Original Research

Effects of diethylstilbestrol on luteinizing hormone-producing cells in the mouse anterior pituitary

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Abstract

Gonadotrophs in the anterior pituitary secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Neonatal diethylstilbestrol (neoDES) treatment affects reproductive function of male and female mice, but the effect of this treatment on the development as well as direct effects on pituitary gonadotrophs have not been ascertained. We investigated LH-secreting gonadotropes and the expression of genes involved in the synthesis and secretion of gonadotropins in the anterior pituitary of neoDES mice using immunohistochemistry and real-time reverse transcription-polymerase chain reaction (RT-PCR). The percentage of LH-secreting gonadotropes in 90-day-old female mice treated neonatally with an oil vehicle (neoOil) was significantly lower than in 30-day-old neoOil females but not in males, indicating a significant reduction after reproductive maturation in females. The percentage of LH-secreting gonadotropes in the medial area of 90-day-old neoDES females was significantly lower than that of 90-day-old neoOil females, ovariectomized neoOil females, and neoOil and neoDES males. The expression of the LH beta (*Lhb*) subunit in the anterior pituitary of 90-day-old neoDES females was similar to that in neoOil females, but it was significantly lower than that observed in 90-day-old males. Ovariectomy increased the expression of the alpha subunit of glycoprotein hormones, FSH beta (*Fshb*) subunit and *Lhb* subunit both in neoOil and neoDES females, suggesting that the anterior pituitary of neoDES female mice is regulated by ovarian hormones via negative feedback. In organ-cultured, anterior pituitaries exposed to DES exhibited no change in the number of LH-secreting gonadotropes but did reduced gene expression. These results suggest that LH-secreting gonadotropes in the female mice are not only directly affected by neoDES but also are influenced by the masculinization of the hypothalamus. That is, neonatal DES exposure can masculinize or defeminize the hypothalamus of female mice. However, the regulation of the pituitary gonadotropins by the hypothalamus could be different from that in intact male mice.

Keywords: Luteinizing hormone, anterior pituitary, diethylstilbestrol

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Introduction

Reproductive function in female mice is regulated by the hypothalamus-pituitary-gonad (HPG) axis. Gonadotrophs, in the anterior pituitary, secrete the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). LH is a heterodimer of the glycoprotein-alpha subunit (αGSU or CGa) and luteinizing hormone beta (LHβ) subunit, whereas FSH is composed of CGa and a FSH beta (FSHβ) subunit.[1] These hormones are secreted into the blood stream, primarily in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus.[2] Gonadal steroid hormones, such as 17β-estradiol (estradiol), progesterone, and testosterone, in addition to peptide hormones, such as activin, inhibin, and follistatin, modulate LH and FSH levels via feedback to the anterior pituitary, as well as to the hypothalamus.[3]

The mRNA for *Cga* first appears in the mouse pituitary at embryonic stage 11.5 days (E11.5), whereas *Lhb* and *Fshb* are detected initially at E16.5 and 17.5, respectively.[4] The immunoreactive gonadotropic cells are observed from E17 in C57BL6 male and female mice and these cells increase in number until the end of gestation in both sexes,[5] indicating that gonadotrophs begin to develop and proliferate during embryonic development. Although estrogens are not necessary for the differentiation of gonadotrophs, they promote proliferation of gonadotrophs in the pituitary.[6,7]

Synthesis and secretion of LH and FSH are regulated by sex hormones via negative feedback mechanisms, where estrogens cause the LH surge, resulting in ovulation and corpora lutea formation in the ovary.[8] In the hypothalamus, sexually dimorphic nuclei of the preoptic areas (SDN-POA) are about five-fold larger in male versus female rats.[8] The volume of the anteroventral periventricular nucleus (AVPV) in female rats is approximately twice as large compared with that in males.[9] The AVPV is important for gonadotropin secretion as it is believed to control the LH surge essential for ovulation in adult females.[10] In male rats, the fetal testes secrete androgens, which are converted into estradiol in the brain by the enzyme aromatase. Estradiol then interacts with estrogen receptors, but not androgen receptors, to induce a masculinized SDN-POA.[10,11] Thus, sex differences in the hypothalamus are established under the influence of sex hormones.

Alterations in the hypothalamus by neonatal estrogen treatment result in the disappearance of the estrous cycle and ovulation.[12,13] Diethylstilbestrol (DES), a synthetic estrogen, can bind both subtypes of estrogen receptor, ERα and ERβ,[14] and prenatal or neonatal DES (neoDES) treatment in female rats significantly enlarges the SDN-POA and is associated with the loss of ovulation.[15] Male mice exposed to high levels of DES neonatally incur testicular weight loss, reduction of sperm counts, and impaired motility of sperm.[16] However, the percentage of LH-producing gonadotropes and the expression of *Lhb* are not altered in the pituitary of 18- or 90-day-old neoDES male rats.[17] In neonatal female mice treated with estradiol benzoate (EB), plasma concentrations of LH and pituitary LH content are reduced until 15 days of age.[18] In neoDES female mice, LH levels in the serum and pituitary are reduced at days six, 12 and 21; however, serum LH concentrations at day 56 are similar to those of control female mice.[19] These results suggest that neonatal estrogen treatment alters hypothalamus and pituitary functions, and thus LH levels in females but not in males. Our previous study demonstrated that in female mice exposed neonatally to 3 μg DES, pituitary expression of *Lhb* was significantly lower than that of control females resulting in the impaired steroidogenesis, hypertrophy, and accumulation of lipid droplets in interstitial cells of the ovary.[20] However, the developmental effects in mice of neonatal DES treatment on LH-secreting gonadotropes have not been extensively investigated. In addition, it is not clear whether neonatal DES affects the pituitary directly or not.

In this study, LH-secreting gonadotropes in the anterior pituitary of neoDES mice were examined by immunohistochemistry. Ontogenic changes in these cells in five-, 30- 60-, and 90-day-old female and male mice were investigated. In addition, expression of genes involved in gonadotropin synthesis and secretion was investigated using real-time reverse transcription-polymerase chain reaction (RT-PCR). Finally, to investigate the direct effects of DES on LH-secreting gonadotropes and gene expression, neonatal mouse pituitary organ-cultured with or without DES was performed.

Materials and methods

Animals and treatments

C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan) were housed under a 12 h light: 12 h dark cycle with lights on at 8:00 and a controlled temperature (23°C). Animals given free access to mouse chow (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water *ad libitum*; they were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care Committee of Yokohama City University.

Female and male pups were given daily subcutaneous injections of 3 μg DES (Sigma Chemical Co., St. Louis, MO, USA), dissolved in 0.02 mL sesame oil or oil vehicle alone, for five days starting on the day of birth (day zero). Pituitaries of five-, 30-, 60-, and 90-day-old female mice (necropsies occurred at the estrous stage at 60 and 90 days), 90-day-old female mice ovariectomized at 80 days treated neonatally with oil vehicle alone (neoOil), 90-day-old female mice ovariectomized at 80 days treated neonatally with DES (neoDES), 30- and 90-day-old neoDES female mice, five-, 30-, and 90-day-old neoOil and neoDES male mice were collected. The estrous stage in each female mouse was determined by obtaining vaginal smears for 14 days. Estrus was defined as the presence of cornified cells. Treatment timelines are shown in Figure 1.

LH immunohistochemistry

For LH immunohistochemistry, pituitaries were collected as described above. Pituitaries were fixed in Bouin's solution overnight, dehydrated, embedded in paraffin, and cut into 6-μm-thick sections. Sections were deparaffinized and hydrated through xylene and a graded alcohol series. Endogenous peroxidase was blocked using 1% (v/v) H_2O_2 in reverse osmotic water (RO); the slides were then washed with phosphate-buffered saline (PBS). Sections were incubated with normal goat serum (VECTASTAIN Elite ABC kit, Vecter Laboratories, Inc., Burlingame, CA, USA) for 15 min at room temperature to reduce nonspecific binding, then incubated with the rabbit polyclonal anti-ovine LHβ antibody diluted with PBS (1:2500 or 1:5000) overnight at 4°C. After washing in PBS, sections were incubated with biotinylated antirabbit IgG (1:200, Vector Laboratories Inc.) for 30 min. After washing in PBS, sections were incubated with avidin-biotin complex (ABC) reagents (Vector Laboratories Inc.) for 30 min. Reaction products were visualized using 3, 3′-diaminobenzidine (DAB, Sigma) for 90 or 120 s. Negative controls were prepared by incubation with a rabbit immunoglobulin fraction (DAKO Cytomation, Glostrup, Denmark) instead of the primary antibody. Sections were counterstained with hematoxylin. Three to six animals were examined for each point.

RNA isolation and real-time RT-PCR

Pituitaries from zero-, five-, 30-, and 90-day-old animal groups were homogenized in TRIzol (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA was purified

with an RNeasy total RNA kit (Qiagen, Hilden, Germany). Total RNA was reverse transcribed with Super Script II reverse transcriptase (Invitrogen) using oligo (dT) primer (Invitrogen). Real-time RT-PCR was carried out with an Applied Biosystems StepOne Real-Time PCR System with Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). To determine the mRNA expression of *Cga* (αGSU mRNA), *Fshb*, *Lhb*, and *gonadotropin-releasing hormone receptor* (*Gnrhr*), an aliquot of cDNA was amplified with specific primers derived from mouse mRNA sequences (Table 1). *Peptidylprolyl isomerase A* (*Ppia*) was chosen as an internal standard to control for variability in amplification due to differences in starting mRNA concentration. Single pituitaries were used from 30- and 90-day-old animals, whereas 2–10 pituitaries were pooled for RNA isolation at each point for zero- and five-day-old animals. Three independent replicates were carried out for each study.

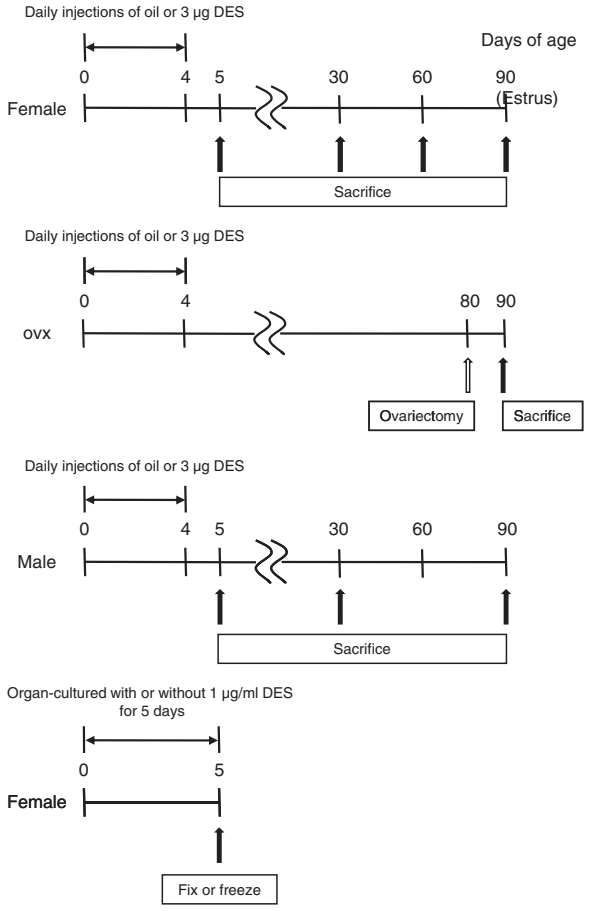

Figure 1 Treatment timelines. Male and female newborn mice were given five daily injections of 3 μg DES (neoDES) or the vehicle alone (neoOil) from day zero (the day of birth). Some female mice were ovariectomized at 80 days. Pituitaries from five-, 30-, 60-, and 90-day-old female neoDES mice, 90-day-old ovx female and neoDES mice, 30- and 90-day-old female neoDES mice, and five-, 30-, and 90-day-old male mice were collected

Organ culture

Pituitaries of zero-day-old female mice were dissected and placed in a 24-well culture plate with 200 μL serum-free DMEM/Nutrient Mixture F12 (1:1, phenol red free, Invitrogen) with or without 1 μg/mL DES (Sigma) and cultured in a humidified incubator at 37°C and 5% CO_2 for five days. For a serum-free medium, 10 μg/mL Dulbecco's modified Eagle's medium/Ham's F-12 medium (Invitrogen) containing 100 units of penicillin and 100 μg/mL streptomycin (Invitrogen) was supplemented with 10 μg/mL insulin (Sigma), 10 μg/mL transferrin (Sigma), 10 ng/mL epidermal growth factor (EGF, Sigma), 0.01 ng/mL cholera toxin (Sigma), and 5 mg/mL bovine serum albumin fraction V (BSA, Sigma). After five days in culture, pituitaries were fixed in Bouin's solution and immunostained for LH-secreting cells or frozen for real-time RT-PCR, as described above. Ten organ-cultured pituitaries were used for LH immunohistochemistry or pooled for RNA isolation, at each time point. Three independent replicates were carried out for real-time RT-PCR. Treatment timelines are shown in Figure 1.

Cell count and data analysis

LH immunoreactive gonadotropes were counted using a light microscope (×40 objective lens). LH immunoreactivity in 200 anterior pituitary cells in four chosen areas from transversal horizontal sections was counted from 30-, 60-, and 90-day-old female and male mice. LH immunoreactivity in 600 anterior pituitary cells in two chosen areas from transversal horizontal sections was counted from five-day-old female and male mice, whereas LH immunoreactivity in 500 anterior pituitary cells in two chosen areas from transversal horizontal sections was counted from organ-cultured pituitaries. Because of the size difference in the pituitary from animals of different ages, different numbers of pituitary cells were counted. So that a comparison could be performed among ages and treatments, the percentage of LH-positive cells versus those identified was calculated.

Data are expressed as the mean ± 1 standard error. For multiple comparisons, treatment groups were compared

Table 1 Primers used for real-time RT-PCR

Gene	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)
Cga	GACTTTATTATTCAGGGTTGCCCA	AGAAGCAACAGCCCATACACTG
Fshb	GACTGCACAGGACGTAGCTGTTTA	CTGAGATGGTGATGTTGGTCAATT
Lhb	TGTCAACGCAACTCTGGCC	GGCAGTACTCGGACCATGCT
Gnrhr	TTCATCAAGACCCACGCAAA	GAGGTAGCGAATGCGACTGTC
Ppia	AGGTCCTGGCATCTTGTCCAT	CCATCCAGCCATTCAGTCTTG

using analysis of variance followed by an appropriate *post hoc* test. Two-tailed Student's *t*-test was used for single comparisons. A statistically significant difference was defined as $P < 0.05$.

Results

LH-positive gonadotropes in the anterior pituitary of 30-, 60-, and 90-day-old neoOil female mice

LH immunoreactivity was observed in the cytoplasm of anterior pituitary cells (Figure 2), and LH-secreting gonadotropes were more abundant in the lateral area compared with the medial area of 30-, 60-, and 90-day-old C57BL6J females and males (Figure 2(C) to (H)). The percentage of LH-positive gonadotropes in the medial area of female mice was significantly lower than that in the lateral area at 30, 60, and 90 days of age (Figure 3(B) and (C)). The percentage of LH-positive cells in the pituitary of 30-day-old female mice was high, whereas the percentage was significantly reduced at 90 days of age (Figure 3(A)). In contrast, there were no observed differences in the lateral area at any ages (Figures 2C to (E) and 3(B)). In the anterior pituitary of 90-day-old neoOil female mice ovariectomized (ovx) at 80 days, the percentage of LH cells in the medial area was significantly reduced compared to the lateral area (Figure 3(B) and (C)). However, the percentage of LH-positive cells in the lateral, medial, or whole areas of the pituitary in ovx-mice was not different when compared with the same regions in intact 90-day-old female mice (Figures 2(E) and (F), and 3).

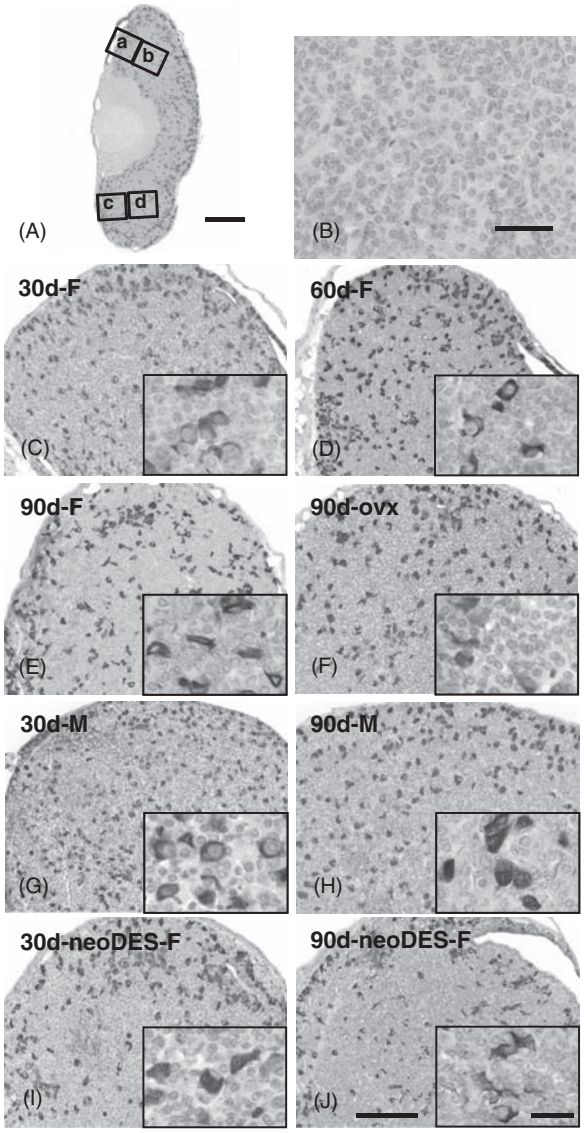

Figure 2 Four areas were counted from the anterior pituitary using a transverse horizontal section (A). The lateral area (a and c) and the medial area (b and d) are shown. Luteinizing hormone immunoreactive gonadotropes in the anterior pituitaries of 30- (30d-F, C), 60- (60d-F, D), and 90-day-old (90d-F, E) C57BL/6J female mice, 90-day-old female mice ovariectomized at 80 days (90d-ovx, F), 30- (30d-M, G) and 90-day-old (90d-M, H) male mice, and 30- (30d-neoDES-F, I) and 90-day-old (90d-neoDES-F, J) neonatally DES-treated female mice. Negative staining for LH immunostaining (B). Scale bar: 500 μm (A); 50 μm (B); 200 μm (C–J). Insets are gonadotropes in the medial area of anterior pituitaries of each mouse. Scale bar: 25 μm

LH-positive gonadotropes in the anterior pituitary of 30- and 90-day-old neoOil male mice

LH-positive gonadotropes in the lateral area were more abundant compared with those in the medial area of 30- and 90-day-old female and male mice (Figure 2(G) and (H)). The percentage of LH-positive cells in the medial

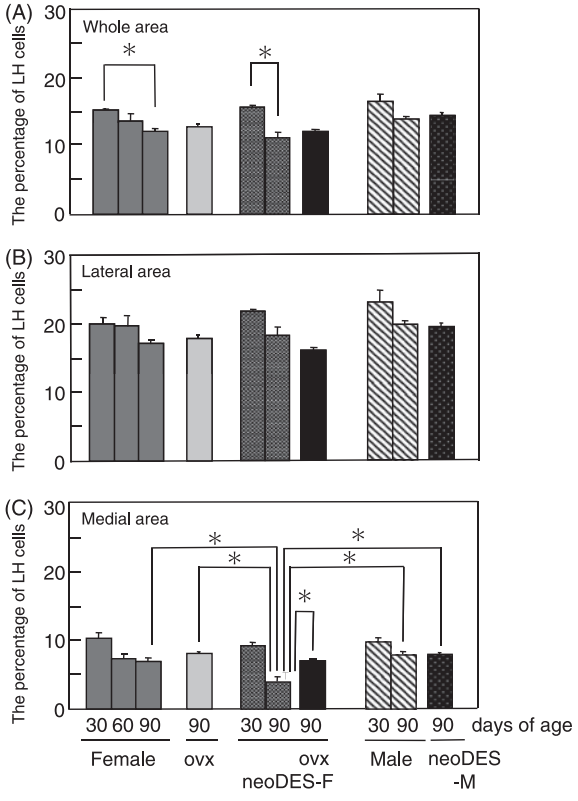

Figure 3 The percentage of LH-positive cells in the whole area (A), lateral area (B), and medial area (C) of 30-, 60-, and 90-day-old female mice ($n = 8$, 7 and 5), 90-day-old female mice ovariectomized at 80 days ($n = 6$), 30- and 90-day-old male mice ($n = 6$ and 5), 30- and 90-day-old neonatally DES-treated (neoDES) female mice ($n = 3$ and 5), 90-day-old neoDES female ovx-mice ($n = 5$), and 90-day-old neoDES male mice ($n = 5$). *$P < 0.05$

area was significantly reduced in the lateral area in 30- and 90-day-old neoOil male mice (Figure 3(B) and (C)).

LH_positive gonadotropes in the anterior pituitary of 30- and 90-day-old neoDES mice

Ninety-day-old neoDES female mice exhibited no estrous cycles. Distribution of LH-positive cells in the pituitary of neoDES female mice was similar to that observed in 90-day-old neoOil females (Figure 2(E) and (J)). The percentage of LH cells in the whole area of the pituitary in 90-day-old neoDES females was significantly lower than that observed in 30-day-old neoDES females (Figures 2(I) and (J), and 3(A)). The percentage of LH-positive cells in the medial area of 90-day-old neoDES females was significantly reduced when compared to the same region in 90-day-old neoOil females, neoOil ovx-mice, 90-day-old neoDES, and neoOil male mice (Figures 2 and 3(C)). However, the percentage in the whole and lateral areas was not different among groups (Figure 3(A) and (B)). The percentage of LH-positive gonadotropes in the medial area of 90-day-old neoDES female mice ovariectomized at 80 days was significantly elevated when compared with the same region in 90-day-old neoDES females (Figure 3(C)).

Expression of genes involved in gonadotropin synthesis and secretion

The expression of *Cga*, *Fshb*, *Lhb*, and *Gnrhr* in the anterior pituitary of 90-day-old neoOil female and male mice was the same (Figure 4(B)), whereas the expression of *Cga*, *Fshb*, and *Lhb* in ovx-mice was significantly elevated compared with that of 90-day-old female neoOil mice (Figure 4(B)). In neoDES female mice, the expression of *Cga*, *Fshb*, *Lhb*, and *Gnrhr* was similar to that observed in neoOil females at 30 and 90 days (Figure 4(A) and (B)), whereas the expression of *Lhb* was significantly reduced when compared to 90-day-old males (Figure 4(B)). In 90-day-old neoDES female mice ovariectomized at 80 days, the expression of *Cga*, *Fshb*, and *Lhb* was significantly increased compared to 90-day-old neoDES females (Figure 4(B)). The expression of *Cga*, *Fshb*, *Lhb*, and *Gnrhr* in 90-day-old neoDES and neoOil males was similar (Figure 4(B)).

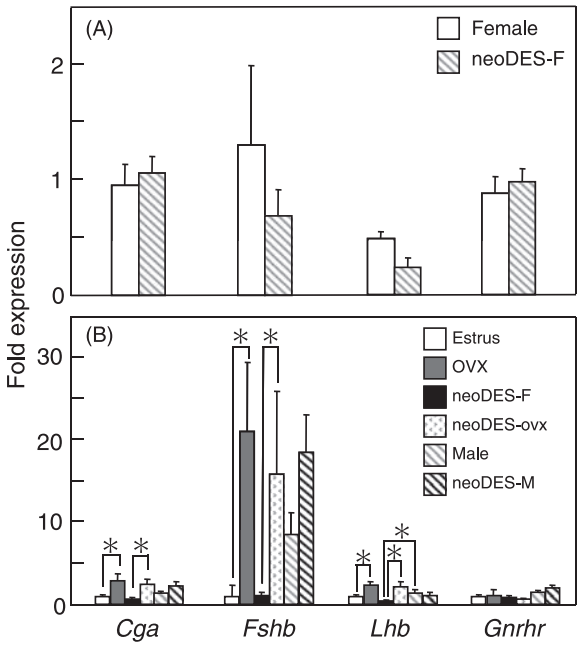

Figure 4 The mRNA expression of genes involved in gonadotropin synthesis and secretion (*Cga*, *Fshb*, *Lhb*, and *Gnrhr*) in 30-day-old intact and neoDES female mice (neoDES-F) (A), 90-day-old oil-treated female mice, 90-day-old female mice ovariectomized at 80 days, 90-day-old neoDES female mice (neoDES-F), 90-day-old neoDES female ovx-mice (neoDES-ovx), 90-day-old male mice, and 90-day-old neoDES male mice (neoDES-M) (B). Data were expressed relative to mRNA expression of 90-day-old female mice at estrous stage (relative expression = 1.0). One pituitary was used and three independent experiments were carried out for each study

LH-positive gonadotropes and genes involved in gonadotropin synthesis and secretion in the anterior pituitary of five-day-old female and male mice

In five-day-old female and male mice, LH immunoreactive cells are scattered throughout the anterior pituitary (Figure 5(B) and (C)), thus 600 anterior pituitary cells in two chosen areas from transversal horizontal sections were counted (Figure 5(A)). The percentage of LH-positive cells in five-day-old female mice was significantly higher compared to age-matched males (Figure 5(D)).

The mRNA expression of *Cga* and *Lhb* in the anterior pituitary of five-day-old neoOil female mice increased significantly compared with that in zero-day-old females (Figure 5(E)), whereas the expression of *Cga* and *Lhb* in five-day-old neoDES female mice was significantly reduced when compared with five-day-old neoOil females (Figure 5(E)). Similar to females, the expression of *Cga* in the anterior pituitary of five-day-old neoOil male mice was elevated compared with zero-day-old males (Figure 5(E)). Pituitary expression of *Fshb* increases in zero-day to five-day-old neoOil female and male mice (Figure 5(E)), but decreases in five-day-old neoDES female and male mice. The expression of *Lhb* in zero-, five-day-old neoOil males and age-matched neoDES males showed the same tendency. The expression of *Gnrhr* did not differ among the treatment groups (Figure 5(E)).

LH-positive gonadotropes and genes involved in gonadotropin synthesis and secretion in organ-cultured anterior pituitaries

To study the potential direct effects of DES on changes in the percentage of LH-positive gonadotropes as well as gene expression, pituitaries from zero-day-old female mice were organ-cultured with or without 1 μg/mL DES for five days, as previously used for the induction of polyovular follicles in organ-cultured neonatal mouse ovaries.[21]

LH immunoreactivity was observed in the cytoplasm of organ-cultured anterior pituitary cells (Figure 6(A) and (B)), whereas negative controls incubated with a rabbit immunoglobulin fraction showed no staining (data not shown). The percentage of LH-positive cells in organ-cultured pituitaries treated with 1 μg/mL DES was similar to those

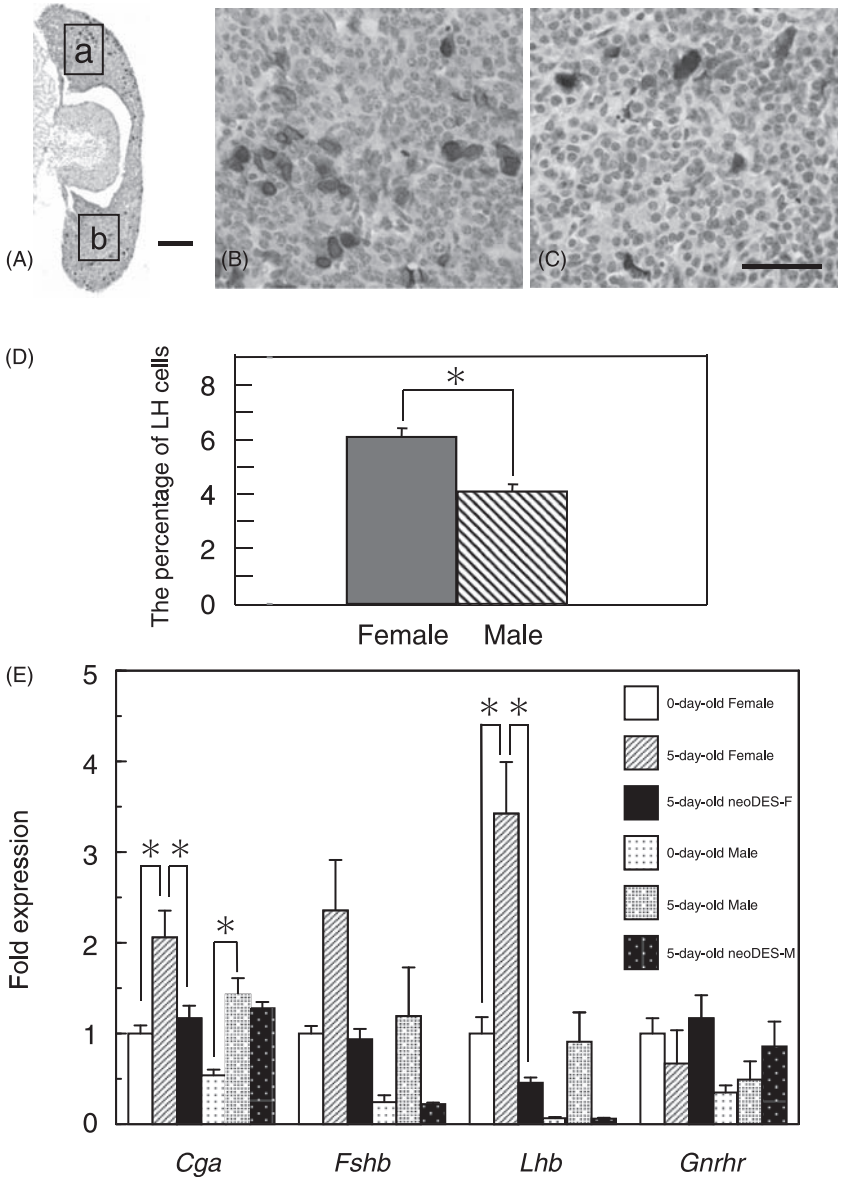

Figure 5 Two areas were counted (a and b) in the anterior pituitary from a transverse horizontal section (A). LH cells in the anterior pituitaries of five-day-old female mice (B) and five-day-old male mice (C). The percentage of LH cells in the pituitary of five-day-old female and male mice (D). The expression of genes involved in gonadotropin synthesis and secretion (*Cga*, *Fshb*, *Lhb*, and *Gnrhr*) in zero-day-old intact male and female mice, five-day-old oil-treated and neoDES male and female mice (E). Data were expressed relative to mRNA expression of zero-day-old female mice (relative expression = 1.0). Two to 10 pituitaries of zero- and five-day-old animals were pooled for RNA isolation and three independent experiments were carried out for each study. Scale bar: 200 μm (A); 50 μm (B and C)

without DES (control medium, Figure 6(C)). The expression of *Cga*, *Fshb*, and *Lhb* in DES-treated pituitaries decreased significantly compared to those cultured without DES (Figure 6(D)). In contrast, DES-treated pituitaries exhibited a significant increase expression in the expression of *Gnrhr* when compared with pituitaries cultured without DES (Figure 6(D)).

Discussion

In this study, the percentage of LH-positive gonadotropes in the medial area was lower as compared with that in the lateral area at all ages examined in both sexes, indicating that LH cells were localized in the lateral and rostral areas of the anterior pituitary as reported previously.[22] In female mice, the percentage of LH-positive cells in the whole area of the pituitary showed a decrease with age, whereas male mice exhibited no age-dependant changes. In addition, the percentage of LH-positive gonadotropes in five-day-old female versus male mice was significantly elevated. Gonadotropic immunoreactive cells have been observed from E17 and reported to increase until the end of gestation in both sexes but were higher in female versus male mice.[5] In the present study, female mice showed higher LH-positive cell populations compared to males from fetal through immaturity, but LH-positive gonadotropes decline in density after reproductive maturation in females, but not in

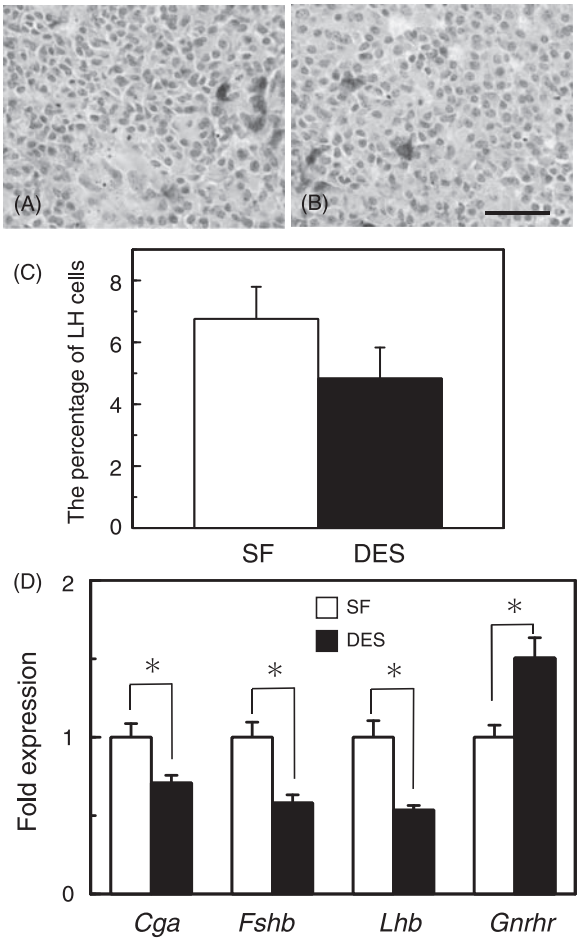

Figure 6 LH-positive cells in the organ-cultured anterior pituitary with (B) or without 1 μg/mL DES (A). The percentage of LH-positive cells in the organ-cultured pituitary with or without 1 μg/mL DES (C, $n = 10$). The expression of genes involved in gonadotropins synthesis and secretion (*Cga*, *Fshb*, *Lhb*, and *Gnrhr*) in the organ-cultured anterior pituitary with or without 1 μg/mL DES (D). Data were expressed relative to mRNA expression of 90-day-old female mice at estrous stage (relative expression = 1.0) as in Figure 4. Ten pituitaries were pooled for RNA isolation at each point and three independent experiments were carried out for each study. Scale bar: 50 μm

males. Since gonadotrophs are capable of GnRH-induced cell movement both *in vitro* and *in situ*,[23] LH cells are able to form cell-type specific networks in the pituitary of both sexes.

The number of LH-positive gonadotropes in the adult female rat is smaller than that of males[24] and basal LH concentrations in the mouse sera are also lower.[25] In this study, sexual dimorphism was not clearly observed based on the percentage of LH-positive cells or in the pituitary expression of *Lhb*. Ovariectomy resulted in significant elevations of *Cga*, *Fshb*, and *Lhb*, similar to a previous report,[3] but the percentage of LH-positive cells in ovx-mice was not changed when compared with 90-day-old intact female mice. These results suggest that estrogens suppress *Lhb* expression via negative feedback on the hypothalamus and/or the pituitary, but appear as it does not influence the number of LH-positive gonadotropes. While the expression of various gonadotropin genes is directly regulated by estradiol,[26] synthesis and secretion of LH in the anterior pituitary are also under the control of GnRH pulses and ovulation associated surges.[26,27] Therefore, the number of LH-positive cells, gene expression, and LH concentrations are not synchronized and could be differentially regulated by ovarian steroids and GnRH in female mice.

Neonatal DES exposure did not affect the distribution of LH-positive gonadotropes; however, the percentage of these cells decreased at 90 days of age. Although our previous studies showed that the expression of *Lhb* in neoDES female mice was significantly reduced when compared to intact female mice,[20] the expression of *Lhb* in neoDES female mice was not significantly changed. That is, the expression of *Lhb* in neoDES female mice was significantly decreased by comparison of two mean values using Student's *t*-test. Thus, both the percentage of LH cells and the expression of *Lhb* in neoDES female mice were reduced compared with those in neoOil female mice at 90 days of age.

No difference either in the percentage of LH-positive cells or in the expression of *Lhb* in neoDES females at 30 days of age was noted. The expression of *Cga* and *Lhb* increased significantly from days 0 to 5 in female mice, and neonatal DES treatment suppressed the increase. Moreover, DES treatment did not change the percentage of LH-positive cells but did decrease the pituitary expression of *Cga*, *Fshb*, and *Lhb* in organ-culture, suggesting that DES directly affects gene expression but not gonadotrope number. GnRH pulses are responsible for the synthesis and secretion of LH and FSH from the anterior pituitary.[27] In this study, LH-positive cells were partially decreased after reproductive maturation in females, suggesting that the GnRH surge could affect their distribution. Since neoDES mice lack a LH surge,[13] a decrease of LH-positive cells in the medial area could be due to altered hypothalamus function following neonatal DES treatment.

The hypothalamus in neoDES females is masculinized.[15] In this study, the percentage of LH-positive cells in the medial area as well as the mRNA expression of *Lhb* in 90-day-old neoDES females were significantly reduced when compared to 90-day-old neoOil females and intact males. The expression of *Gnrhr* in the pituitary did not vary among groups, suggesting that the sensitivity to GnRH may not be altered. Therefore, it is possible that the masculinized or defeminized hypothalamus induced by neonatal DES could be a different response that seen in the intact male hypothalamus that displays a change in the regulation of LH cell number and *Lhb* expression. In the hypothalamus, the kisspeptin system is considered an important regulator of GnRH neurons.[28,29] Kiss1 neurons in the AVPV are more abundant in females than males and are thought to be responsible for governing the GnRH/LH surge system.[28,29] Kiss1 neurons in the arcuate nucleus (ARC) also modulate the tonic pulsatile release of GnRH.[28] Neonatal exposure to EB or an estrogen receptor α selective agonist decreases KISS-immunoreactivity in AVPV and ARC of the female rat,[30] suggesting that neonatal DES can affect *Kiss1* expression in both neuronal populations. In addition, kisspeptin regulates gonadotropin gene expression through the activation of Kiss1R signaling in LβT2 cells and primary mouse pituitary cells,[31] suggesting

that the kisspeptin system has a functional role in the pituitary in addition to the regulation of GnRH neurons. However, it has not been fully documented how the reproductive axis is regulated by the kisspeptin system in either males or females. It is likely a complicated system; therefore, neonatal DES can masculinize or defeminize the hypothalamus in females, but details concerning the regulation of the LH secreting cells are still unclear.

Ovariectomy resulted in a significant increase in pituitary mRNA for *Cga*, *Fshb*, and *Lhb* in neo-DES female mice, as also observed in neoOil female mice, suggesting that the anterior pituitary of neoDES female mice is regulated by ovarian hormones via negative feedback. Indeed, serum E2 concentrations in neoDES mice are similar to those observed in intact females.[20] Thus, the HPG axis in neoDES mice is functioning to some degree. *Lhb* and *Fshb* gene expressions also are regulated by GnRH, accompanied by several transcription factors such as SF-1, Egr-1, and Ptx1.[26] Neonatal DES exposure alters SF-1 expression in the ovary[32]; therefore, it is possible that DES can also affect those transcription factors in the pituitary.

A significant depression in pituitary LH content is found in neoDES female mice at six, 12, and 21 days of age, and reduced serum LH concentrations also are observed at days six and 12; however, both are recovered at day 56.[19] Although serum LH concentrations were not measured in this study, a decrease of the number of LH-positive gonadotropes and mRNA *Lhb* expression in neoDES mice may not affect serum LH concentrations. LH secretion is stimulated by GnRH pulses,[33] but, potential changes in GnRH pulses from the neonatally DES-exposed hypothalamus are not clear. Therefore, further molecular level studies should be conducted to examine such potential changes.

In male mice, neonatal DES treatment did not change pituitary mRNA expression of *Cga*, *Fshb*, *Lhb*, and *Gnrhr*, consistent with a previous report.[17] Therefore, it is unlikely that DES affects the percentages of LH-positive cells or the expression of *Lhb* in male mice. However, neonatal EB exposure reduces hypothalamic *Kiss1* expression and LH concentrations in the male rat,[34] suggesting that molecular studies are also necessary for a further understanding the effects of DES in the male hypothalamus.

In conclusion, our present results suggest that LH-positive cells in adult female mice are not only directly affected by DES exposure during neonatal period but also influenced by the masculinized hypothalamus. Finally, the anterior pituitary of female mice exposed neonatally to DES is capable of responding to ovarian hormones via negative feedback, suggesting that the HPG axis is functioning albeit differently than that of an intact female. Since disruption of the HPG axis is associated with a number of reproductive disorders in women, further study examining, at a molecular level, the hypothalamus of neonatally DES-exposed mice, could be insightful.

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