Effects of sex steroids on the regulation of pituitary gonadotropins in the pituitary and hypothalamus

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Abstract

Sex steroids play a pivotal role in the control of the hypothalamic-pituitary-gonadal axis. In this study, we examined how the sex steroids estradiol (E2), progesterone (P4), and dihydrotestosterone (DHT) influence the synthesis of three pituitary gonadotropin subunits in a pituitary gonadotroph cell model (LβT2 cells) *in vitro* and ovary-intact rats *in vivo*. The effects of sex steroids on *Kiss1* gene expression in the hypothalamus were also examined in ovary-intact rats. E2 increased common glycoprotein alpha (*Cga*) and luteinizing hormone beta (*Lhb)* subunit promoter activity as well as their mRNA expression. Although gonadotropin subunit promoter activity was not modulated by P4, *Cga* and *Lhb* mRNA expression was increased by P4. DHT inhibited *Cga* and *Lhb* mRNA expression with a concomitant decrease in their promoter activity. During the 2-week administration of exogenous E2 to ovary-intact rats, the estrous cycle, which was determined by vaginal smears, was disrupted. P4 or DHT administration completely eliminated the estrous cycle in rats. Protein expression of all three gonadotropin subunits within the pituitary gland was inhibited by E2 or P4 treatment *in vivo*; however, DHT reduced *Cga* expression but did not modulate *Lhb* or follicle-stimulating hormone beta subunit expression. E2 administration significantly repressed *Kiss1* mRNA expression in a posterior hypothalamic region that included the arcuate nucleus. P4 and DHT did not modulate *Kiss1* mRNA expression in this region. In contrast, P4 administration significantly inhibited *Kiss1* mRNA expression in a posterior region of the hypothalamus that included the anteroventral periventricular nucleus. Our findings suggest that sex steroids have different effects in the hypothalamus and pituitary gland.

Introduction

The pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) play pivotal roles in the maintenance of female reproductive function by stimulating follicular development and steroid biosynthesis in the ovary. LH and FSH are under the control of gonadotropin-releasing hormone (GnRH), which is released in a pulsatile manner from the hypothalamus. Gonadotropins are also released in a pulsatile manner in response to GnRH pulses, which are altered during the reproductive cycle [1]. At present, it is generally agreed that hypothalamic kisspeptin neurons, which are located in two different areas of the hypothalamus, monitor the serum levels of sex steroids and modulate the secretion of GnRH [2]. In rodents, kisspeptin neurons located in the anteroventral periventricular nucleus (AVPV) are involved in the surge secretion of GnRH, which induces a surge in LH after oocyte maturation with a concomitant increase in the serum level of estradiol (E2). However, kisspeptin neurons in the arcuate nucleus (ARC) control the basal pulsatile secretion of GnRH and are reinforced by a decrease and/or depletion of E2 [1]. This concept was evidenced by the observation that the expression of the *Kiss1* gene (which encodes kisspeptin) in the AVPV is upregulated by E2, whereas its expression in the ARC region is repressed by E2 [3, 4]. Thus, kisspeptin neurons in the AVPV are a center of E2-induced positive feedback and induce a surge of GnRH/LH, while those in the ARC regulate the negative feedback mechanism of E2.

E2 produced from ovarian follicles plays an indispensable role in regulating the hypothalamic-pituitary-gonadal (HPG) axis and maintains female reproductive function. In addition to E2, other sex steroids

such as progesterone (P4) and androgens also participate in the control of the HPG axis by acting on the hypothalamus [5, 6]. These sex steroids play a role in the feedback mechanism of the hypothalamus by modulating the secretory pattern of GnRH; however, sex steroids also influence the pituitary gland directly and regulate gonadotroph function, as evidenced by the expression of receptors for E2, P4, and androgens in gonadotrophs [7]. E2 increases luteinizing hormone beta (*Lhb*) subunit mRNA synthesis in pituitary tissue, but it has no effect on follicle-stimulating hormone beta (*Fshb*) subunit expression [8]. Another study reported that E2 and P4 stimulate FSH secretion and synthesis in ovine pituitary cultures [9]. In a study of GnRH-deficient males, Finkelstein et al. provided direct evidence showing that E2 inhibits gonadotropin secretion at the pituitary level [10]. These previous studies revealed the complexity of sex steroid action on pituitary gonadotropins, which can be both stimulatory and inhibitory depending on study design, the presence or absence of GnRH, or other experimental conditions. The effects of E2 on gonadotropin secretion have been reviewed comprehensively [7, 11]. The principal effect of P4 is to decrease the frequency of gonadotropin pulses, presumably mediated by the action of the hypothalamus on GnRH pulse secretion. After ovulation and when P4 concentrations are high, LH pulse frequency slows markedly [12]. Although studies on the direct action of P4 are limited compared with those of E2, P4 is reported to augment the E2-induced stimulatory or inhibitory effect on gonadotropin secretion in different parts of the menstrual cycle [13]. Much like the effects of E2, androgens also inhibit gonadotropin secretion when administered to normal human males and rats, and these inhibitory effects are considered to occur mainly at the hypothalamic level [10]; however, evidence for the direct action of androgens on pituitary gonadotropins is limited.

In this study, we determined the action of sex steroids on the HPG axis. We examined the effects of the sex steroids E2, P4, and androgens on the expression of gonadotropins. To examine androgens, we applied dihydrotestosterone (DHT), a potent stimulator of the androgen receptor. We assessed the direct action of these sex steroids on pituitary gonadotrophs using an immortalized murine pituitary gonadotroph-derived model, LβT2 cells. Furthermore, we administered these sex steroids to ovary-intact rats and investigated the changes in gonadotropin subunit expression in the anterior pituitary gland and also examined the alteration in *Kiss1* gene expression in hypothalamic regions.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: fetal bovine serum (Invitrogen, Carlsbad, CA); kisspeptin-10 (AnaSpec, Fremont, CA); and Dulbecco's modified Eagle's medium, water-soluble β-E2, water soluble P4, 5a-DHT, and penicillin–streptomycin (Sigma-Aldrich Co., St. Louis, MO).

Cell culture

LβT2 cells (kindly provided by Dr. P. Mellon, University of California, San Diego, CA) were plated on 35-mm tissue culture dishes and incubated in high-glucose Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were used for experiments at 24 h after plating. For stimulation, the cells were incubated with or without (control) the test reagents (E2, P4, DHT) at the indicated concentrations for 48 h in high-glucose Dulbecco's modified Eagle's medium (no phenol red) containing 1% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin.

In vivo experiments using ovary-intact rats

Seven-week-old female Wistar rats (The Jackson Laboratory Japan, Inc., Yokohama, Japan) were maintained under a 12-h light/dark cycle at 20°C–25°C with food (CE-2; CLEA Japan, Tokyo, Japan) and water available *ad libitum*. The rats were housed two per cage. Vaginal smears were assessed daily to evaluate their estrous cycle. After observation for 1 week, a pellet containing 0.25 mg E2 or 50 mg P4 was implanted subcutaneously. while the rats were anesthetized using isoflurane inhalation (Abbott Laboratories, North Chicago, IL). For DHT administration, the rats received a daily subcutaneous injection of 25 mg/kg DHT in 140 μL sesame oil (Fujifilm, Tokyo, Japan) to produce a supraphysiological androgen level, based on a previous study [14]. Control rats were treated with 140 μL sesame oil daily. Two weeks later, the rats were euthanized while under isoflurane anesthesia, and the pituitary gland and hypothalamus were removed and subjected to quantitative real-time (RT)-PCR and western blot analyses. Blood samples taken at euthanization were used for a hormone assay. This protocol was approved by the Ethics Committee of the Experimental Animal Center for Integrated Research at Shimane University (IZ31-51).

Luciferase assay

Reporter constructs, which were generated by fusing − 846/0 of the human common glycoprotein alpha (CGA) gene (*Cga*-Luc), − 797/+5 of the rat *Lhb* gene (*Lhb*-Luc), or − 2000/+698 of the rat *Fshb* gene (*Fshb*-Luc) to firefly luciferase (Luc) cDNA in pXP2, were generously provided by Dr. U. Kaiser(Brigham and Women's Hospital, Boston, MA)[15, 16]. The cells were transiently transfected via electroporation with 2.0 μg/well gonadotropin subunit-Luc and 0.1 μg pRL-TK (Promega, Madison, WI), which expresses *Renilla* luciferase. An empty vector (pCI-neo) served as the mock control. After incubation with the indicated concentration of E2, P4, and DHT for 6 h, the cells were washed with ice-cold phosphate-buffered saline and lysed with Passive Lysis Buffer (Promega). After centrifugation at 15,000 rpm at 4°C, firefly and *Renilla* Luc activity was measured in the supernatant using the Dual-Luciferase Reporter Assay System and a TD-20/20 luminometer (both from Promega). Firefly Luc activity was normalized to that of *Renilla* Luc to correct for transfection efficiency, and the results were expressed as the level (fold) of increase compared with the unstimulated control.

RNA preparation, reverse transcription, and quantitative RT-PCR

Total RNA was extracted from the cultured cells, anterior pituitary tissue, or hypothalamic tissue using TRIzol-LS (Invitrogen). To obtain cDNA, 1.0 μg total RNA was reverse transcribed using oligo-dT primers (Promega) and prepared using a First-Strand cDNA Synthesis Kit (Invitrogen) in reverse transcription buffer. The preparation was supplemented with 10 mM dithiothreitol, 1 mM each dNTP, and 200 U RNase inhibitor/human placenta ribonuclease inhibitor (Cat. No. 2310; Takara, Tokyo, Japan) in a final reaction volume of 10 μL. The reaction was incubated at 37°C for 60 min. *Cga*, *Lhb*, and *Fshb* subunits and *Kiss1* mRNA levels were determined by using RT-PCR (ABI Prism 7000; Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's protocol (User Bulletin No. 2) as well as Universal ProbeLibrary probes and Fast Start Master Mix (Roche Diagnostics, Mannheim, Germany). The PCR primers were designed based on the published sequences of *Cga* [17], *Lhb* and *Fshb* [18], and *Kiss1* [19]. *Gapdh* mRNA was used to normalize the amount of cDNA added per sample. For each set of primers, a no-template control was included. The thermal cycling conditions were as follows: 10 min denaturation at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were monitored by melting curve analysis (55°C–95°C). To determine PCR efficiency, a 10-fold serial dilution of cDNA was performed as described previously [20]. The PCR conditions were optimized to generate > 95% efficiency, and only those reactions with between 95% and 105% efficiency were included in subsequent analyses. Relative differences in cDNA concentrations between the baseline and experimental conditions were calculated using the comparative threshold cycle (Ct) method [21]. Briefly, for each sample, ΔCt was calculated to normalize expression to the internal control (*Gapdh*) by using the following equation: ΔCt = ΔCt(gene) – Ct(*Gapdh*). To determine differences between the experimental and control conditions, ΔΔCt was calculated as ΔCt(sample) – ΔCt(control). Relative mRNA levels were calculated using the following equation: fold difference = $2^{-\Delta\Delta Ct}$.

Western blot analysis

Extracts from the anterior pituitary or hypothalamus were lysed on ice with radioimmunoprecipitation assay buffer (phosphate-buffered saline, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing 0.1 mg/mL phenylmethyl sulfonyl fluoride, 30 mg/mL aprotinin, and 1 mM sodium orthovanadate, scraped for 20 s, and centrifuged at 14,000 × *g* for 10 min at 4°C. The protein concentration in cell lysates was measured using the Bradford method. Denatured protein (15 μg per well) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to standard protocols and transferred onto polyvinylidene difluoride membranes (Hybond-P PVDF; Amersham Biosciences, Little Chalfont, UK). The membranes were blocked for 2 h at room temperature in Blotto (5% milk in Tris-buffered saline). The membranes were incubated with an anti-rabbit polyclonal GCA/hCGα antibody (1:500 dilution; ab1965005, Abcam, Cambridge, UK), anti-rabbit polyclonal LHβ antibody (1:500 dilution; ab180787, Abcam), anti-rabbit monoclonal FSHβ antibody (1:1,000 dilution; ab180489, Abcam), or anti-mouse monoclonal kisspeptin antibody (1:200 dilution; sc101246, Santa Cruz Biotechnology, Dallas, TX) in Blotto overnight at 4°C and washed three times for 10 min with Tris-buffered saline/1% Tween. A subsequent incubation with horseradish peroxidase-conjugated secondary antibodies (anti-mouse antibody, 1:15,000 dilution; anti-rabbit antibody, 1:20,000 dilution) was performed for 1 h at room

temperature in Blotto, and additional washes were performed as needed. Following enhanced chemiluminescence detection (Amersham Biosciences), the membranes were exposed to an X-ray film (Fujifilm, Tokyo, Japan). Tissues from rat ovaries or brains were used as a positive control, and an anti-β-actin antibody was used as an internal control. For comparisons of protein expression levels, images were analyzed by densitometry (ImageJ; National Institutes of Health, Bethesda, MD), and the intensities of protein bands were normalized to those of β-actin to correct for protein loading.

Hormone measurement

Blood was collected at sacrifice and the serum was isolated and stored at − 20°C. Serum LH was measured using a Rat LH ELISA Kit (Elabscience, Houston, Texas). Optical density was obtained with a spectrophotometer at a wavelength of 450 nm, and the concentration of samples was calculated based on a standard curve that ranged from 1.56 to 100 mIU/mL. Values are reported as mIU/mL of whole blood.

Statistical analysis

All experiments were repeated independently at least three times. Each experiment in each experimental group was performed using triplicate (luciferase assay) or duplicate samples (quantitative RT-PCR, western blotting). When mRNA expression was determined, two samples were assayed in duplicate. From four sets of data, the mean ± standard error of the mean (SEM) was calculated. Averages from three independent experiments were analyzed statistically. Data are expressed as mean ± SEM values. Statistical analysis was performed using Student's *t*-test in the experiments comparing two stimulation groups. One-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test was conducted to analyze the experiments that determined the effects of two doses of stimulant on target gene expression, and two-way ANOVA was applied to the experiments that tested combined stimulation by two stimulants. Statistical significance was assessed at a threshold of $P < 0.05$. All analyses were performed using Prism 6.07 Software (GraphPad Software, San Diego, CA).

Results

Effects of E2 on gonadotropin subunit gene expression in LβT2 cells

The effects of E2 on gonadotropin subunit gene expression were examined using a mouse pituitary gonadotroph cell model comprising LβT2 cells. First, the effects of E2 on the promoter activity of *Cga*, *Lhb*, and *Fshb* were assessed. At 1 μM E2, *Cga* subunit promoter activity was significantly increased by 1.73 ± 0.30-fold compared with non-stimulated cells (Fig. 1A). *Cga* mRNA expression was also significantly increased by the same concentration of E2 (Fig. 1D). Furthermore, 1 μM E2 significantly increased *Lhb* subunit promoter activity by 1.5 ± 0.14-fold compared with non-stimulated cells (Fig. 1B). *Lhb* mRNA expression was also significantly increased by 1.26 ± 0.1-fold at the same concentration of E2

(Fig. 1E). However, E2 stimulation did not increase *Fshb* subunit promoter activity or its mRNA expression (Fig. 1C and F).

Effects of P4 on gonadotropin subunit gene expression in LβT2 cells

Next, the effects of P4 on *Cga*, *Lhb*, and *Fshb* expression were examined using LβT2 cells. The promoter activity of the three gonadotropin subunits was not significantly changed by P4 stimulation. Even when P4 concentration was increased, neither *Cga*, *Lhb*, nor *Fshb* promoters were activated (Fig. 2A–C). In contrast, P4 significantly increased the mRNA expression of the *Cga* and *Lhb* subunits by 1.78 ± 0.11-fold and 1.53 ± 0.22-fold, respectively, compared with non-stimulated cells (Fig. 2D and E). However, P4 did not modulate *Fshb* mRNA expression (Fig. 2F).

Effects of DHT on gonadotropin subunit gene expression in LβT2 cells

The effects of DHT on the expression of gonadotropin subunits was examined using LβT2 cells. *Cga* promoter activity was significantly inhibited in the presence of 1 μM DHT (Fig. 3A). *Cga* mRNA expression was also significantly repressed at the same concentration of DHT by 0.28 ± 0.22-fold compared with non-stimulated cells (Fig. 3D). Furthermore, *Lhb* promoter activity and mRNA expression were significantly inhibited by 1 μM DHT by 0.52 ± 0.22-fold and 0.16 ± 0.03-fold, respectively (Fig. 3B and E). However, DHT had no effect on the promoter activity and mRNA expression of *Fshb* (Fig. 3C and F).

Effects of sex steroid administration on the estrous cycle in ovary-intact rats

Next, E2, P4, or DHT was administered to ovary-intact rats and their estrous cycle was determined by vaginal cytology. In normal rats, three sequential estrous cycles were observed in a 2-week period (Fig. 4A). When the rats were implanted with an E2 pellet, the estrous cycle was disrupted, but the estrus and/or proestrus stages remained (Fig. 4B). In contrast, after the start of P4 administration, the estrus cycle was completely eliminated (Fig. 4C). Similarly, the estrus cycle was not observed after DHT administration to rats (Fig. 4D).

Effects of sex steroid administration on pituitary gonadotropin subunit expression in ovary-intact rats

After the administration of E2, P4, or DHT to ovary-intact rats for 2 weeks, changes in pituitary gonadotropin subunit expression were determined by western blot analysis using specific antibodies for each gonadotropin subunit. Both E2 and P4 had an inhibitory effect on CGA, LHβ, and FSHβ subunit expression at the protein level (Fig. 5A). Quantitative analysis revealed that both E2 and P4 inhibited the

expression of all three gonadotropin subunits. However, DHT stimulation did not modulate their expression (Fig. 5B–D). Serum LH levels in rats after sex steroid administration showed a similar pattern to their subunit expression. E2 or P4 treatment similarly inhibited the serum levels of LH; however, DHT did not change the circulating levels of LH *in vivo* (Fig. 6).

Effects of sex steroid administration on Kiss1 gene expression in the rat hypothalamus

Finally, we examined the expression of the *Kiss1* gene, which encodes kisspeptin, within the hypothalamus. Because Kiss1 neurons are located in two different areas, the ARC and AVPV regions of the hypothalamus, *Kiss1* gene expression in the anterior hypothalamus (which includes the AVPV region) and posterior hypothalamus (which includes the ARC region) was examined by quantitative RT-PCR analysis. *Kiss1* gene expression within the anterior hypothalamus was significantly repressed by 0.77 ± 0.04-fold in E2-treated rats compared with non-treated animals. Similarly, P4 treatment repressed *Kiss1* gene expression significantly by 0.43 ± 0.03-fold. However, DHT significantly increased *Kiss1* gene expression by 1.78 ± 0.13-fold in this region (Fig. 7A).

In the posterior hypothalamus, which includes the ARC region, the changes in *Kiss1* gene expression induced by sex steroids were a little distinct from those in the anterior hypothalamus. E2 inhibited *Kiss1* gene expression in the posterior hypothalamus by 0.42 ± 0.00-fold compared with non-treated animals. The E2-induced repression of *Kiss1* gene expression was more prominent in the posterior hypothalamus. However, P4 and DHT treatment did not modulate *Kiss1* gene expression in the posterior region of the hypothalamus (Fig. 7B).

Discussion

In this study, we examined the direct action of the sex steroids E2, P4, and DHT on pituitary gonadotrophs using LβT2 cells, a mouse gonadotroph cell model. Sex steroid administration induces changes in gonadotropin subunit expression within the pituitary gland, and we also assessed *Kiss1* gene expression in the hypothalamus *in vivo* using ovary-intact rats.

In LβT2 cells, E2 increased the promoter activity of the *Cga* and *Lhb* subunits as well as their mRNA expression; however, E2 did not increase *Fshb* subunit expression. P4 also increased *Cga* and *Lhb* gene expression, but had no effect on the *Fshb* gene, although their promoter activity was not modulated by P4. DHT, a potent androgen, inhibited *Cga* and *Lhb* promoter activity and mRNA expression, but had no effect on *Fshb*. These observations indicate that although hypothalamic GnRH is a principal regulator of gonadotropin subunit gene expression, the sex steroids E2 and P4 can also stimulate *Cga* and *Lhb* gene expression by themselves. Although Cga and *Lhb* gene expression was increased by E2 or P4 stimulation, their effects on promoter activity was limited or absent. Therefore, it is plausible that the increase in the expression of these two gonadotropin subunits by E2 and P4 partly depends on a decrease in their degradation.

In our examination of the effects of sex steroids on gonadotropin subunit expression using the LβT2 gonadotroph cell model, E2 and P4 concentrations below 1 μM had no effect on gonadotropin subunit promoter activity or mRNA expression. Only a higher dose of female sex steroids had significant positive effects on *Cga* and *Lhb*. This suggests that the number or sensitivity of steroid receptors might be altered by multiple passages of this cell model. However, we found that unlike DHT, E2 and P4 did not have a negative effect on gonadotropin synthesis. The female sex steroids E2 and P4 positively affected gonadotropin *Cga* and *Lhb* subunit expression at the level of pituitary gonadotropins; however, the androgen DHT repressed *Cga* and *Lhb* expression. These observations suggest that androgens have an opposite effect to female sex steroids on gonadotropin *Cga* and *Lhb* gene expression. Considering the observations that *Cga* expression was increased by E2 and P4, while it was repressed by DHT, female sex steroids may positively regulate physiological gonadotropin LH and FSH expression, but androgens may repress their synthesis because physiological gonadotropic hormones are composed of CGA and unique β subunits. If CGA synthesis was repressed by DHT, physiological LH and FSH synthesis might be concomitantly repressed even if their specific β subunit expression was unchanged. In the current study using LβT2 cells, *Fshb* subunit promoter activity as well as its gene expression was not modulated by direct stimulation with E2, P4, or DHT, indicating that the *Fshb* subunit is not as sensitive as the *Cga* and *Lhb* subunits to sex steroids. However, previous studies have reported the regulatory action of these sex steroids on *Fshb* subunit expression in LβT2 cells [22–24].

In the LβT2 cell line *in vitro*, we observed the direct effects of sex steroids on gonadotropin subunit expression because these cells were not exposed to any other stimuli such as growth factors or biologically active peptides. In addition, LβT2 gonadotrophs are not influenced by neighboring pituitary cells as in the anterior pituitary gland. Therefore, the results obtained from this cell line might simply reflect the characteristics of gonadotrophs and their response to sex steroids. However, the actions of sex steroids on gonadotropin expression *in vivo* are considered to be more complicated because sex steroids may also affect hypothalamic regions that control the production of pituitary hormones. By using ovary-intact rats *in vivo*, we revealed that the estrous cycle, which was assessed by vaginal smears, was disrupted by exogenous E2 administration. In addition, exogenous P4 or DHT administration completely eliminated the estrous cycle. Because E2 and P4 had positive effects on gonadotropin *Cga* and *Lhb* subunit gene expression in clonal LβT2 gonadotroph cells, it seems that E2 or P4 do not prevent gonadotropin synthesis by themselves at the pituitary level. On the contrary, it makes sense that DHT administration eliminated the estrous cycle *in vivo* because DHT itself prevents gonadotropin *Cga* and *Lhb* subunit expression *in vitro*. When we determined the expression levels of each gonadotropin subunit in the anterior pituitary gland in ovary-intact rats that were administered sex steroids, both E2 and P4 inhibited the expression of the CGA, LHβ, and FSHβ subunits at the protein level to a similar degree. Serum LH levels were also reduced by E2 or P4 administration in rats. In contrast, DHT did not modulate gonadotropin subunit expression or serum LH levels *in vivo*. It is reasonable to think that the disruption or elimination of the estrous cycle in E2- or P4-treated rats depends on the decrease of gonadotropin subunit expression and concomitant decrease of LH serum levels *in vivo*; however, the estrous cycle was abolished by DHT administration, even though DHT did not reduce gonadotropin subunit expression or

LH levels *in vivo*. It is unclear why DHT administration abolished the rat estrous cycle, but if DHT itself affects the morphology of vaginal epithelial cells and prevents their morphological change to that of the estrous phase, the estrous cycle could not be identified accurately according to the morphology of vaginal cells.

The rat estrous cycle is regulated by the HPG axis, and accumulating evidence supports a principal role for Kiss1 neurons in the control of GnRH release [29]. Kiss1 neurons in the ARC region of the hypothalamus are involved in the E2-induced negative feedback mechanism, while those in the AVPV play a pivotal role in the E2-induced positive feedback mechanism. In the present study, we examined the changes in *Kiss1* gene expression in tissues from the anterior or posterior parts of the hypothalamus, which include the AVPV and ARC regions, respectively. *Kiss1* gene expression in the anterior part of the hypothalamus was significantly repressed by E2 and P4 treatment. However, *Kiss1* gene in the posterior part of the hypothalamus was suppressed by E2 but not by P4. The observation that P4 inhibited *Kiss1* gene expression in the AVPV but not in the ARC indicates that P4 treatment disrupts the normal positive feedback mechanism and prevents GnRH/LH surge-induced ovulation. It is plausible that in addition to the decrease in gonadotropin subunit expression, the rat estrous cycle was eliminated partly because the LH surge and subsequent ovulation were not induced by 1-week administration of P4. A progestin-primed ovarian stimulation protocol has been applied recently to patients who underwent oocyte retrieval during *in vitro* fertilization and embryo transfer procedures [30]. In this protocol, women who underwent *in vitro* fertilization are administered progestin orally during controlled ovarian stimulation to prevent a spontaneous LH surge and concomitant induction of ovulation. The prevention of *Kiss1* expression in the anterior hypothalamus, which includes the AVPV region, might explain the mechanism by which P4 inhibits the LH surge. DHT administration disrupted the estrous cycle without decreasing gonadotropin subunit expression in ovary-intact rats. In addition, *Kiss1* gene expression in hypothalamic tissues from the anterior and posterior parts was not decreased by DHT treatment. Rather, its expression was increased in the anterior hypothalamus by DHT. These observations suggest that the basal release of kisspeptin and kisspeptin-regulated GnRH secretion are not altered by DHT, which was supported by our observation that gonadotropin subunit expression as well as serum levels of LH were not altered by DHT administration. As described above, DHT-induced morphological changes in vaginal smears might lead to the mischaracterization of the estrous cycle.

This study determining *Kiss1* gene expression using hypothalamic tissues was reliable because *Kiss1* gene expression was repressed by E2 treatment in the posterior hypothalamus, which includes the ARC region and which was quite similar to the effect observed in the ARC region of the hypothalamus [4]. In addition, we found that E2 was a much more potent repressor of *Kiss1* gene expression in this region compared with the other sex steroids P4 and DHT. *Kiss1* gene expression is known to be upregulated by E2 in the AVPV region of the hypothalamus [4]. We did not observe a clear increase in *Kiss1* gene expression in the anterior hypothalamus following E2 administration, rather it was slightly repressed. This is probably because we did not accurately determine the local expression of *Kiss1* only in the AVPV region and we might have evaluated other neuronal populations that also express *Kiss1*.

In this study, we evaluated the effects of sex steroids using a gonadotroph cell model *in vitro* and in the rat anterior pituitary and hypothalamus *in vivo*. Both E2 and P4 significantly increased *Cga* and *Lhb* gene expression in LβT2 gonadotroph cells. In contrast, DHT repressed *Cga* and *Lhb* gene expression. By the administration of E2 to ovary-intact rats, the estrous cycle was disrupted, while it was completely eliminated by P4 and DHT administration. The expression of the three gonadotropin subunits was inhibited by E2 and P4 but not by DHT. Hypothalamic *Kiss1* gene expression was repressed by E2 in the anterior (including the AVPV) and posterior (including the ARC) hypothalamus. P4 administration strongly inhibited *Kiss1* gene expression only in the AVPV-containing anterior hypothalamus. DHT administration had no effect on *Kiss1* gene expression in the hypothalamus. Our findings indicate that female and male sex steroids affect the pituitary and hypothalamus, but they have divergent effects at different levels of the HPG axis. It is plausible that sex steroids, especially E2 and P4, strongly affect the hypothalamus and modulate gonadotropin secretion and regulate reproductive functions.

Declarations

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Availability of supporting data

The datasets used and/or analyzed during this study are available from corresponding authors on reasonable request.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical approval

All applicable international, national, and\or institutional guidelines for the care and used of animals were followed.

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Figures

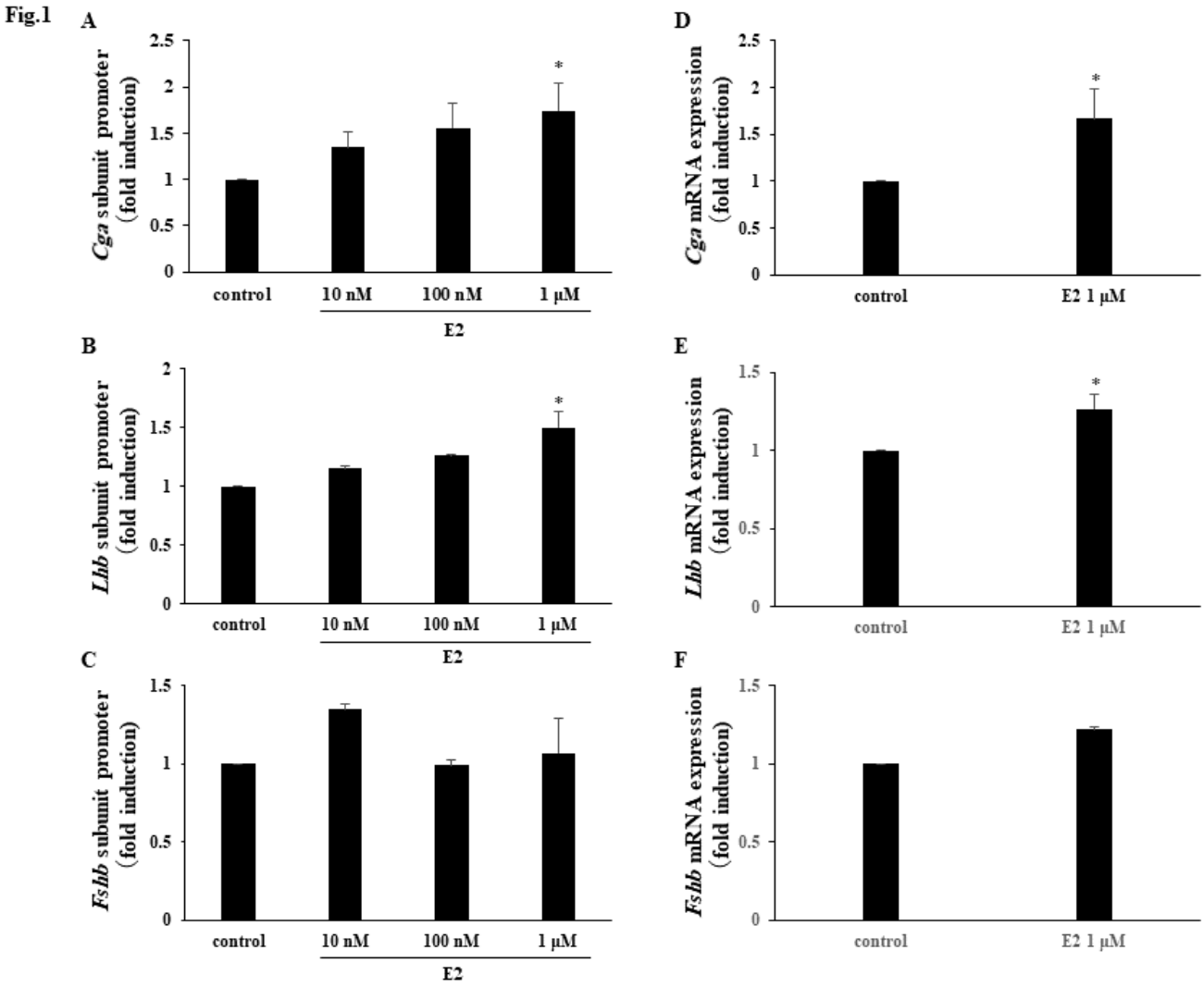

Figure 1

Effects of E2 on gonadotropin subunit promoter activity and mRNA expression. (A, B, C) LβT2 cells were transfected with 2.0 μg *Cga*-Luc (A), *Lhb*-Luc (B), or *Fshb*-Luc (C) and pRL-TK (0.1 μg). At 48 h after transfection, the cells were treated with the indicated concentrations of E2 for 6 h. A luciferase assay was performed to examine *Lhb* and *Fshb* promoter activity, which was normalized to *Renilla* luciferase activity, and is expressed as the fold difference of activation over the unstimulated controls. (D, E, F) LβT2 cells were stimulated with 1 μM E2 for 24 h. After stimulation, mRNA was extracted and reverse transcribed, and the mRNA levels of the *Cga* (D), *Lhb* (E), and *Fshb* (F) subunits were measured by quantitative RT-PCR. Samples from each experimental group were run in duplicate and normalized to the mRNA levels of the housekeeping gene *Gapdh*. The results are expressed as fold induction over unstimulated cells. Data are expressed as the mean ± SEM (three independent experiments were performed using triplicate samples). **P* < 0.05 vs. control.

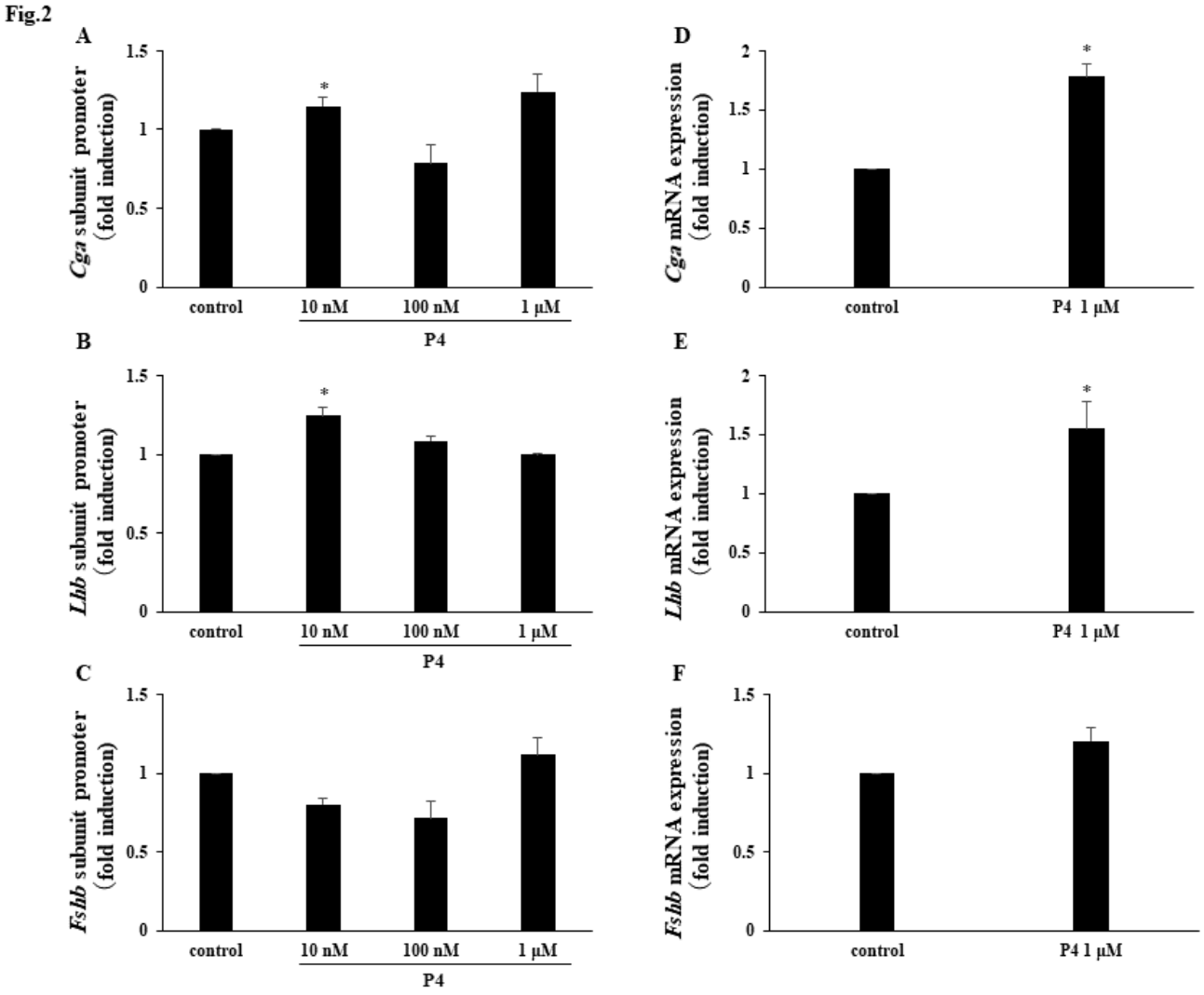

Figure 2

Effects of P4 on gonadotropin subunit promoter activity and mRNA expression. (A, B, C) LβT2 cells were transfected with 2.0 μg *Cga*-Luc (A), *Lhb*-Luc (B), or *Fshb*-Luc (C) and pRL-TK (0.1 μg). At 48 h after transfection, the cells were treated with the indicated concentrations of P4 for 6 h. A luciferase assay was performed to examine *Lhb* and *Fshb* promoter activity, which was normalized to *Renilla* luciferase activity, and is expressed as the fold difference of activation over the unstimulated controls. (D, E, F) LβT2 cells were stimulated with 1 μM P4 for 24 h. After stimulation, mRNA was extracted and reverse transcribed, and the mRNA levels of the *Cga* (D), *Lhb* (E), and *Fshb* (F) subunits were measured by quantitative RT-PCR. Samples from each experimental group were run in duplicate and normalized to the mRNA levels of the housekeeping gene *Gapdh*. The results are expressed as fold induction over unstimulated cells. Data are expressed as the mean ± SEM (three independent experiments were performed using triplicate samples). **P* < 0.05 vs. control.

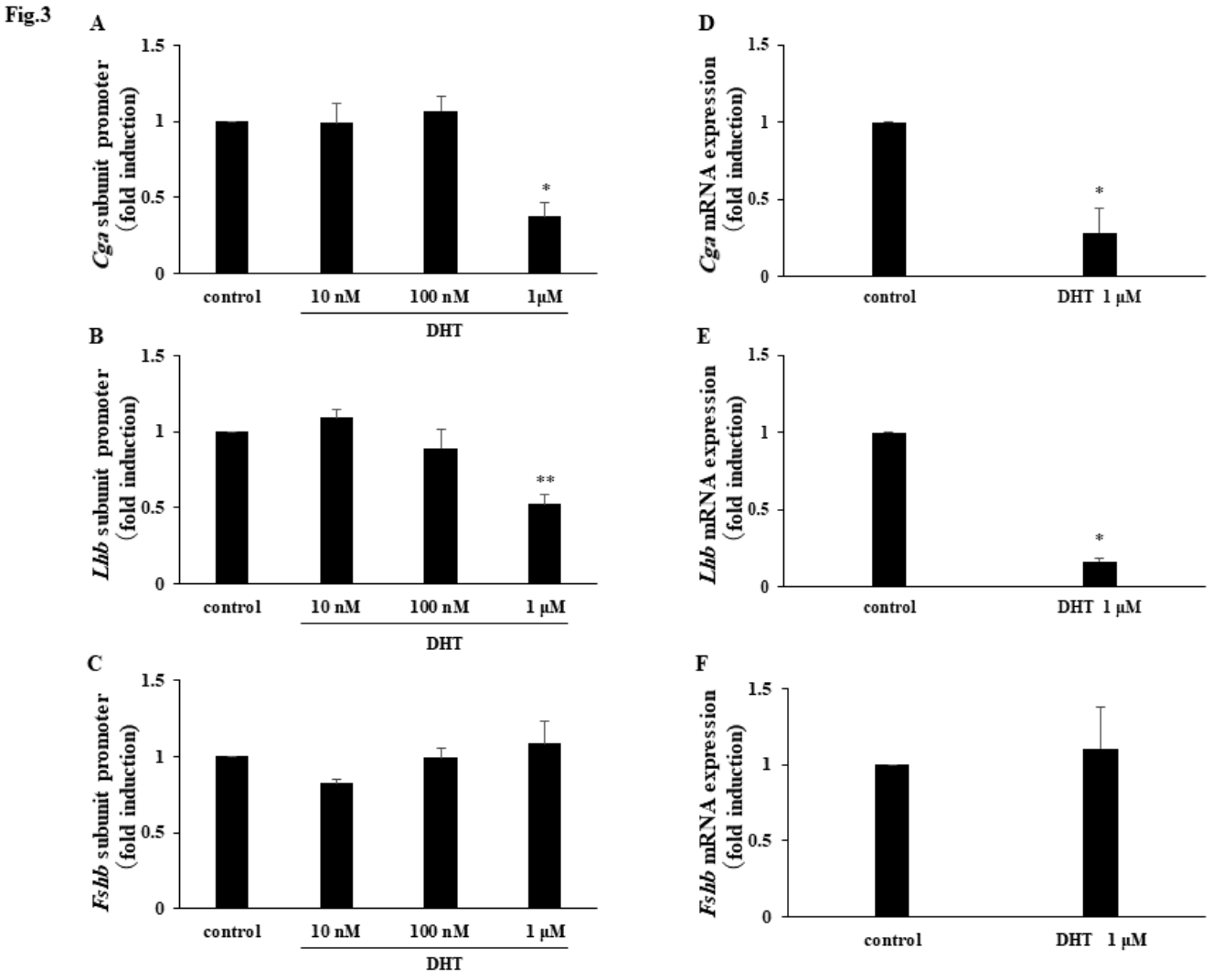

Figure 3

Effects of DHT on gonadotropin subunit promoter activity and mRNA expression. (A, B, C) LβT2 cells were transfected with 2.0 μg *Cga*-Luc (A), *Lhb*-Luc (B), or *Fshb*-Luc (C) and pRL-TK (0.1 μg). At 48 h after

transfection, the cells were treated with the indicated concentrations of DHT for 6 h. A luciferase assay was performed to examine *Lhb* and *Fshb* promoter activity, which was normalized to *Renilla* luciferase activity, and is expressed as the fold difference of activation over the unstimulated controls. (D, E, F) LβT2 cells were stimulated with 1 μM DHT for 24 h. After stimulation, mRNA was extracted and reverse transcribed, and the mRNA levels of the *Cga* (D), *Lhb* (E), and *Fshb* (F) subunits were measured by quantitative RT-PCR. Samples from each experimental group were run in duplicate and normalized to the mRNA levels of the housekeeping gene *Gapdh*. The results are expressed as fold induction over unstimulated cells. Data are expressed as the mean ± SEM (three independent experiments were performed using triplicate samples). **P* < 0.05 vs. control.

Fig.4

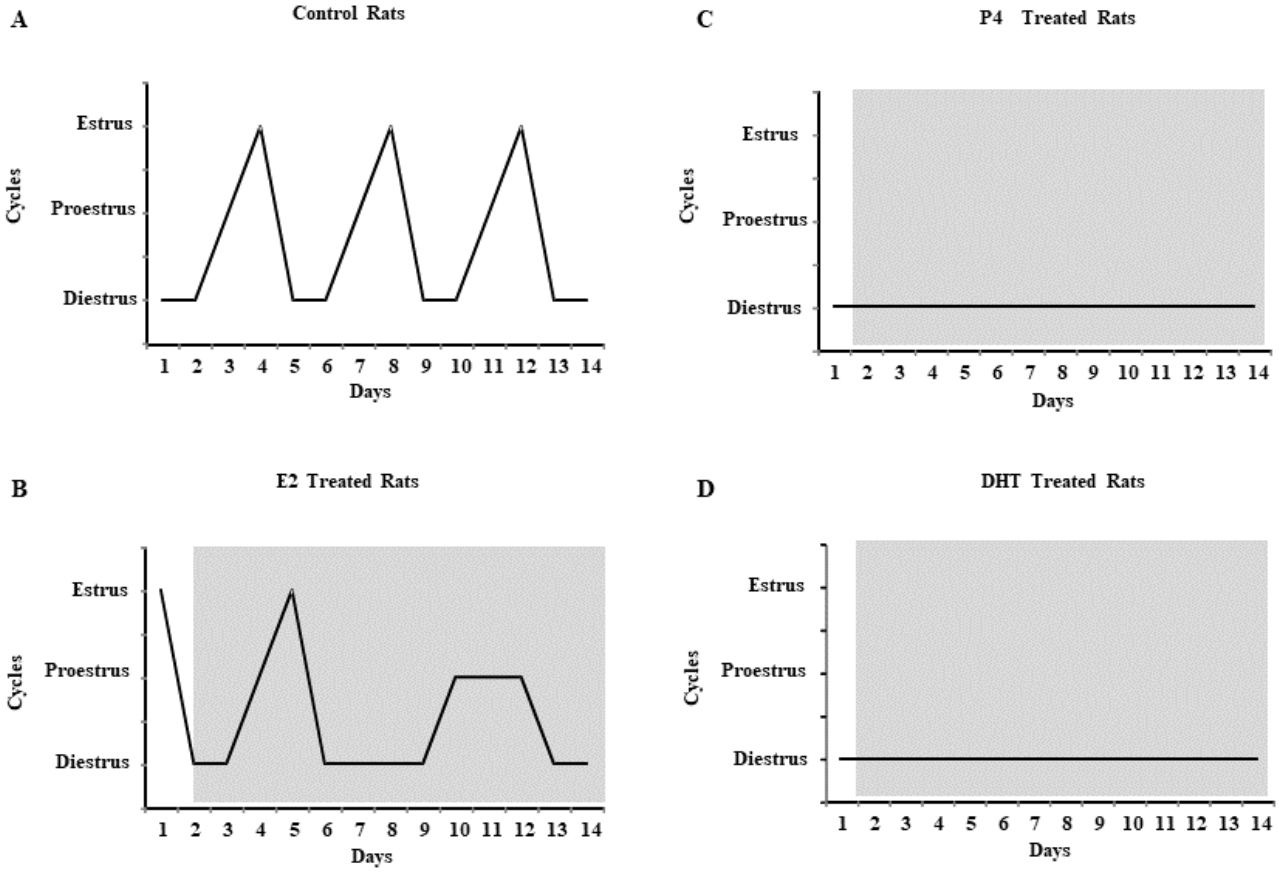

Figure 4

Estrous cycle in steroid hormone-treated rats. Seven-week-old female rats were implanted with a pellet containing 0.25 mg E2 (B) or 50 mg P4 (C) or treated by daily subcutaneous injection of 25 mg/kg DHT. Control rats were treated with 140 μL sesame oil daily (A). Vaginal smears were collected daily to

evaluate the estrous cycle for 2 weeks. Representative cycles in control and steroid hormone-treated rats are shown.

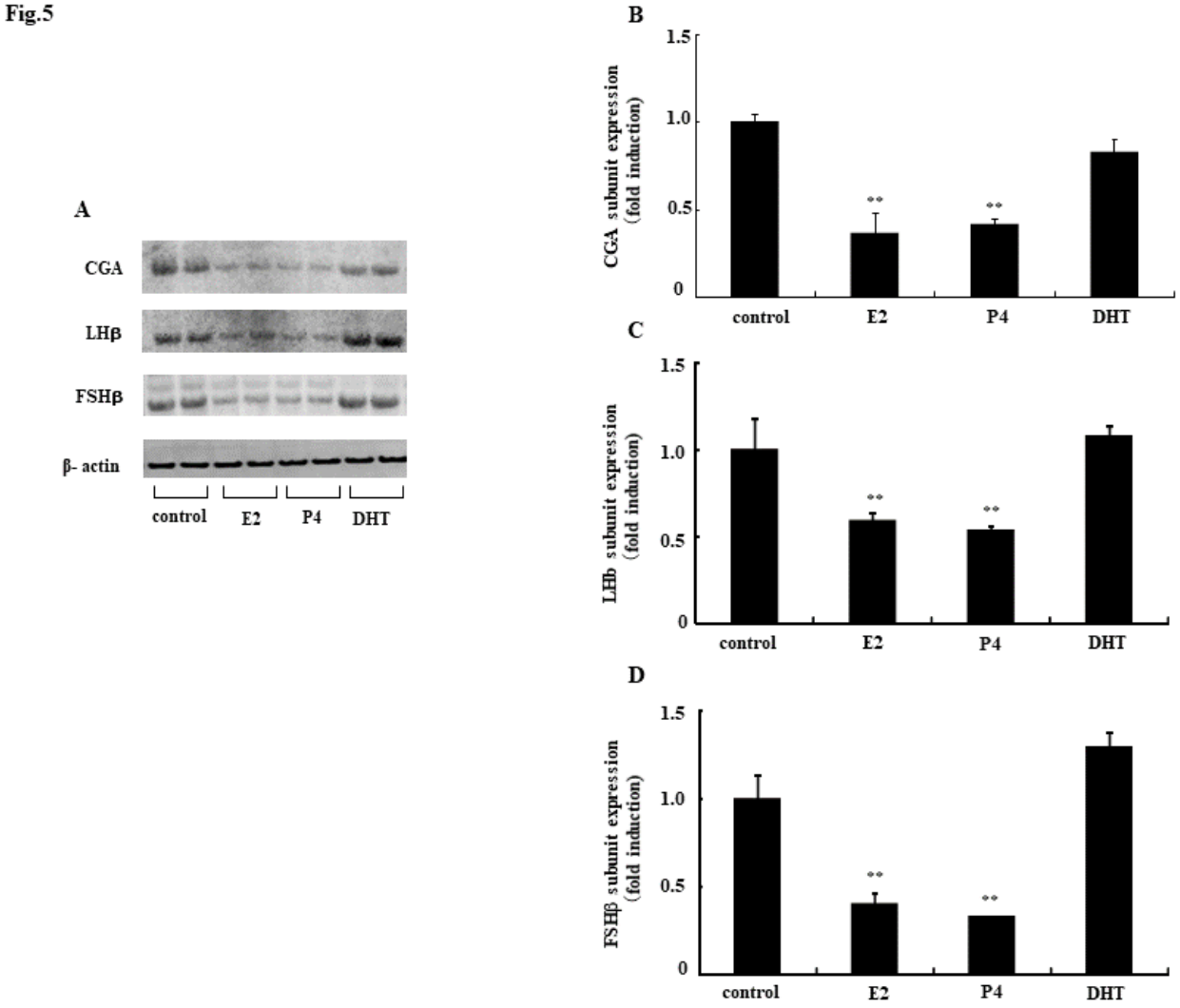

Figure 5

Effects of E2, P4, and DHT administration on gonadotropin subunit expression in ovary-intact rats. Seven-week-old female rats were implanted with a pellet containing 0.25 mg E2 or 50 mg P4 (C) or treated by daily subcutaneous injection of 25 mg/kg DHT. Control rats were treated with 140 μL sesame oil daily (A). After 2-week administration of sex steroids, the rats were euthanized, and the anterior pituitary gland was removed. Lysates (15 μg protein) from the anterior pituitary were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting and incubation with specific anti-CGA, -LHβ, and -FSHβ subunit antibodies. The bands were visualized using a horseradish peroxidase-conjugated secondary antibody (A).Scanning densitometry of bands was performed using ImageJ to determine differences in CGA (B), LHβ (C), and FSHβ (D) subunit protein levels, with normalization to β-actin levels. The results are expressed as fold stimulation over the unstimulated group/control. Values are the mean ± SEM of fold stimulation from independent experiments. $^{**}P < 0.01$ vs. control.

Fig.6

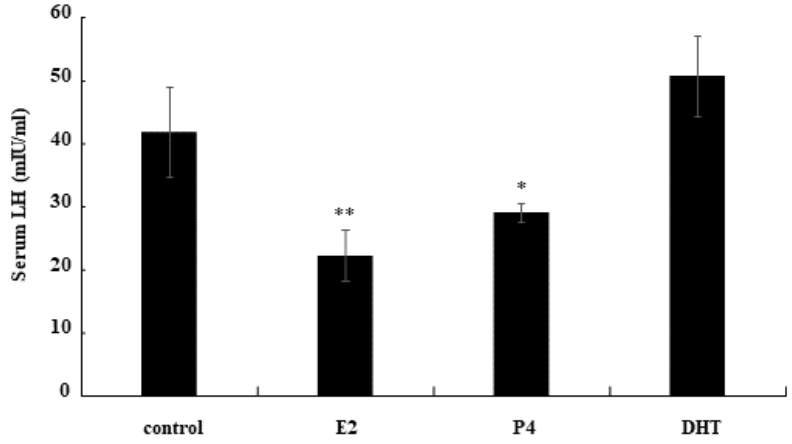

Figure 6

Effects of E2, P4, and DHT administration on serum LH levels in ovary-intact rats. Seven-week-old female rats were implanted with a pellet containing 0.25 mg E2 or 50 mg P4 (C) or treated by daily subcutaneous injection of 25 mg/kg DHT for 2 weeks, and then blood was collected at sacrifice. Control rats were treated with 140 μL sesame oil daily (A). Serum LH levels were measured using a rat LH enzyme-linked immunosorbent assay. Values are the mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control.

Fig.7

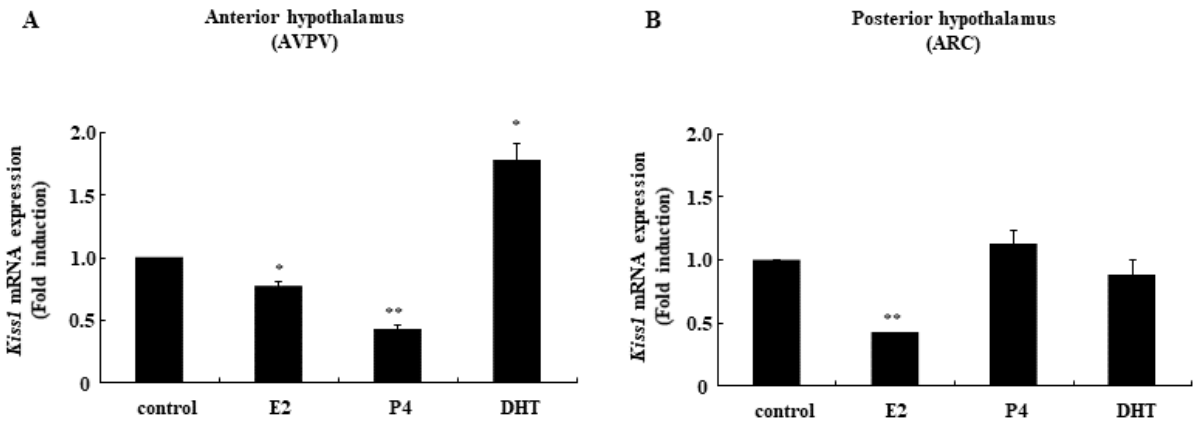

Figure 7

Effects of E2, P4, and DHT administration on gonadotropin subunit expression in ovary-intact rats. Seven-week-old female rats were implanted with a pellet containing 0.25 mg E2 or 50 mg P4 (C) or treated by daily subcutaneous injection of 25 mg/kg DHT. Control rats were treated with 140 μL sesame oil daily (A). After 2-week administration of sex steroids, the rats were euthanized, and the anterior hypothalamus (which includes the AVPV region) and posterior hypothalamus (which includes the ARC region) were removed. mRNA was extracted from the anterior and posterior hypothalamic tissues and reverse transcribed, and then *Kiss1*mRNA levels were measured by quantitative RT-PCR. Samples for each experimental group were run in duplicate and normalized to the mRNA levels of *Gapdh* as a housekeeping gene. The results are expressed as fold induction over unstimulated cells and presented as the mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control.