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Significance of Neonatal Testicular Sex Steroids to Defeminize Anteroventral Periventricular Kisspeptin Neurons and the GnRH/LH Surge System in Male Rats[1]

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ABSTRACT

The brain mechanism regulating gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) release is sexually differentiated in rodents. Kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) have been suggested to be sexually dimorphic and involved in the GnRH/LH surge generation. The present study aimed to determine the significance of neonatal testicular androgen to defeminize AVPV kisspeptin expression and the GnRH/LH surge-generating system. To this end, we tested whether neonatal castration feminizes AVPV kisspeptin neurons and the LH surge-generating system in male rats and whether neonatal estradiol benzoate (EB) treatment suppresses the kisspeptin expression and the LH surge in female rats. Immunohistochemistry, in situ hybridization, and quantitative real-time RT-PCR were performed to investigate kisspeptin and *Kiss1* mRNA expressions. Male rats were castrated immediately after birth, and females were treated with EB on postnatal Day 5. Neonatal castration caused an increase in AVPV kisspeptin expression at peptide and mRNA levels in the genetically male rats, and the animals showed surge-like LH release in the presence of the preovulatory level of estradiol (E2) at adulthood. On the other hand, neonatal EB treatment decreased the number of AVPV kisspeptin neurons and caused an absence of E2-induced LH surge in female rats. Semiquantitative RT-PCR analysis showed that neonatal steroidal manipulation affects *Kiss1* expression but does not significantly affect gene expressions of neuropeptides (neurotensin and galanin) and enzymes or transporter for neurotransmitters (gamma-aminobutyric acid, glutamate, and dopamine) in the AVPV, suggesting that the manipulation specifically affects *Kiss1* expressions. Taken together, our present results provide physiological evidence that neonatal testicular androgen causes the reduction of AVPV kisspeptin expression and failure of LH surge in genetically male rats. Thus, it is plausible that perinatal testicular androgen causes defeminization of the AVPV kisspeptin system, resulting in the loss of the surge system in male rats.

GPR54, Kiss1, Kiss1r, *luteinizing hormone, metastin, sexual differentiation*

INTRODUCTION

The brain mechanism regulating gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) surge is known to be sexually differentiated in rodents [1, 2]. Positive estrogen feedback to induce GnRH/LH surge is evident in female rats, whereas male rats never show LH surges, even with a preovulatory level of estradiol (E2) [3]. The LH surge-generating system has been implicated to be disrupted by neonatal E2 converted from testosterone because a single estradiol benzoate (EB) injection during the neonatal period, such as at Day 0 or Day 5 of age, causes persistent estrus and failure of ovulation in female rats [4, 5], and because 5α-dihydrotestosterone, an unaromatizable androgen, did not affect estrous cyclicity in neonatal female rats [6]. Corbier [7] found that male rats castrated within 1 h after birth showed an afternoon increase in LH levels compared with the morning level, whereas neonatal castration at 24 h postnatally failed to feminize the afternoon increase in LH. Actually, circulating levels of androgen are much higher in male than female rats from prenatal Day 18 to Day 1 after birth [8].

Kisspeptin (first named metastin)-Kiss1r (previously called GPR54) signaling [9, 10] plays a critical role in regulating reproductive function via stimulation of GnRH/LH release in mammals [11–15], and loss of *Kiss1r* function in human and rodents causes hypogonadotropic hypogonadism [16, 17]. Several bodies of evidence suggest that kisspeptin neurons are sexually dimorphic. Our work [18] and a number of other studies [19–21] showed that cell bodies of kisspeptin neurons were distributed in the anteroventral periventricular nucleus (AVPV) and the hypothalamic arcuate nucleus (ARC) in female rats and mice. On the other hand, males show a different distribution of kisspeptin neurons, which are expressed in the ARC but are few in the AVPV. Interestingly, the AVPV has long been suggested to be a sexual dimorphic nucleus and a center for GnRH/LH surge [22, 23]. Electrolytic lesion of a small periventricular column of cells located immediately caudal to the organum vasculosum of the lamina terminalis (OVLT), which is equivalent to the AVPV, or of medial preoptic nucleus (MPO) results in persistent estrus and failure of the steroid-induced LH surge [24], whereas E2 implantation in the anterohypothalamic area, including the AVPV in the ovariectomized (OVX) rat, showed a surge-like increase in LH release [25]. Hence, AVPV kisspeptin neurons are considered to be a target of estrogen-positive feedback action to induce GnRH/LH surge. Indeed, estrogens positively regulate AVPV kisspeptin and *Kiss1* mRNA expression in female rodents [18, 20]. The elegant work of Kauffman et al. [19] showed that neonatally androgenized female rats display a malelike pattern of reduced *Kiss1* expression in the AVPV. Recently, Navarro et al. [26] demonstrated that neonatal EB treatment suppresses

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whole hypothalamic *Kiss1* mRNA expressions in male and female rats. Furthermore, neonatal treatment of estrogenic substances or estrogen receptor-α (ESR1) agonist in female rats causes reduction of AVPV kisspeptin neurons [27, 28]. All of these findings provide circumstantial evidence that estrogens converted from fetal or neonatal testicular androgens cause loss of the AVPV kisspeptin expression and GnRH/LH surge mechanism in male rats. However, there has been no direct evidence for the role of neonatal testicular androgen in defeminization of AVPV kisspeptin neurons in genetically male rats. The neonatal steroidal milieu could not merely be the determinant for neuronal phenotype. Genetic factors have also been considered to be another determinant for sex difference in neurons because sex chromosome genes have been implied to cause sexual dimorphisms of behaviors and neuronal phenotypes of dopaminergic or vasopressin neurons [29, 30]. Therefore, it is of great interest to clarify the role of neonatal testicular androgen in defeminizing AVPV kisspeptin expression by removing neonatal steroids.

Another issue to be addressed is whether neonatal steroids affect other AVPV neurons besides kisspeptin, because a number of neurotransmitters or neuropeptides in the AVPV, such as glutamate [31–34], gamma-aminobutyric acid (GABA) [35, 36], galanin [37, 38], neurotensin [39], and dopamine [22, 40, 41], have been suggested to be involved in the induction of the GnRH/LH surge. Ottem et al. [34] have demonstrated that ERα-immunoreactive GABA/glutamate neurons in the AVPV show sexual dimorphism and suggested that these dual-phenotype neurons are the main targets of the positive estrogen feedback action on GnRH neurons. Galanin, neurotensin, and dopaminergic neurons in the AVPV are also acknowledged to be sexually dimorphic and larger in female rats than in male rats [37, 40–42], and neonatal testosterone causes masculinization of AVPV dopaminergic neurons [43]. Therefore, it is noteworthy to investigate the effects of neonatal steroidal manipulation on gene expressions of these neuronal markers in the AVPV to determine whether the manipulation specifically affects *Kiss1* expression.

The present study aimed to determine whether androgen released from neonatal testes defeminizes AVPV kisspeptin neurons, and then the GnRH/LH surge-generation system. To this end, we tested whether castration at birth feminizes AVPV gene expression of kisspeptin and/or the other neuropeptides, enzymes, and transporters for neurotransmitters that may result in feminization of the estrogen-positive feedback system in genetic male rats. In addition, we examined whether the attenuation of AVPV kisspeptin caused by neonatal estrogen is associated with the absence of a GnRH/LH surge system in female rats.

MATERIALS AND METHODS

Animals

Wistar-Imamichi-strain male and female rats were housed under controlled temperature (23°C ± 2°C) and light (lights on, 0500–1900 h) conditions with standard rodent chow (CE-2; CLEA, Tokyo, Japan) and water ad libitum. The present studies were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Neonatal Treatments

The day of parturition was designated Day 0 postpartum, and litters were adjusted to 10 rats per dam on Day 1. Male rats were castrated under hypothermic anesthesia within 2 h after birth to serve as the Neo Cast group. Female rats received a single subcutaneous injection of EB (25 μg/50 μl; Sigma, St. Louis, MO) dissolved in peanut oil on Day 5 to serve as the Neo EB group. The animals were weaned on Day 20 and then maintained until at least 8 wk of age. Vaginal smears were checked in Neo EB females to determine estrous cyclicity from Day 30 until the day they were ovariectomized (OVX). Neonatal treatments did not affect body weight gain until they were used for adult experiments (data not shown).

Adult Treatments

Adult female rats (ages 10–12 wk; 250–300 g body weight) that showed at least two consecutive 4-day estrous cycles, as well as adult male rats (ages 10–12 wk; 300–350 g body weight) without any neonatal treatments were gonadectomized to serve as the Ad OVX and Ad Cast groups, respectively. Six days before blood or brain tissue sampling, some animals in the Neo Cast, Neo EB, Ad Cast, and Ad OVX groups were implanted with subcutaneous Silastic tubing (1.57-mm inner diameter; 3.18-mm outer diameter; 25 mm in length; Dow Corning, Midland, MI) filled with estradiol-17β (Sigma) dissolved in peanut oil at 20 μg/ml to produce a negative-feedback level of plasma E2 [44] for 4 days. Tubing was then replaced with Silastic tubing (1.0-mm inner diameter; 1.5-mm outer diameter; 20 mm in length; Dow Corning) containing crystalline E2 to produce a positive-feedback level of plasma E2 for 2–3 days. This high E2 level has been confirmed to induce daily LH surges in OVX rats [45]. Remaining animals in each group were kept without E2 treatments to serve as Non-E2-treated controls. All surgical procedures at adulthood were performed under ketamine or isoflurane anesthesia.

Blood Sampling to Detect E2-Induced LH Surge

Blood samples were collected from animals (n = 4–6 in each group) with adult E2 treatments in each group through a silicon cannula (0.5-mm inner diameter; 1-mm outer diameter; Shin-Etsu Polymer, Tokyo, Japan) that had been inserted into the right atrium on the day before blood sampling. To detect LH surges, samples were collected every 1 h from 1100 h for 24 h in unanesthetized and unrestrained conditions by an automatic blood sampling system (DraQ; EICOM, Kyoto, Japan).

In Situ Hybridization

Kiss1 in situ hybridization in rats was conducted as described previously [18, 46]. Briefly, animals (n = 4–7 in each group) were deeply anesthetized with sodium pentobarbital and perfused with 4% paraformaldehyde between 1300 and 1600 h. Serial 50-μm coronal sections containing AVPV and ARC were obtained. *Kiss1*-specific digoxigenin (DIG)-labeled probe was used for nonradioactive free-floating in situ hybridization. Every second section through the whole AVPV and the rostral part of the periventricular hypothalamic nucleus (Pe; from 0.36 mm anterior to 0.48 mm posterior to the bregma) or every fourth section through the ARC (from 1.8 to 3.96 mm posterior to the bregma) was taken from each rat brain, according to a rat brain atlas [47]. Sections were hybridized with DIG-labeled antisense cRNA probe (position 33–348; GenBank accession no. AY196983) synthesized from the rat hypothalamus using a labeling kit (Roche Molecular Biochemicals, Mannheim, Germany). To visualize the DIG labeling, sections were incubated with an alkaline phosphatase-conjugated anti-DIG1 antibody (1:1000; Roche) for 2 h at 37°C and then with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate solution. Numbers of *Kiss1*-expressing cells were counted under a light microscope, and the sum of the cell number in brain sections (eight sections for AVPV; 11 sections for ARC) was obtained.

Immunohistochemistry

Kisspeptin immunohistochemistry was conducted as described previously [18]. To detect AVPV kisspeptin-immunoreactive cells, animals (n = 4–5 in each group) were given a lateral ventricular injection of colchicine (75 μg per 15 μl) 2 days before killing, then deeply anesthetized with sodium pentobarbital and perfused with 5% acrolein (Tokyo Kasei Kogyo, Tokyo, Japan) between 1300 and 1600 h. Every second section through the whole AVPV and the rostral part of the Pe (from 0.36 mm anterior to 0.48 mm posterior to the bregma) or every fourth section through the ARC (from 1.8 to 3.96 mm posterior to the bregma) was taken from each rat brain, according to a rat brain atlas [47]. Sections containing AVPV or ARC were stained with mouse anti-rat kisspeptin monoclonal antibody (1:50 000; no. 254; Takeda), which was produced by Dr. H. Matsumoto of Takeda Pharmaceutical Co. and is not commercially available. The antibody was raised against a C-terminal 17-amino acid sequence (36–52) of rat kisspeptin [15]. The specificity of the antibody was already confirmed by competitive binding assay, Western blotting, and double staining with kisspeptin immunohistochemistry and *Kiss1* in situ hybridization [15, 48]. According to the previous study [48], rat kisspeptin showed the highest affinity to the anti-rat kisspeptin monoclonal antibody (Takeda no. 254) but showed a lower affinity to human kisspeptin and amidated

tetrapeptide of rat kisspeptin C-terminal (GLRY-amide) and did not crossreact with neuropeptide Y, adrenomedullin, rat/mouse RF-amide-related peptide (RFRP)-1, human RFRP-3, human prolactin-releasing peptide, rat neuropeptide FF, and nonamidated rat kisspeptin C-terminal tetrapeptide (GLRY). Numbers of kisspeptin-immunopositive cells were counted under a light microscope, and the sum of the cell number in brain sections (eight sections for AVPV; 11 sections for ARC) was obtained.

Tissue Dissection for Discrete Brain Areas

Animals (n = 4–7 in each group) were decapitated between 1300 and 1500 h, and each brain sample including the AVPV and medial preoptic area (mPOA) was punched out with 18-gauge stainless-steel tubing from the coronal brain section for quantitative and semiquantitative RT-PCR. The AVPV tissue samples were named OVLT-AVPV because they included whole OVLT and AVPV, the rostral part of Pe, and small portions of the diagonal band of Broca and MPO (Supplemental Fig. S1, all Supplemental Data are available online at www.biolreprod.org). The mPOA tissues included part of the horizontal limb of the diagonal band (HDB), the median preoptic nucleus, and most of the MPO, so they were named HDB-MPO. The OVLT-AVPV and HDB-MPO tissue slices extended from 0.6 mm anterior to 0.6 mm posterior to the bregma, respectively. The ARC tissues included whole ARC and medium eminence (ME) regions and small portions of the medial ventral part of ventromedial hypothalamic nucleus, the ventromedial part of the dorsal tuberomammillary nucleus, the ventral part of the dorsomedial hypothalamic nucleus, and the ventral part of the premammillary nucleus. The ARC-ME region was dissected out from the brain with the following brain atlas coordination [47]: anterior and posterior ends were 1.8 and 3.96 mm posterior to the bregma, respectively. The coronal sections of the rest of the brain were stained with thionin and observed under a microscope to verify whether each brain region had been correctly dissected out.

Quantitative Analysis of Kiss1 and Kiss1r mRNA Expressions by Real-Time RT-PCR

Expressions of *Kiss1* and *Kiss1r* mRNA in these above-mentioned brain regions were determined by real-time RT-PCR (TaqMan) with ABI PRISM 7900HT (Applied Biosystems, Foster City, CA) as described previously [15, 46, 48]. The cDNA from each RNA sample was synthesized with oligo(deoxythymidine) primer using the SuperScript III First-Strand Synthesis System for RT-PCR according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The following cycling protocol was used: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Forward and reverse primers and TaqMan probe for *Kiss1*, *Kiss1r*, and *Actb* were described elsewhere [15, 48, 49]. The copy number of *Kiss1* and *Kiss1r* transcripts was normalized to the ratio to the copy number of *Actb* transcripts for each sample.

Semiquantitative Analysis of mRNA Expressions of Kiss1, Neuropeptides, and Enzymes or Transporters for Neurotransmitters in the AVPV by RT-PCR

Total RNA isolation in all samples (n = 4 in each group) was processed using ISOGEN reagents (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's instructions. A total of 2 μg of RNA was used to reverse transcribe using high-capacity cDNA of the reverse transcription kit RT (Applied Biosystems) in accordance with the instructions. All primer sequences and PCR products are described in Supplemental Table S1. Tyrosine hydroxylase (*Th*) is a marker of dopaminergic neurons in the AVPV [40]. Vesicular glutamate transporter 2 (*vGlut2*), the gene of which is officially named solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 (*Slc17a6*), is a marker of hypothalamic glutamatergic neurons [50]. GAD65 (*Gad65*; official symbol *Gad2*) and GAD67 (*Gad67*; official symbol *Gad1*) are isoforms of glutamic acid decarboxylase (GAD), a marker of GABA neurons. Neurotensin (*Nts*), galanin (*Gal*), *Gnrh*, and *Kiss1* were also analyzed by RT-PCR. β-Actin (*Actb*) was used as the internal control. The RT-PCR for the mRNAs for *Gad1*, *Gad2*, and *Actb* was performed under the following conditions: 95°C for 5 min, and 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min using AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR for *Th*, *Nts*, *Gal*, *Slc17a6*, and *Gnrh* was performed under the following conditions: 95°C for 5 min, and 35 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min. The mRNA levels of *Kiss1*, *Th*, *Nts*, *Gal*, *Gad1*, *Gad2*, *Slc17a6*, and *Gnrh* were semiquantified using the Image J program (version 1.40g; http://rsb.info.nih.gov/ij/). These values were determined by subtracting the background gel intensity value (obtained by measuring the area of the gel devoid of PCR product) from that of each amplicon. The intensity was then expressed as a value relative to that of the *Actb* amplicon. Results were averaged from four sets of independent experiments.

LH Assay

Luteinizing hormone concentrations in 25 μl of plasma samples were measured by a rat LH radioimmunoassay (RIA) kit provided by the National Hormone and Peptide Program (Baltimore, MD) and were expressed in terms of National Institute of Diabetes and Digestive and Kidney Diseases rat LH RP-3. The least detectable level was 0.32 ng/ml for 25 μl of plasma samples. Intraassay and interassay coefficients of variation were 5.6% at 2.3 ng/ml and 6.4% at 1.4 ng/ml, respectively.

Statistical Analysis

Statistical differences in cell numbers expressing *Kiss1* mRNA or kisspeptin among Ad OVX, Ad Cast, Neo Cast, and Neo EB groups were determined by two-way ANOVA (main factors, experimental groups, and adult E2 treatment) followed by Bonferroni test. Statistical differences in mRNA levels of *Kiss1* and *Kiss1r* analyzed by quantitative real-time RT-PCR and of *Kiss1*, *Th*, *Nts*, *Gal*, *Gad1*, *Gad2*, *Slc17a6*, and *Gnrh* analyzed by semiquantitative RT-PCR among Ad OVX, Ad Cast, Neo Cast, and Neo EB groups were also determined by two-way ANOVA (main factors, experimental groups, and adult E2 treatment) followed by Bonferroni test. Hourly changes in LH levels in each group treated with preovulatory levels of E2 were analyzed by two-way ANOVA with repeated measures (main factors, experimental groups, and clock time), and then differences in LH levels at each clock time and the basal LH levels (at 1100 h) in the same group were determined by Bonferroni test.

RESULTS

Effects of Neonatal Steroidal Manipulations on E2-Induced LH Surge

Hourly changes in plasma LH levels for 24 h in all groups treated with preovulatory levels of E2 in adulthood are shown in Figure 1. The analysis for the LH concentrations using a two-way ANOVA with repeated measures revealed the significant effects of experimental groups ($F[3,12] = 15.291$; $P < 0.05$) and clock time ($F[23,276] = 7.862$; $P < 0.05$) and interaction of these factors ($F[69,276] = 3.360$; $P < 0.05$). Plasma LH levels were increased in the afternoon and peaked at 1600–1700 h in Neo Cast and Ad OVX rats. As a result, the LH levels at 1600 h in Neo Cast and at 1700 h in Ad OVX were significantly higher ($P < 0.05$, Bonferroni test) compared with the levels at 1100 h within the group. On the other hand, LH levels remained low throughout the sampling period in Ad Cast males and Neo EB female rats, so LH levels at any time were not significantly different from those at 1100 h in the same group.

Effects of Neonatal Steroidal Manipulation on Kiss1 mRNA-Expressing Neurons in AVPV and ARC

Figure 2 shows AVPV and ARC *Kiss1* mRNA-expressing cells in all groups with (for AVPV) or without (for ARC) adult E2 treatment. A large number of *Kiss1* mRNA-expressing cells were found in the AVPV and the rostral part of the Pe in Neo Cast (Fig. 2A), whereas few *Kiss1*-expressing cells were detected in Ad Cast males (Fig. 2B). Many AVPV *Kiss1*-expressing cells were found in Ad OVX females (Fig. 2C), whereas cells in Neo EB rats were scattered in the AVPV (Fig. 2D). In the ARC, *Kiss1* mRNA-expressing cells were abundant in all groups without adult E2 treatment (Fig. 2, E–H), yet few were detected with adult E2 treatment (data not shown).

Analysis of the numbers of *Kiss1 mRNA*-expressing cells in the AVPV using a two-way ANOVA showed the significant effects of experimental groups ($F[3,28] = 22.104$; $P < 0.001$)

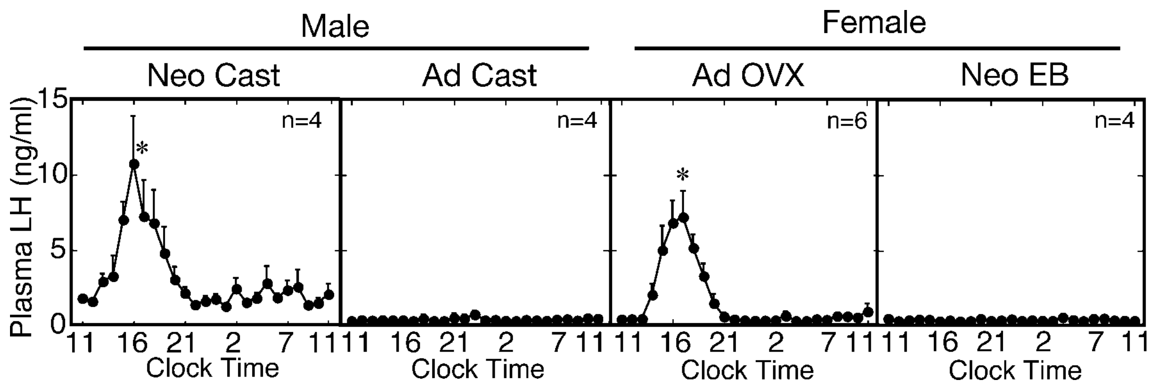

FIG. 1. Effects of neonatal steroidal manipulation on E2-induced LH surge at adulthood. Hourly changes in plasma LH levels were determined in freely moving conscious rats from 1100 h for 24 h. Values are means ± SEM (n = 4–6). *$P < 0.05$ vs. LH levels at 1100 h within each group (two-way ANOVA with repeated measures followed by Bonferroni test). Neo Cast, male rats castrated on the day of birth; Ad Cast, male rats castrated at adulthood; Ad OVX, female rats ovariectomized at adulthood; Neo EB, female rats injected with EB 5 days after birth and ovariectomy at adulthood.

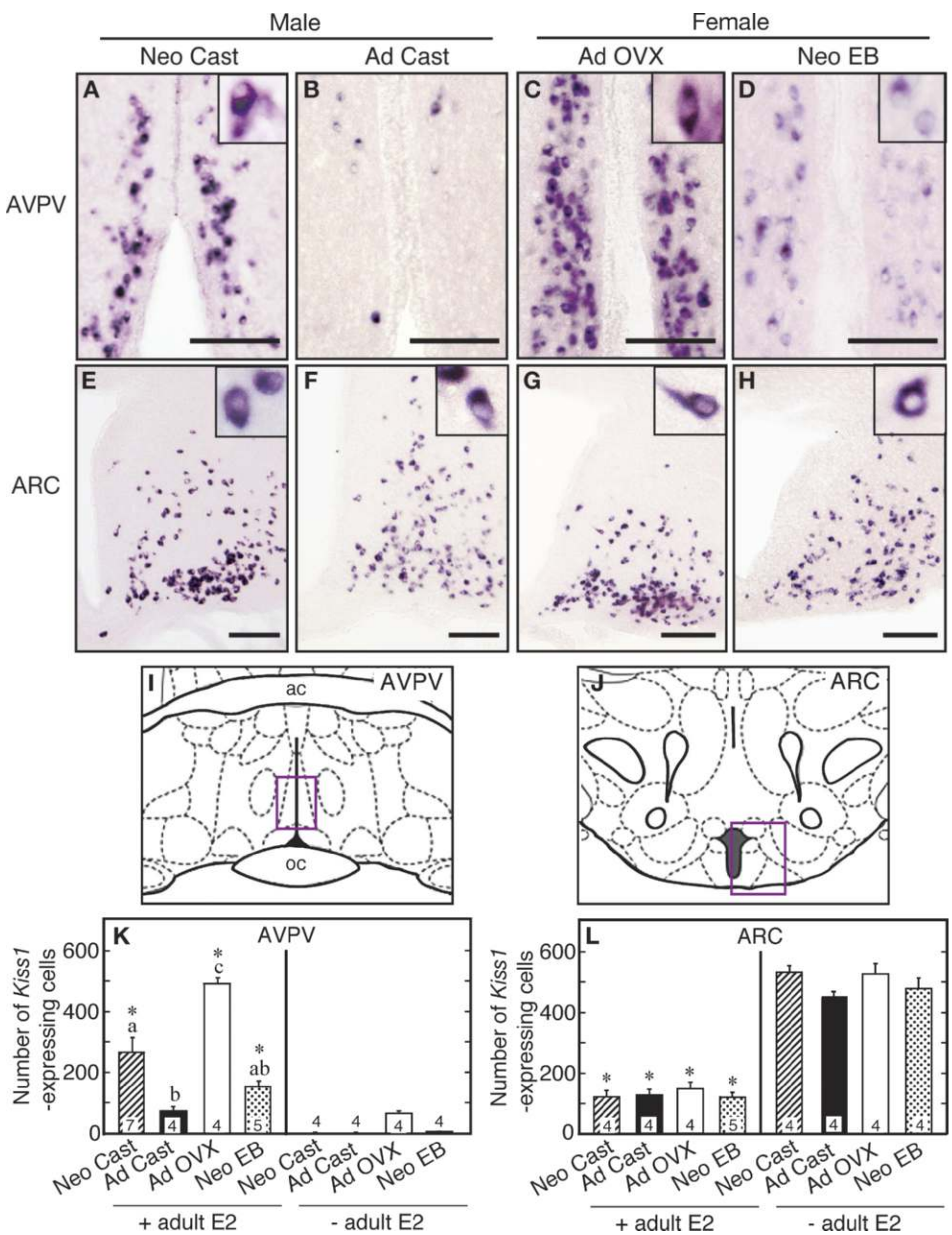

FIG. 2. Effects of neonatal steroidal manipulation on *Kiss1* mRNA expressions in the AVPV and ARC in the presence or absence of E2 treatment at adulthood. **A**–**D**) *Kiss1* mRNA in situ hybridization in the AVPV of a representative animal in each group treated with E2 at adulthood. **E**–**H**) *Kiss1* mRNA in situ hybridization in the ARC of a representative animal without adult E2 treatment. Insets in **A** and **C**–**H** show *Kiss1* mNRA-expressing cells; original magnification ×3. **I** and **J**) schematic drawings of the brain sections including the AVPV (**I**) and ARC (**J**). The squares show the areas shown in **A**–**D** (for AVPV) and **E**–**G** (for ARC). **K** and **L**) Number of *Kiss1* mRNA-expressing cells in the AVPV and ARC. Hatched, solid, open, and dotted columns indicate Neo Cast, Ad Cast, Ad OVX, and Neo EB groups, respectively. Values are means ± SEM. Values with same letters are not significantly different ($P < 0.05$, two-way ANOVA followed by Bonferroni test) within the group with or without E2 treatment at adulthood. Values with asterisks are significantly different ($P < 0.05$, two-way ANOVA followed by Bonferroni test) from corresponding animals without adult E2 treatment. The number in or on each column indicates the number of animals used. The coordination of brain sections in these representative animals is around 0–0.12 mm anterior from the bregma. Bars = 200 μm. See Figure 1 for abbreviations for the groups.

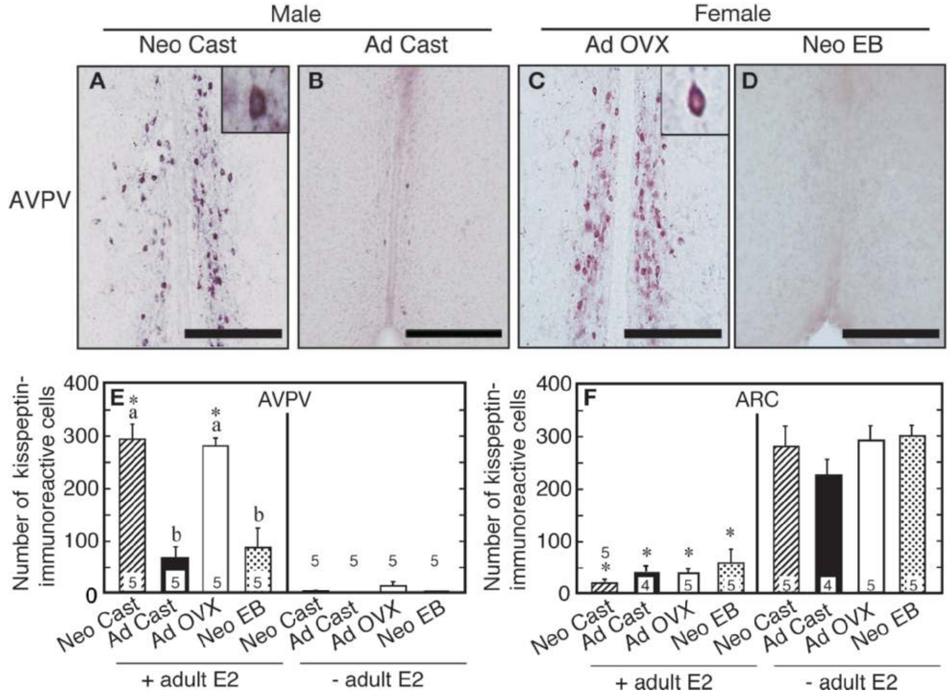

FIG. 3. Effects of neonatal steroidal manipulation on kisspeptin expressions in the AVPV and ARC in the presence or absence of E2 treatment at adulthood. **A–D**) Kisspeptin-immunopositive cells detected by immunohistochemistry in the AVPV of a representative animal in each group treated with E2 at adulthood with intracerebroventricular injection of colchicine. Insets in **A** and **C** show kisspeptin-immunopositive cells; original magnification ×3. **E** and **F**) number of kisspeptin-immunopositive cells in the AVPV and ARC in each group with or without E2 treatment at adulthood. Values are means ± SEM. Values with same letters are not significantly different ($P < 0.05$, two-way ANOVA followed by Bonferroni test) within the group with the same adult E2 treatment. Values with asterisks are significantly different ($P < 0.05$, two-way ANOVA followed by Bonferroni test) from corresponding animals without adult E2 treatment. The number in or on each column indicates the number of animals used. The coordination of brain sections in these representative animals is around 0–0.12 mm anterior from the bregma. Bars = 200 μm. See Figure 1 for abbreviations for the groups.

and adult E2 treatment ($F[1,28] = 102.951$; $P < 0.001$) and a significant interaction of these factors ($F[3,28] = 11.1$; $P < 0.001$). The analysis showed the significant effects of adult E2 treatment on the numbers of ARC *Kiss1*-expressing cells ($F[1,27] = 322.072$; $P < 0.001$). The number of AVPV *Kiss1* mRNA-expressing cells in Neo Cast or Ad OVX with adult E2 treatment was significantly higher ($P < 0.05$, Bonferroni test) than in Ad Cast animals (Fig. 2K), although the number in Neo Cast males was significantly lower than in Ad OVX rats ($P < 0.05$, Bonferroni test). The number in Neo EB was significantly lower than in Ad OVX females and was comparable to Ad Cast males. Few *Kiss1*-positive cells were found in the AVPV of any groups in the absence of adult E2 treatment, and AVPV *Kiss1*-expressing cells in Neo Cast, Ad OVX, and Neo EB animals with adult E2 treatment were significantly more numerous ($P < 0.05$, Bonferroni test) than without E2 treatment.

The number of ARC cells expressing *Kiss1* mRNA in rats treated with E2 at adulthood was significantly lower than without adult E2 treatment ($P < 0.05$, Bonferroni test; Fig. 2L). No significant difference was found between groups in the number of ARC *Kiss1*-expressing cells in the presence or absence of E2 treatment in adulthood.

Effects of Neonatal Steroidal Manipulation on Kisspeptin-Immunoreactive Neurons in AVPV and ARC

Figure 3, A–D, indicates kisspeptin-immunoreactive cells in the AVPV in all groups with adult E2 treatment. A large number of kisspeptin-immunoreactive cells were found in the AVPV and the rostral part of the Pe in both Neo Cast and Ad OVX rats (Fig. 3, A and C), whereas few were detected in Ad Cast males and Neo EB females (Fig. 3, B and D). In the ARC, kisspeptin-immunoreactive cells were hardly found in all groups treated with E2 at adulthood (data not shown).

Analysis of the numbers of AVPV kisspeptin-immunopositive cells with two-way ANOVA showed the significant effects of experimental groups ($F[3,32] = 7.442$; $P < 0.01$) and adult E2 treatment ($F[1,32] = 63.129$; $P < 0.001$) and a significant interaction of these factors ($F[3,32] = 6.649$; $P < 0.01$). The analysis showed no significant effects of experimental groups on the numbers of ARC kisspeptin-immunoreactive cells but did show a significant effect of adult E2 treatment on them ($F[1,30] = 191.511$; $P < 0.001$).

The numbers of AVPV kisspeptin-immunoreactive cells in Neo Cast and Ad OVX rats with adult E2 treatment were significantly higher ($P < 0.05$, Bonferroni test) compared with those in Ad Cast and Neo EB animals with adult E2 treatment, as well as to those in Neo Cast and Ad OVX rats without adult E2 treatment (Fig. 3E). The ARC kisspeptin-immunoreactive cells in each group were significantly fewer in rats with adult E2 treatment than without it ($P < 0.05$, Bonferroni test; Fig. 3F). No significant difference was found in the number of ARC kisspeptin-immunoreactive cells between groups.

Quantitative Analysis of Kiss1 *and* Kiss1r *mRNA Levels in OVLT-AVPV, ARC-ME, and HDB-MPO Regions*

Kiss1 and *Kiss1r* mRNA levels in the OVLT-AVPV, ARC-ME, and HDB-MPO in all groups gtreated with or without E2 at adulthood are shown in Figure 4. The analysis for *Kiss1* mRNA levels in the OVLT-AVPV and ARC-ME regions using a two-way ANOVA showed the significant effects of experimental groups (OVLT-AVPV, $F[3,30] = 6.112$; $P < 0.05$; ARC-ME, $F[3,30] = 19.25$; $P < 0.001$) and adult E2 treatment (OVLT-AVPV, $F[1,30] = 16.799$; $P < 0.001$; ARC-ME, $F[1,30] = 260.59$; $P < 0.001$) and a significant interaction of these factors (OVLT-AVPV, $F[3,30] = 5.408$; $P < 0.05$; ARC-ME, $F[3,30] = 10.524$; $P < 0.001$). OVLT-AVPV *Kiss1* mRNA levels in Neo Cast and Ad OVX rats with adult E2 treatment were significantly higher ($P < 0.05$, Bonferroni test) compared with those in Ad Cast and Neo EB animals with adult E2 treatment, as well as those in Neo Cast and Ad OVX rats without adult E2 treatment (Fig. 4A). ARC-ME *Kiss1* mRNA levels in all groups with adult E2 treatment were not significantly different but were significantly lower ($P < 0.05$, Bonferroni test) than those in corresponding groups without adult E2 treatment. ARC-ME *Kiss1* mRNA levels in Neo Cast and Ad OVX rats without adult E2 treatment were significantly higher ($P < 0.05$, Bonferroni test) than in Ad Cast and Neo EB

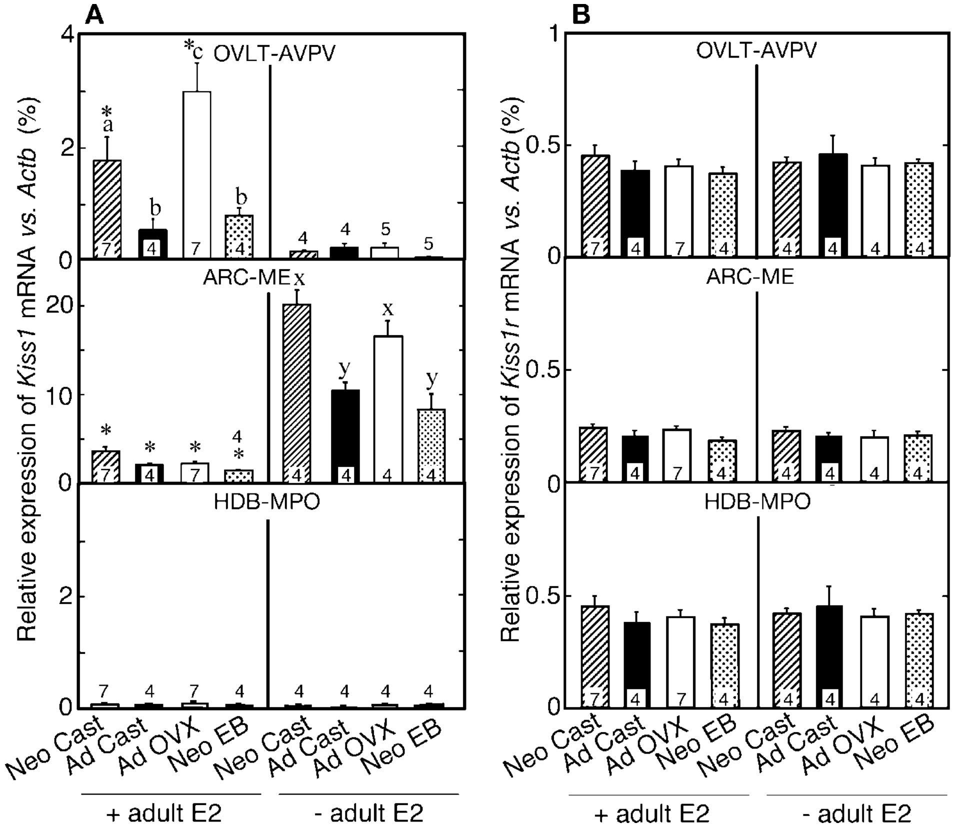

FIG. 4. Effects of neonatal steroidal manipulation on *Kiss1* (**A**) and *Kiss1r* (**B**) mRNA levels in the OVLT-AVPV, ARC-ME, and HDB-MPO regions in each group in the presence or absence of E2 treatment at adulthood. The mRNA levels were determined by quantitative real-time RT-PCR and indicated as a relative expression to *Actb*. Values are means ± SEM. Values with same letters are not significantly different ($P < 0.05$, two-way ANOVA followed by Bonferroni test) within the group with the same adult E2 treatment (with or without E2 treatment at adulthood). Values with asterisks are significantly different ($P < 0.05$, two-way ANOVA followed by Bonferroni test) from corresponding animals without adult E2 treatment. The number in or on each column indicates the number of animals used. See Figure 1 for abbreviations for the groups.

animals. *Kiss1* mRNA levels in the HDB-MPO and *Kiss1r* mRNA levels in all of the brain regions determined were not significantly different among groups, regardless of adult E2 treatment (Fig. 4).

Effects of Neonatal Steroidal Manipulations on mRNA Expressions of Kiss1, Th, Nts, Gal, Gad1, Gad2, Slc17a6, *and* Gnrh *in OVLT-AVPV Region*

The mRNA expressions of several neuropeptides and enzymes or a transporter for neurotransmitters in the OVLT-AVPV region were determined by RT-PCR. The analysis for *Kiss1* mRNA levels in the OVLT-AVPV using a two-way ANOVA showed no significant effects of experimental groups ($F[3,24] = 2.158$; $P > 0.05$) and no significant interaction of these factors ($F[3,24] = 2.191$; $P > 0.05$), but it evidenced the significant effect of adult E2 treatment ($F[1,24] = 26.723$; $P < 0.001$). The *Kiss1* mRNA expressions were sexually dimorphic in the presence of E2 at adulthood: *Kiss1* mRNA levels were significantly higher ($P < 0.05$, Bonferroni test) in Ad OVX females compared with Ad Cast males (Fig. 5). *Kiss1* mRNA expression in Neo Cast males was rescued, and the levels were comparable to those in Ad OVX rats with adult E2 treatment. Neo EB treatment partially reduced *Kiss1* mRNA levels, but the value was not significantly different from Ad OVX females and Ad Cast males. On the other hand, *Kiss1* mRNA was undetectable in most animals in all groups in the absence of adult E2 treatment, resulting in a significant difference found between the presence and absence of adult E2 treatment in Neo Cast, Ad OVX, and Neo EB groups. No significant difference was found in *Kiss1* mRNA levels among any groups without adult E2 treatment.

Th and *Nts* mRNA levels in the presence of adult E2 treatment were relatively higher in Ad OVX females than those in Ad Cast males. The *Th* and *Nts* mRNA expressions were partially rescued in Neo Cast males, and those in Neo EB were partially regulated down. However, the levels were not significantly different ($P > 0.05$, Bonferroni test) among any groups. A similar expression pattern was observed in *Th* and *Nts* mRNA levels in all groups without adult E2 treatment.

The other neuropeptides and enzymes or a transporter for neurotransmitters did not show a sexually dimorphic pattern, regardless of adult E2 treatment. *Gal* mRNA levels were comparable between Neo Cast, Ad Cast, Ad OVX, and Neo EB groups, regardless of adult E2 treatment. *Gal* mRNA expression in any group with adult E2 treatment was relatively lower than that in the absence of adult E2 treatment, but no significant effect of adult E2 was found. *Gad1* mRNA levels were significantly lower in Neo EB animals compared with other groups in the absence of adult E2. The *Gad1* mRNA levels were not significantly different among any groups in the presence of adult E2. No significant difference was found in mRNA expressions of *Gad2*, *Slc17a6*, and *Gnrh* in the presence or absence of adult E2 treatment.

DISCUSSION

The present study showed that AVPV kisspeptin expression was feminized in genetically male rats by removing the testes on the day of birth. In this neonatally castrated male model, exogenous E2 was able to induce the surge-like LH release, as shown in female rats. Considering the limited number of AVPV kisspeptin neurons and absence of E2-induced LH surge in normal male rats [18, 21, 51], the present results provide direct physiological evidence that neonatal testicular androgens but not genetic factors cause the reduction in AVPV kisspeptin expression and the absence of LH surge in genetically male rats. The EB treatment at 5 days of age attenuated the AVPV kisspeptin neurons in female rats and abolished the LH surge-generating system. Kauffman et al.

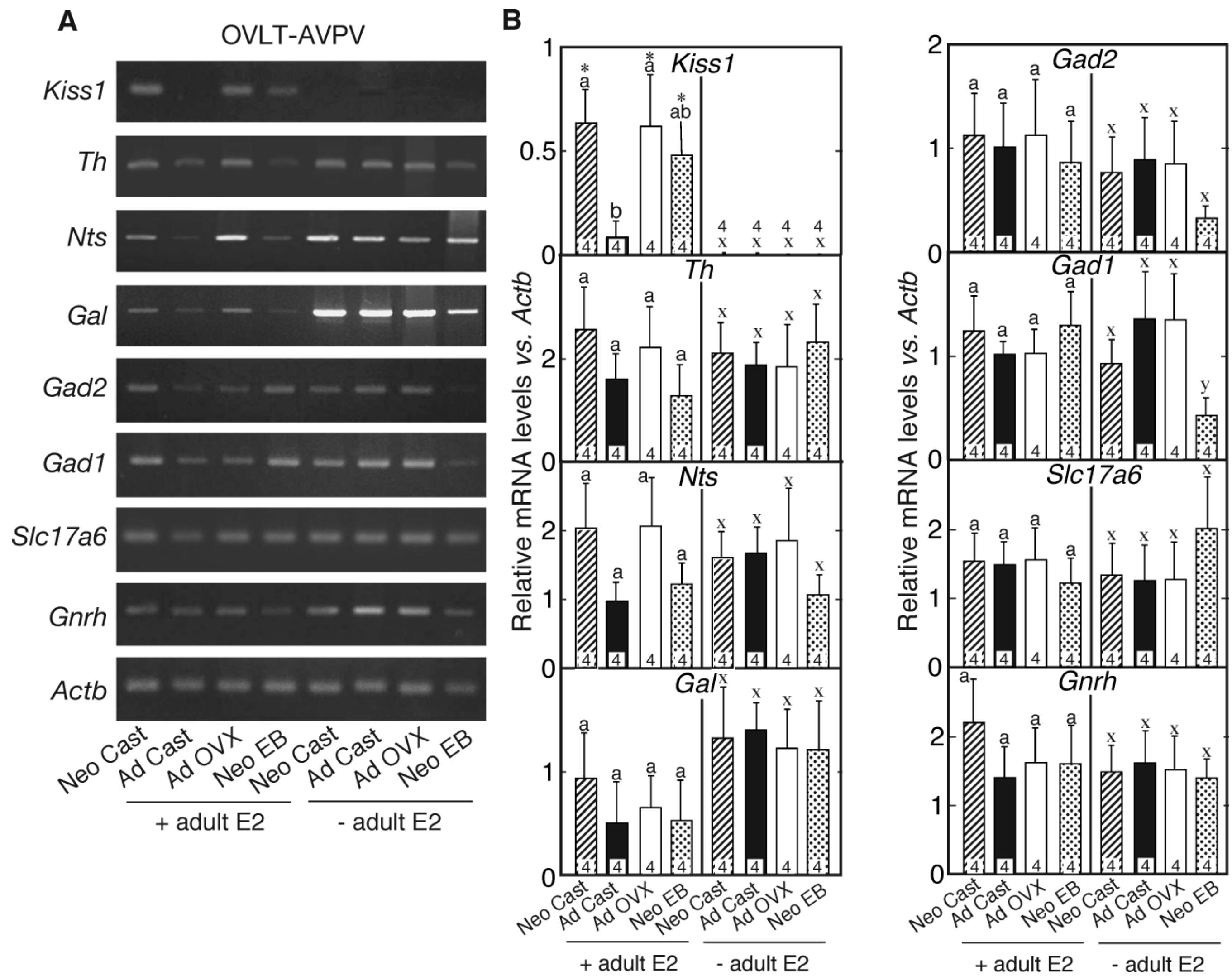

FIG. 5. Effects of neonatal steroidal manipulation on mRNA expressions of *Kiss1*, *Th*, *Nts*, *Gal*, *Gad1*, *Gad2*, *Slc17a6*, *Gnrh*, and *Actb* in the OVLT-AVPV region in each group in the presence or absence of E2 treatment at adulthood. The mRNA levels of *Kiss1*, *Th*, *Nts*, *Gal*, *Gad1*, *Gad2*, *Slc17a6*, and *Gnrh* were determined semiquantitatively by RT-PCR with the primers shown in Supplemental Table S1 followed by analysis with Image J from the National Institutes of Health. The values are indicated as a relative expression to *Actb*. Values are means ± SEM. Values with same letters are not significantly different ($P > 0.05$, two-way ANOVA followed by Bonferroni test) within the group with the same adult E2 treatment (with or without E2 treatment at adulthood). See Figure 1 for abbreviations for the groups.

[19] first showed that the number of kisspeptin neurons and *Kiss1* mRNA levels at adulthood remained low in female rats with neonatal androgen treatment on the day of birth. Further, Gonzalez-Martinez et al. [52] showed downregulation of kisspeptin-immunoreactive cells in the AVPV in α-fetoprotein knockout female mice compared with wild-type females. Taken together with the present results, perinatal E2 converted from androgen is responsible for the defeminization of AVPV kisspeptin neurons, and then most probably resulting in the lack of an LH surge-generating system. This idea is also supported by our previous results showing that blockade of endogenous central kisspeptin disrupts preovulatory and E2-induced LH surge [15].

The present study has some limitations due to the techniques. One is inconsistency between the number of *Kiss1*-expressing cells and kisspeptin-expressing cells: the number of cells expressing kisspeptin was less than the number of cells expressing *Kiss1* mRNA (Figs. 2 and 3). These inconsistencies could be attributed to the difference in targets between in situ hybridization (*Kiss1* mRNA) and immunohistochemistry (mature amidated kisspeptin) [48]. Our previous study showed that the anti-rat kisspeptin antibody (Takeda no. 254) used in the present study recognizes mature rat kisspeptin (1–52) but not its prepropeptide form [48]. Thus, it is plausible that some of the *Kiss1* mRNA-containing cells do not express mature kisspeptin peptides. Another limitation of the present study is the usage of colchicine to detect kisspeptin immunoreactivity in the AVPV. The blockade of axonal transport by the colchicine treatment may have caused an extraphysiological accumulation of kisspeptin peptides in the cell body, which has caused inconsistent results between the two techniques. Further studies will be required to clarify this point.

Besides kisspeptin, there are a number of neuropeptides or neurotransmitters located in the AVPV, playing critical roles in the induction of LH surge [31, 36, 38, 39]. The present semiquantitative analysis revealed that gene expressions of neuropeptides and enzymes or a transporter for neurotransmitters except for *Kiss1* in the OVLT-AVPV region were not significantly affected by the neonatal steroidal manipulation.

OVLT-AVPV *Kiss1* mRNA expression showed sexual dimorphism (high in Ad OVX female rats vs. low in Ad Cast males) in the presence of E2 at adulthood. Further, *Kiss1* mRNA and kisspeptin peptide were highly expressed in both Neo Cast males and Ad OVX rats in the presence of E2, which induced the surge-like LH release. *Th* and *Nts* gene expressions in the OVLT-AVPV showed a modest sexual dimorphism in the presence of E2 at adulthood, as reported previously [37, 40], but the effect of neonatal steroidal treatment was not significant. Likewise, the present results showed no apparent effects of neonatal steroidal manipulation on gene expressions of *Gad1/2* and *Slc17a6* in the OVLT-AVPV. *Gad1/2* and *Slc17a6* mRNA expressions were constantly detected in the OVLT-AVPV, regardless of the neonatal and adult steroidal milieu, suggesting that both sexes equally have components (SLC17A6 and GAD1/2) for glutamatergic and GABAergic neurons in the AVPV, which are widely acknowledged to play an important role in induction of LH surge [31, 33–36, 53–57]. These findings suggest that the components (kisspeptin, TH, neurotensin, galanin, GAD1/2, and SLC17A6, as well as GnRH) required for the GnRH/LH surge-generating system seem to be equipped in the OVLT-AVPV region of female rats, but that male rats lack at least one component—the AVPV kisspeptin neurons. This may be one reason why GnRH/LH surge generation is absent in the male rat.

A number of studies have indicated that the exogenous kisspeptin challenge can induce surge-like LH release in male rodents [12, 13, 58], suggesting that male rats retain the responsiveness to kisspeptin to release a surge amount of LH. The GnRH neurons have been reported to express *Kiss1r* and are thus considered to be a key action site for central kisspeptin [13, 21]. In fact, *Kiss1r* mRNA expressions in brain regions including the HDB-MPO, which contains the majority of GnRH neuronal cell bodies, were similar among all groups. This suggests that there is no sex difference in central kisspeptin receptor expressions, regardless of the neonatal and adult steroidal milieu. Therefore, the sex difference in the surge-generating system is likely due to the difference in kisspeptin neuronal activities and not to the difference in the responsiveness of the GnRH-releasing system to kisspeptin. Thus, low expression of AVPV kisspeptin neurons is likely responsible for the lack of positive estrogen feedback to induce the LH surge in male rats.

It still remains to be determined how AVPV *Kiss1* expression is reduced by fetal or neonatal androgen/estrogen. Navarro et al. [59] showed that *Kiss1* mRNA levels in the whole hypothalamus, including both AVPV and ARC areas, did not differ between male and female rats on the day of birth. Apoptosis may be a mechanism causing sexual dimorphism of kisspeptin expressions in the AVPV. Neonatal androgen treatment reportedly increased apoptotic cells in the AVPV-POA region in female rats [60]. Forger et al. [61] showed that Bax-dependent cell death is required for the sexual differentiation of the AVPV cell number, and that the sex difference in the AVPV dopaminergic cell number was not affected by *Bax* gene deletion. Neonatal male rats showed higher Bax expression and caspase-3 activation in the AVPV on the day of birth than female rats [62]. Thus, the neonatal estrogens converted from androgens may cause Bax-mediated apoptosis in AVPV kisspeptin neurons. There is still another possibility that AVPV *Kiss1* gene expression is specifically suppressed throughout life by certain mechanisms, such as epigenetic regulation of gene expression after neonatal exposure to estrogen. Indeed, in vitro DNA methyltransferase expression is regulated by progesterone and estrogen in human endometrial cells [63]. In addition, neonatal methylation of human glucocorticoid receptor gene (*NR3C1*) has been reported to be increased by prenatal exposure to maternal depression [64].

In summary, the current study demonstrates that AVPV kisspeptin expressions are feminized at peptide and mRNA levels in genetically male rats by castration on the day of birth. Neonatally castrated genetic male rats proved capable of responding to estrogen to show the LH surge, which is induced only in female rats in nature. Thus, neonatal testicular androgen may cause the reduction of AVPV kisspeptin neurons and then the absence in LH surge in genetically male rats. The present study also shows that neonatal estrogen causes a reduction of AVPV kisspeptin neurons and the loss of the E2-induced LH surge in female rats. Although no direct evidence of a mechanism mediating sex difference in the GnRH/LH surge-generating system was found, our results make it plausible that fetal or neonatal testicular androgen causes defeminization of the AVPV kisspeptin system, resulting in the loss of the surge system in male rats.

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