate a GnRH/LH surge is sexually differentiated, occurring only in females (3, 4, 18, 50, 51), we argue that *Kiss1* neurons in the AVPV of females serve as the cellular conduit for integrating and relaying circadian and steroid hormone signals to GnRH neurons to produce the preovulatory GnRH/LH surge.

In contrast to the AVPV/PeN, the ARC showed no gender-based differences in either the number of identifiable *Kiss1* neurons or the content of *Kiss1* mRNA per cell. Both males and females displayed relatively high numbers of *Kiss1* neurons in the ARC after gonadectomy and low numbers of *Kiss1* neurons after sex hormone (E or T) replacement. An earlier report in mice noted anecdotally that the number of apparent kisspeptin-expressing cells in the ARC was similar in males and females (44), although this observation was neither quantitatively or statistically corroborated nor controlled for sex differences in circulating levels of adulthood hormones. We have argued that *Kiss1* cells in the ARC provide tonic stimulatory input to GnRH neurons and that *Kiss1* neurons in the ARC mediate the negative feedback regulation of gonadotropin secretion that occurs in both sexes (24). The classical work of Halasz and Gorski (52) suggests that the ARC comprises the essential anatomical circuitry that mediates the negative feedback regulation of gonadotropin secretion. In both sexes, removal of sex steroid feedback (by gonadectomy) increases the expression of *Kiss1* in the ARC and is associated with a concomitant increase in gonad-

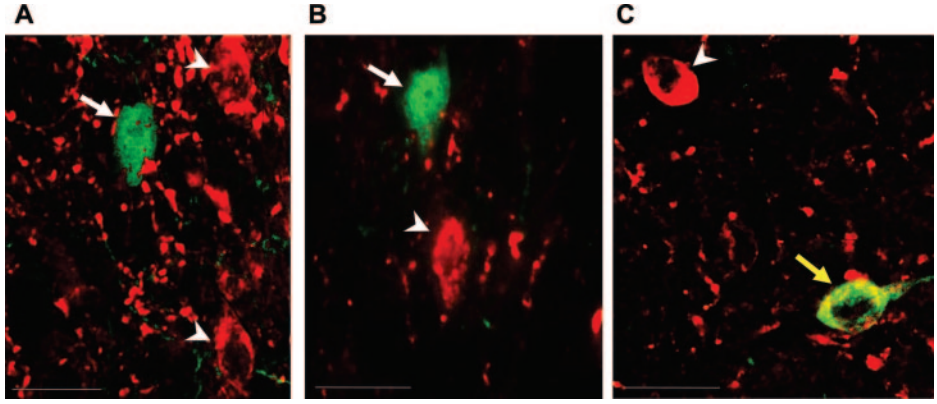

FIG. 6. Representative photomicrographs showing the low degree of coexpression of kisspeptin (*red*) and TH (*green*) proteins in the AVPV/PeN of female rats (OVX + E). A and B, Examples of no colocalization of kisspeptin and TH in AVPV neurons. C, Two kisspeptin cells in the AVPV, one single-labeled and one colabeled with moderate TH immunoreactivity (colabeled area appears *yellow*). *Scale bars*, 10 microns. *White arrowheads*, kisspeptin-containing cells; *white arrows*, single-label TH cells; *yellow arrow*, double-labeled cell.

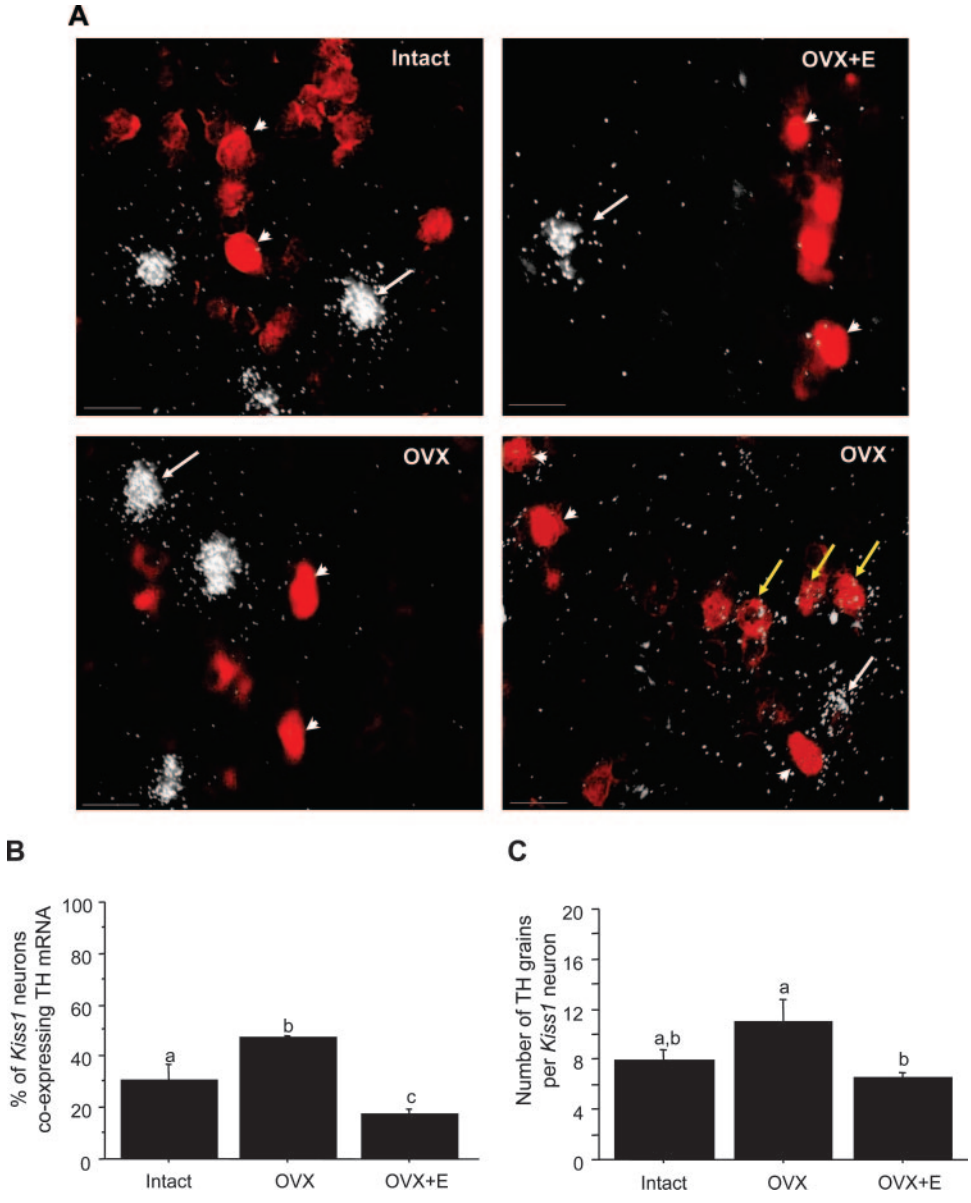

FIG. 7. A, Representative photomicrographs showing the degree of coexpression of *Kiss1* mRNA and *TH* mRNA in the AVPV/PeN region. *Kiss1* cells were visualized with Vector Red substrate, and *TH* mRNA was marked by the presence of *silver grains*. *Scale bars*, 10 microns. *White arrowheads*, example *Kiss1* mRNA-containing cells; *white arrows*, example *TH* mRNA cell clusters; *yellow arrows*, example *Kiss1* neurons in which *TH* mRNA was moderately coexpressed. B, Quantitative analysis showing the mean (+SEM) percentage of *Kiss1*-mRNA neurons in the AVPV/PeN that express *TH* mRNA in intact, OVX, and OVX + E female rats. C, Mean (+SEM) number of *silver grains* (representing *TH* mRNA) on double-labeled *Kiss1* neurons in the AVPV/PeN in intact, OVX, and OVX + E female rats. (For comparison, note that the large clusters of *silver grains* of the single-labeled TH cells shown in A contain between 50 and 100 *silver grains*.) Values with *different letters* differ significantly from each other ($P < 0.05$).

otropin secretion, whereas steroid hormone treatment inhibits the expression of *Kiss1* in this region and is associated with reduced gonadotropins (26, 30, 31). The fact that *Kiss1* neurons in the ARC are direct targets for inhibition by sex steroids (T in males and E in females) and that the population of *Kiss1* neurons is sexually undifferentiated provide a compelling, albeit circumstantial, case for *Kiss1* neurons in this region serving as the central player of this negative feedback system.

The AVPV contains a previously described, sexually dimorphic population of TH-positive (*i.e.* dopaminergic) neurons (2, 3), which is larger in females than males. We considered the possibility that these TH-containing cells might be the same as the newly discovered population of *Kiss1* neurons in the AVPV, whose expression is also sexually dimorphic. However, based on double-labeling assays for mRNAs and proteins, we reject this hypothesis. Few neurons within these two distinct populations showed robust coexpression of both markers. Although both cell types were located throughout the AVPV/PeN region, *TH*-expressing cells tended to be more prominent in the rostral and caudal AVPV and caudal PeN, whereas most *Kiss1* cells were in the caudal AVPV and rostral and middle PeN. In areas where the two cell types were both present, *Kiss1* cells tended to be closer to the third ventricle, whereas *TH* cells were often (but not always) located more laterally. Although there was a modest degree of coexpression between *TH* and *Kiss1* when sex steroids were low or absent, only half of the *Kiss1* cells coexpressed *TH* mRNA, and those *Kiss1* cells that were double labeled contained relatively little *TH* mRNA compared with the more intensely stained, single-labeled TH cells in the AVPV/PeN. Thus, although there was a slight overlap in the anatomical distribution of these two cell populations and a low degree of colabeling for some *Kiss1* cells, we conclude that in the rat, *Kiss1* neurons and the previously described sexually dimorphic *TH*-expressing cells in the AVPV/PeN represent two separate, sexually differentiated populations.

Furthermore, within this region, at least in rats, there appear to be two separate TH populations, one with neurons that contain abundant *TH* mRNA but do not coexpress *Kiss1* and another population containing low amounts of *TH* mRNA that colabel with some (but not all) *Kiss1* cells. We note that our results in the rat are in marked contrast to the mouse, where virtually all *Kiss1* neurons in the AVPV coexpress *TH* mRNA (53). This species difference between the mouse and rat stresses the importance of being cautious when making generalizations about rodents, based on observations in one particular species.

Curiously, the *Kiss1* and TH populations in the rat AVPV are inversely regulated by gonadal steroids; the expression of *Kiss1* in the AVPV/PeN is highest in the presence of E, whereas the expression of *TH* mRNA in this region is lowest in this same steroidal environment (and up-regulated in the absence of E) (19). It is unclear whether this pattern reflects a cooperative functioning between the two populations, with *Kiss1* neurons being activated at the time of the LH surge and TH neurons coincidentally inhibited. If so, the decrease in TH release at the time of the LH surge may serve to reduce inhibitory signaling to GnRH neurons that, when combined with enhanced kisspeptin release, would produce a larger net stimulatory effect on GnRH release. Under conditions when E is absent, the number of identifiable *Kiss1* cells in the AVPV/PeN decreases by 40–50%. It is possible that the additional (up-regulated) weakly stained TH neurons observed in the AVPV/PeN under OVX conditions represent the same subpopulation of *Kiss1* cells in this region that stop expressing *Kiss1* mRNA in the absence of E. Further studies are required to address this possibility and the respective roles of the two independent, sexually differentiated populations in the rat AVPV/PeN and to determine whether (and how) *Kiss1* and TH interact with one another. It also remains to be determined whether the sexually dimorphic *Kiss1* neurons are the same as the recently identified sexually dimorphic GABA/glutamate neurons in the AVPV that have also been implicated in GnRH regulation (46).

As shown in Table 1, mean basal LH levels (in adulthood) tended to be lower in androgenized females than intact diestrous females, although the magnitude of this reduction was somewhat modest (0.36 ± 0.06 *vs.* 0.68 ± 0.15). We also observed that 1 wk post-OVX, levels of LH and FSH in androgenized females were significantly lower than in normal males and females. Both these observations are consistent with earlier reports showing that neonatally androgenized females exhibit lower basal levels of LH in adulthood as well as a reduced LH response to castration relative to normal males and females (54–56). The lower basal levels of LH in the androgenized females before gonadectomy presumably reflect increased E levels (and hence, increased negative feedback) because these animals are known to be in constant estrus in adulthood. The reason for the reduced LH response to gonadectomy is unknown but may involve either changes in central circuits mediating negative feedback or transient effects of chronic elevation of E induced by neonatal androgenization. Further studies are required to understand the role of neonatal sex steroids in the development of mechanism(s) underlying steroidal negative feedback in adulthood.

In summary, the expression of *Kiss1* in the AVPV/PeN (but not the ARC) is sexually differentiated, with females displaying much higher degrees of expression than males, even under identical hormonal conditions as adults. Sexual differentiation of *Kiss1* in the AVPV/PeN occurs by virtue of the organizational effects of gonadal hormones present during the perinatal critical period. Females treated neonatally with androgen display a male-like pattern of *Kiss1* expression in the AVPV/PeN as adults. Furthermore, the sexually dimorphic population of *Kiss1* neurons in the AVPV/PeN appears to be separate and distinct from the large, sexually dimorphic population of TH-expressing (dopaminergic) neurons in this region of the brain. These observations suggest the gender-specific display of the GnRH/LH surge mechanism reflects sexual differentiation in the development of *Kiss1* neurons in the AVPV/PeN.

Note Added in Proof

Since this manuscript was submitted, a paper by Wintermantel *et al*. (37) was published showing that an unidentified population of ERα-expressing neurons in the AVPV directly innervates GnRH neurons, adding further credence to our proposition that *Kiss1* neurons in the AVPV (all of which express ERα) drive the preovulatory GnRH/LH surge.

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