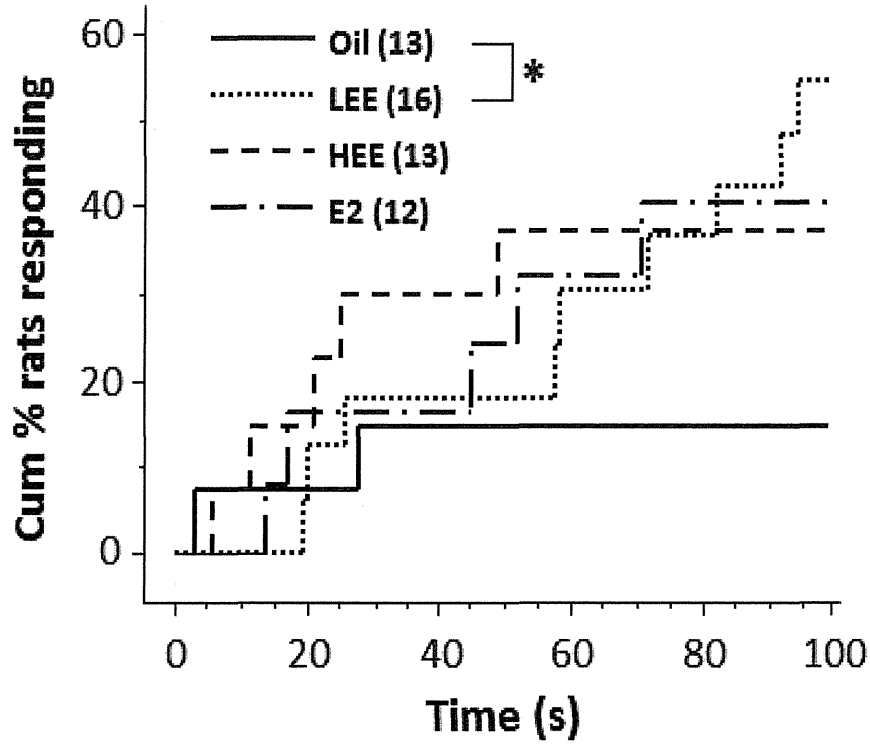

Fig 2. Impairment of avoidance learning in gonadally intact low dose ethinyl estradiol (LEE)-treated female rats. The line plot shows effects of LEE (0.02 mg/kg), HEE (2 mg/kg), and 17β-estradiol (E2; 20 mg/kg) treatment within 24 h after birth on the cumulative percentage of rats that displayed a transfer response in the passive avoidance test. Six-week-old gonadally intact animals were used in this experiment. Numerals in parentheses indicate the number of rats in each group. * $P < 0.05$, log-rank comparison.

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Results

Avoidance learning in EE-treated rats

The results of the passive avoidance test are shown as survival curves in Figs 2 and 3, demonstrating the time-course change in the cumulative percentage of animals producing a transfer response (also see S1A–S1C Fig). The LEE-treated rats showed a shorter latency to enter the dark/shock compartment in the first (gonadally intact) experiment (Oil vs. LEE; $P < 0.05$, log-rank comparison; Fig 2). In the second experiment, no significant differences were found between groups in the non-EB-injected condition (Fig 3A). On the other hand, in the EB-injected condition, the LEE-treated rats again showed a tendency toward a shorter latency to enter the shock compartment (Oil vs. LEE; $P = 0.057$, log-rank comparison; Fig 3B).

Effects of neonatal exposure to EE on expression levels of ERα in the cortex and hippocampus

ERα protein levels in the cortex of LEE-, HEE-, and E2-treated females were significantly lower than in oil-treated females in the non-EB-injected group (Fig 4A; $P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively). In contrast, with EB estrogen replacement, there was no significant difference in ERα protein levels in the cortex among the neonatal treatment groups (Fig 4B).

ERα protein levels in the hippocampus of E2-treated females were significantly lower than in oil-treated females (Fig 5A; $P < 0.05$) in the non EB-injected group. In the EB-injected

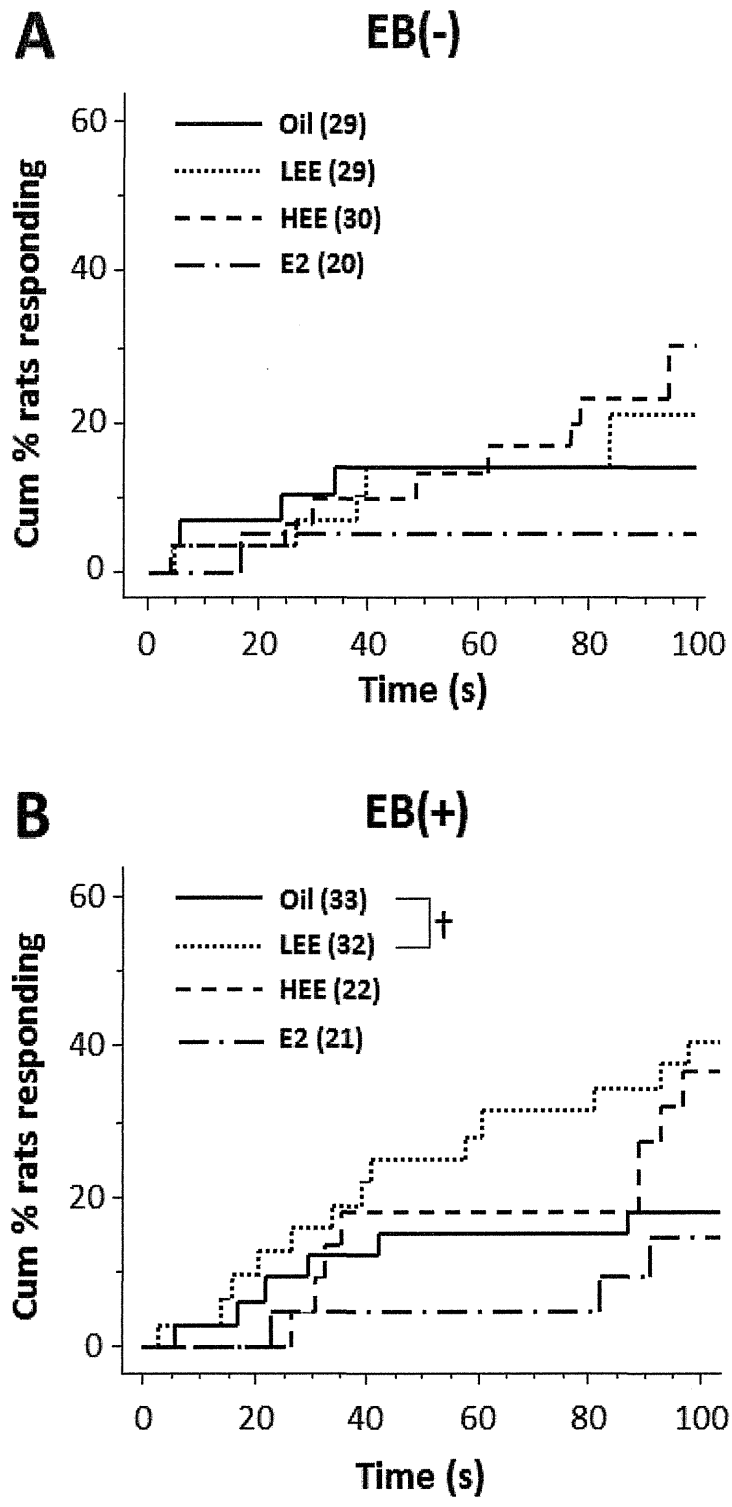

Fig 3. Impairment of avoidance learning in ovariectomized (OVX) low dose ethinyl estradiol (LEE)-treated female rats. The line plots show the effect of LEE (0.02 mg/kg), HEE (2 mg/kg), and 17β-estradiol (E2; 20 mg/kg) treatment within 24 h after birth on the cumulative percentage of rats that displayed a transfer response in the passive avoidance test. All rats were OVX at 10 weeks of age; at 15–17 weeks, the animals were either not injected (A) or injected with EB (B) 1 day before the session. Numerals in parentheses indicate the number of rats in each group. †$P < 0.1$, log-rank comparison.

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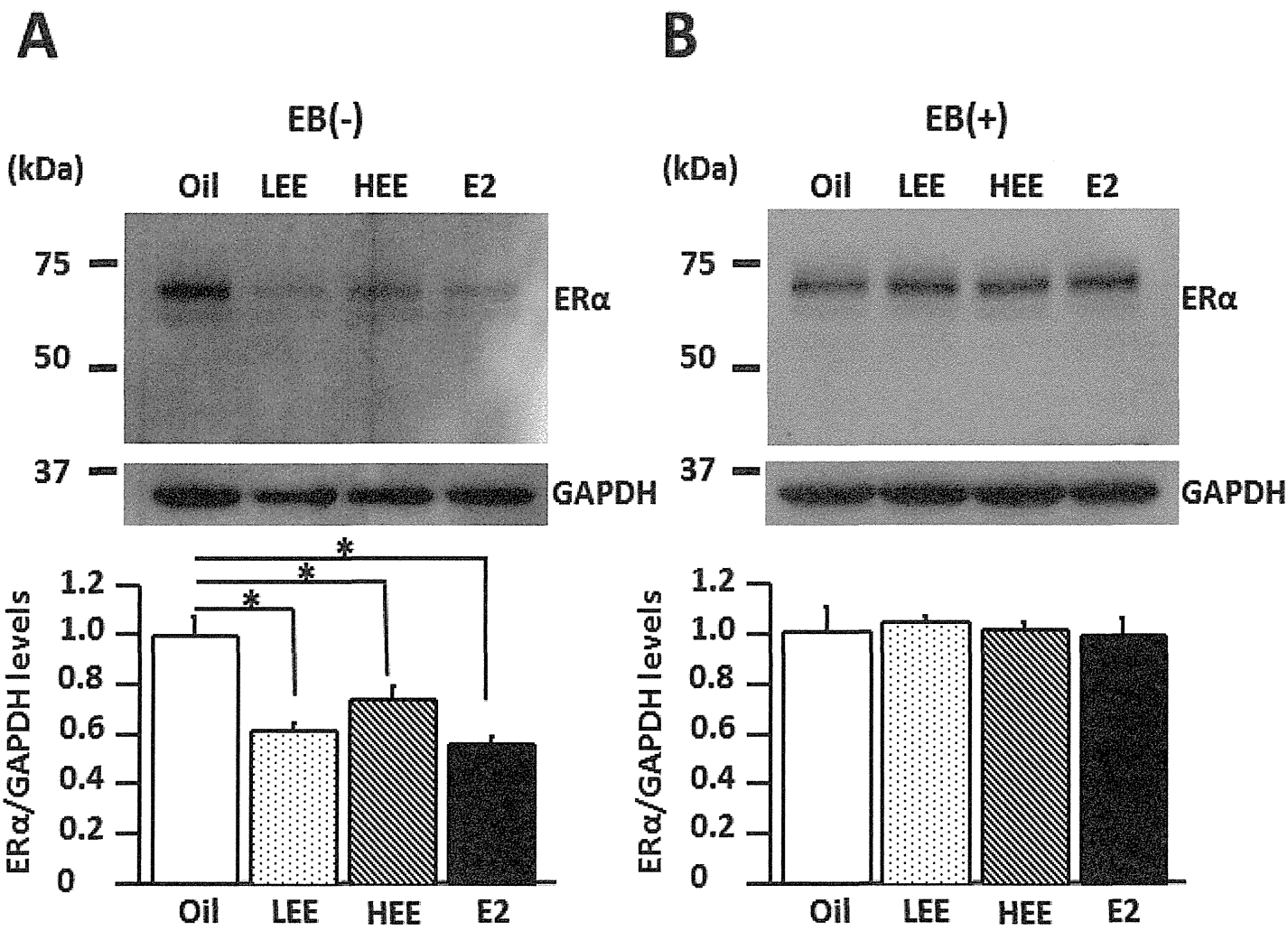

Fig 4. Neonatal exposure to ethinyl estradiol (EE) decreased the expression level of estrogen receptor alpha (ERα) in the female rat cortex. A. Representative western blot images showing the ERα expression in the cortex of EE-treated 15–18-week-old ovariectomized (OVX) rats injected with estradiol benzoate (+) or without injection (-). B. The levels of ERα in the cortex. *$P < 0.05$, Tukey-Kramer test; n = 6/group.

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group, ERα protein levels in the hippocampus of LEE- and HEE-treated females were significantly lower than in oil-treated females (Fig 5B; $P < 0.01$ and $P < 0.001$, respectively).

Discussion

The results of this study show that a single exposure to 0.02 mg/kg of EE within 24 h after birth lowered performance in the passive avoidance learning test. Additionally, a single exposure to 0.02 mg/kg and 2 mg/kg of EE within 24 h after birth reduced the expression levels of ERα in the hippocampus in the OVX/EB group, and in the cortex in the OVX/No EB group.

A single injection of LEE within 24 h after birth, which terminates the estrus cycle early after sexual maturation [20], induced a shorter latency to enter the dark/shock compartment in the passive avoidance-learning test in gonadally intact female rats. The results of this study are similar to those that demonstrated impaired learning performance in the passive avoidance test in female mice pre- and postnatally exposed to 40 μg/kg/day BPA [6]. Unlike that study, though, we limited off-target (i.e., non-estrogenic) disruptive effects by using the rapidly

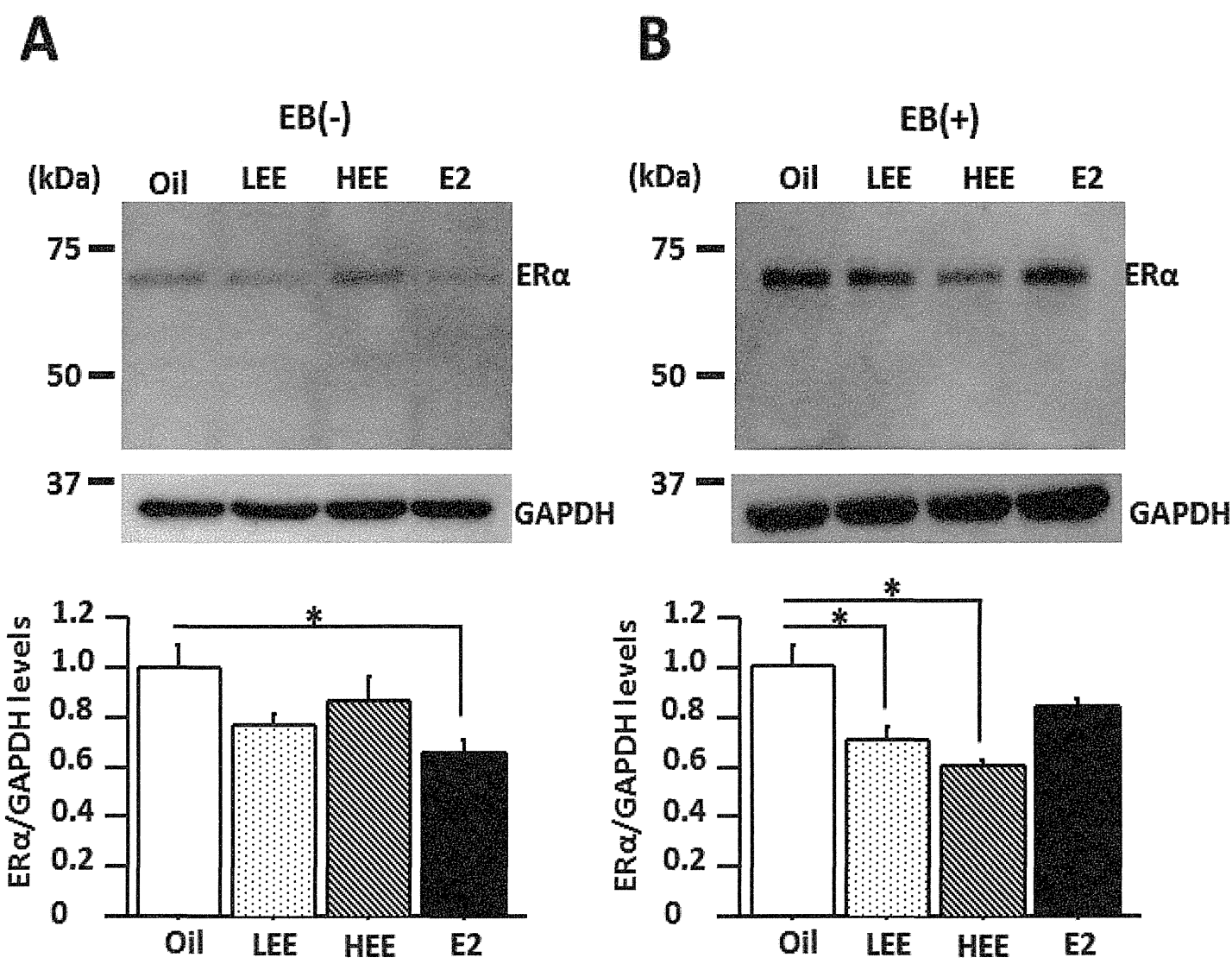

Fig 5. Neonatal exposure to ethinyl estradiol (EE) decreases the expression level of estrogen receptor alpha (ERα) in the hippocampus of adult female rats. A. Representative western blot images showing ERα expression in the hippocampus of EE-treated ovariectomized OVX rats injected with (+) estradiol benzoate or without injection (-) EB. B. Levels of ERα expression in the hippocampus. *$P < 0.05$, Tukey-Kramer test; n = 6/group.

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metabolized and excreted estrogenic agent EE. Thus, our data strongly suggest that exposure to a single dose of EE within 24 h after birth reduced avoidance learning through an estrogen-mediated process. This study also reveals a tendency towards shorter latencies to enter the shock compartment in the passive avoidance learning test of the LEE OVX group that received EB after maturation (on the day before the passive avoidance learning test) as a normal/fertile female model. In using OVX groups, we showed that some of the learning impairment in the intact female was caused by a direct action of neonatal EE on the brain, and that this effect was stronger in the presence of circulating estrogen. Further, this study also indicates that a high dose of an estrogenic agent is not necessarily more potent than a low dose. The learning behavior was affected by a dose lower than the level at which sexual behavior was affected in our previous experiments (see the Introduction). In the general dose-response model, an effect increases with increasing dose; however, estrogen-like substances have been reported to show

an inverted U-shape curve, wherein the effect is greater at low-moderate doses than at high doses [26,17,27]. This study also indicates that the effect of exposure to low-dose estrogen-like substances during critical developmental periods can persist late into life.

In the hippocampus, expression levels of ERα were significantly reduced in the EE-exposure group only when EB was injected 24 h before brain sampling; therefore, the injection of estradiol may have acted to trigger a latent effect of neonatal EE exposure on the hippocampus. This study demonstrates for the first time that a single exposure within 24 h after birth affects ERα in the hippocampus, even after maturation, in the normal fertile female model, but not the menopause model. These results also highlight the importance of ovarian estrogen in both reproductive and normal daily brain function. Estrogen increases dendritic spine density on pyramidal cells, as well as N-methyl-D-aspartate (NMDA) receptor expression. It also enhances the magnitude of long-term potentiation (LTP) through ERα [11–13]. In females, it also improves performance on hippocampus-dependent learning tasks such as passive avoidance [11,13]. Along with the trend towards a shorter latency to enter the shock compartment of the OVX and EB-injected LEE group in the passive avoidance learning test, the lower performance in the passive avoidance learning test in the present study could have been partly caused by the reduction of ERα expression in the hippocampus. On the other hand, the passive avoidance test was only affected in the LEE group, though ERα expression in hippocampus was affected in both the LEE and HEE groups, and in the fertile female model. This difference between the results of learning behavior and ERα expression suggest possible compensatory changes that occurred in other regulatory systems, such as ERβ expression, synaptic modification, or hippocampal NMDA receptor expression [28,12,29]. In future studies, it will be necessary to consider such variables. Furthermore, in the menopause model, a single injection of EE within 24 h after birth had no effect on the expression levels of ERα in the hippocampus after maturation. The potential confound of ovarian estrogen production, and possibly position in the estrus cycle, will also need to be addressed in future work.

In contrast to the hippocampus, EE exposure reduced ERα expression in the cortex in the menopause model. On the other hand, no significant difference was observed in cortical ERα expression between the groups that received EB injections 24 h before brain sampling. Therefore, EB injection 24 h before brain sampling apparently masked the effect of the single neonatal exposure to EE on the reduction of ERα expression in the cortex. Exposure to estrogenic agents within 24 h after birth reduced ERα expression after maturation, and changed the response to estrogen; its effect may have varied in a site-specific manner. In the study conducted by Andrea Gore and colleagues in 2011 [14], sequential exposure to EB (1 mg/kg) and methoxychlor (100 mg/kg) 7–19 days after birth also increased expression levels of ERα in the POA of female rats, while no change was found in the mediobasal hypothalamus. Here, we found that the effect of estrogen exposure during the developmental period varied between the hippocampus and cortex. These results re-affirm the importance and complexity of assessing the risk of exposure to estrogen-like substances during the developmental period.

Supporting Information

S1 Fig. Distribution of latencies for transfer response in the passive avoidance test. Each dot indicates the latency of the individual. A: gonadally intact, B: ovariectomized (OVX), C: OVX and replaced with estradiol benzoate (EB).
(PDF)

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Author Contributions

Conceived and designed the experiments: TJN MY YK MK. Performed the experiments: TS TJN CK YG YM MK. Analyzed the data: TS TJN CK YG YM MK. Contributed reagents/materials/analysis tools: TJN YG YM MY YK MK. Wrote the paper: TS TJN CK YK MK.

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The Critical Hormone-Sensitive Window for the Development of Delayed Effects Extends to 10 Days after Birth in Female Rats Postnatally Exposed to 17alpha-Ethynylestradiol[1]

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ABSTRACT

Neonatal exposure to estrogens is known to cause delayed effects, a late-occurring adverse effect on adult female reproductive functions, such as early onset of age-matched abnormal estrous cycling. However, the critical period in which neonates are sensitive to delayed effects inducible by exogenous estrogen exposure has not been clearly identified. To clarify this window, we examined the intensity and timing of delayed effects using rats exposed to ethynylestradiol (EE) at various postnatal ages. After subcutaneous administration of a single dose of EE (20 μg/kg, which induces delayed effects) on Postnatal Day (PND) 0, 5, 10, or 14 in Wistar rats, hypothalamic and hormonal alterations in young adults and long-term estrous cycling status were investigated as indicators of delayed effects. In young adults, peak luteinizing hormone concentrations at the time of the luteinizing hormone surge showed a decreasing trend, and KiSS1 mRNA expression of the anterior hypothalamus and number of KiSS1-positive cells in the anteroventral periventricular nucleus were significantly decreased in the PND 0, 5, and 10 groups. The reduction in KiSS1 mRNA and KiSS1-postive cells was inversely correlated with age at time of exposure. These groups also exhibited early onset of abnormal estrous cycling, starting from 17 wk of age in the PND0 group and 19 wk of age in the PND5 and 10 groups. These indicators were not apparent in the PND14 group. Our results suggest that PND0–PND10 is the critical window of susceptibility for delayed effects, and PND14 is presumed to be the provisional endpoint of the window.

AVPV, critical window, delayed effects, estrous cycle, ethynylestradiol, KiSS1, postnatal day, sexual differentiation

INTRODUCTION

Exposure to various estrogenic chemicals during critical periods of development is widely known to cause irreversible damage to the programming of hypothalamus-pituitary-gonadal (HPG) axis in a wide range of species, leading to persistent impairment of and various adverse effects on reproductive functions later in life [1, 2]. Neonatal exposure to large amounts of estrogenic compounds has a serious influence on the sexual differentiation of the brain, causing various irreversible abnormalities, such as masculinized sexual behavior, malformation of the reproductive tract, and cessation of cyclic ovulation, effects commonly known as masculinization or defeminization [3–5]. On the other hand, shorter and lower-dose exposures also cause multiple adverse effects on female reproductive functions, but the timing and types of effects are reported to be different from those observed in masculinization. For instance, neonatal exposure to chemicals such as p-t-octylphenol, methoxychlor, and the synthetic estrogen diethylstilbestrol (DES) is reported to induce early onset of age-matched abnormal estrus cycling, advanced reproductive senescence, and increased uterine carcinogenic risk in young adult or aging rats [2, 6, 7]. These late-occurring effects that develop after the pubertal period are known as delayed effects or delayed reproductive dysfunction in previous reports [7–10].

Previously, we investigated delayed effects caused by neonatal exposure to single, relatively low doses of 17α-ethynylestradiol (EE) in rats [8, 11, 12]. EE is one of the synthesized estrogens widely utilized in the study of endocrine disruption and is known to have the characteristics of rapid excretion and lower binding affinity for α-fetoprotein in neonatal blood [13]. Because these characteristics were suitable for limiting exposure time and penetrating the blood-brain barrier, EE was thus selected as the model compound. In these studies, we revealed that the prior attenuation of the luteinizing hormone (LH) surge and reduction of KiSS1 mRNA levels in the hypothalamic kisspeptin neurons occur approximately 2 mo earlier than the onset of abnormal estrous cycling, suggesting that these alterations could be early indicators of delayed effects [11]. In the rat brain, kisspeptin neurons are located mainly in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC), and each of these areas is considered to play a site-specific role: the AVPV triggers

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secretion of gonadotropin-releasing hormone (GnRH)/LH surge leading to ovulation, and the ARC provokes GnRH pulsatile secretion, which is responsible for follicular development [14–16]. In fact, attenuated KiSS1 expression in the AVPV and reduced amplitude of LH surge are reported to synchronize in middle-aged rats [17, 18], and the circadian-regulating function of AVPV KiSS1 expression, which may direct or indirectly control the timing of the LH surge, was also implicated in previous reports [19, 20]. These indicate the critical roles of AVPV KiSS1 signals in the regulation of physiological aspects of the LH surge and subsequent reproductive functions mediated by LH; therefore, it could be said that the AVPV-specific KiSS1 depression and reduced LH surge observed in our previous study might be an early key event in serial, late-occurring reproductive dysfunction in delayed effect.

Sexual differentiation of the central nervous system (CNS) is a crucial process for the establishment of the hypothalamic neural network and attainment of various reproductive functions in adulthood. This process generally occurs early in life; late embryonic to early postnatal ages are relevant in mammals, consistent with the period during which fetuses and neonates are exposed to endogenous maternal or their own gonadal hormones for proper development [21, 22]. Therefore, inappropriate exposure to estrogenic chemicals during this critical period could disrupt sexual differentiation and adult reproductive functions. However, the precise window during which the fetal or neonatal brain is sensitive to estrogen exposure has not been clearly defined. Yoshida et al. [6] reported that the subcutaneous injection of p-t-octylphenol during both Postnatal Day (PND) 1–5 and 1–15 induced the early onset of abnormal estrous cycling (persistent estrus) in young adult Donryu rats; however, the time needed for persistent estrus to develop in all animals within the group was shorter in the PND1–PND15 group (1.5 mo) than in the PND1–PND5 group (6 mo). Another study has shown that a single injection of estradiol valerate (EV) at PND14 induced severe cycle disruption starting at PND50 in Wistar rats, and the frequency of cycling during the period from PND 71 to 90 was reduced by 72% in prepubertal, EV-treated rats compared to rats in the control group [23]. These results indicate that the critical window of sensitivity in neonatal brains might extend to late postnatal ages, after the completion of CNS sexual differentiation [24, 25]; however, to our knowledge, studies that intend to directly determine the critical window of postnatal sensitivity to estrogens have not been performed in the past, and whether there is a clear endpoint for the critical window remains unknown.

In the present study, to clarify the critical window of sensitivity to estrogenic compounds for delayed effects in rats, we investigated and compared the intensities and timing of delayed effects induced by a single subcutaneous injection of EE at PND 0, 5, 10, and 14. We examined 1) artificially induced LH surges and KiSS1 mRNA expression at 11 wk of age in the AVPV and ARC and 2) the long-term estrous cycling status until 40 wk of age; these parameters have previously been shown to be early and late indicators, respectively, of delayed effects [8, 11].

MATERIALS AND METHODS

Animal Treatment and Chemicals

Pregnant Wistar Hannover GALAS rats were obtained from CLEA Japan, Inc. at Gestational Day 14 (n = 37) in order to obtain female neonates for the experiments. The dams and their offspring were housed individually in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (24°C ± 1°C; relative humidity, 55% ± 5%; 12L:12D) with a basal diet (CRF-1; Oriental Yeast Co.) and tap water available ad libitum. CRF-1 is a standard diet that includes soy protein and is known to contain a relatively low level of estrogens. All the neonates were randomized within 24 h after birth, and 10 neonates were allocated to each dam with a female predominance in order to exclude genetic biases. After randomization, dams were assigned to five groups (seven to eight dams/group). Neonates in each group received a single subcutaneous injection of sesame oil at PND0 (control group) or EE 20 μg/kg at PND 0, 5, 10, or 14 (referred to as group PND0, PND5, PND10, and PND14, respectively). EE (CAS No. 57-63-6; purity > 98%) was purchased from Sigma. The dose of EE selected for injection was capable of inducing delayed effects with reference to a previous report [8]. On PND21, the rats were weaned, and 30 female rats per group were housed two to four per cage. Starting on PND23, we checked for vaginal opening (VO) every day; the day of VO and body weight on the day of VO were recorded. After checking for VO, estrous cycle was monitored by vaginal smear for five consecutive days every week, and observations of clinical signs, body weight, and mortality were made throughout the experiment. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences (Japan).

Animal Treatment for Short-Term Analysis in Young Adults

A total of 10 out of 30 rats were used for the short-term analysis. At 10 wk of age, rats with normal estrous cycles received an ovariectomy (OVX) under isoflurane anesthesia. One week after OVX, all the animals were subcutaneously treated with 2 μg of estradiol benzoate at 0900 for three consecutive days and 500 μg of progesterone (P4) at 1100 of the last day of estradiol benzoate treatment for artificial LH surge priming. All the animals were decapitated (n = 5/group) or transcardially perfused with 4% paraformaldehyde (PFA) (Nacalai Tesque, Inc.) under deep anesthesia with pentobarbital sodium (Kyoritsu Seiyaku Corporation) (n = 5/group) between 1600 and 1700 of the surge priming day. In decapitated animals, blood samples were collected for serum hormone measurement, and hypothalami were dissected out for real-time PCR analysis as described in a previous report [11]. Dissected hypothalami were macroscopically divided using the optic chiasm as a boundary into anterior and posterior hypothalamus, which included the AVPV and the ARC, respectively. The hypothalamic samples were immediately frozen in liquid nitrogen and stored at −80°C until the time of RNA isolation. For perfused animals, brains were removed immediately and postfixed in 4% PFA overnight at 4°C, then immersed in 20% sucrose/PBS solution at 4°C until the tissues sank. The fixed brains were embedded in optimal cutting temperature compound (Sakura Finetek Japan Co. Ltd.) and stored at −80°C until sectioning. In all cases, uteri and vaginas were also resected. These tissues and ovaries removed by OVX were fixed in 10% neutral buffered formalin and routinely processed and stained with hematoxylin and eosin (HE) for histopathological examination.

Long-Term Observation of Estrous Cycle

A total of 16 out of 30 rats in each group were maintained until 40 wk of age for the long-term monitoring of estrous cycles. A decision on the cycle pattern was made with every 5-day observation. Regular 4- or 5-day cycles were deemed normal cycles, and other patterns were uniformly judged to be abnormal estrous cycles. In particular, animals showing proestrus or estrus continuously for 5 days or showing diestrus or metestrus continuously for 5 days were designated as having persistent estrus or persistent diestrus, respectively. Estrous cycles were recorded every week throughout the experiment. At 40 wk of age, all the animals were necropsied under isoflurane anesthesia, and the ovaries, uteri, and vaginas were resected from each animal. The weight of the ovary and uterus were also recorded. All the organs were fixed in 10% neutral buffered formalin and routinely processed and stained with HE for histopathological examination.

Quantitative Real-Time PCR

Total RNA was extracted from the hypothalamus lysates using ISOGEN (Nippon Gene Co. Ltd.), and reverse transcription reactions were performed using 1 μg of total RNA with High Capacity Reverse Transcription kits (Applied Biosystems). Following the manufacturer's instructions, real-time PCR (7900HT Fast Real-time PCR System; Applied Biosystems) was performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) as primer-probe sets for the following genes: *Kiss1* (KiSS1, Rn00710914_m1), *Kiss1r* (KiSS1R, Rn00576940_m1), and *Gnrh1* (GnRH, Rn00562754_m1). The expression level of each gene was calculated by the relative standard curve method and normalized against endogenous GAPDH (TaqMan Rodent GAPDH Control Reagent; Applied Biosystems).

Sectioning and Quantitative Analysis of KiSS1 mRNA-Positive Cells in In Situ Hybridization

In order to evaluate the whole area of the AVPV and ARC, we made sequential coronal sections (20 μm thick) of the AVPV from approximately 0.12 mm anterior to 0.36 mm posterior to the bregma, and of the ARC from approximately 1.92 mm posterior to 3.72 mm posterior to the bregma [26] using a cryostat (CM1850UV; Leica). After sectioning, all the tissue sections were immediately mounted on coated slides (Matsunami Glass Ind., Ltd.) and then completely air dried and stored at −80°C until staining. Because one section from every six sections from the AVPV and every 15 sections from the ARC was utilized for KiSS1 in situ hybridization (ISH), a total of four sections for the AVPV and six sections for the ARC per rat were examined to quantitate KiSS1-positive cells. For positive cell counting, an Olympus BX51 microscope with an Olympus digital camera DP73 (Olympus) was utilized, and all KiSS1-positive cells with visible reactions observed in the left half of the brain were manually counted at high magnification in a blinded fashion. The total numbers of KiSS1-positive cells in each group were statistically compared. It was confirmed preliminarily that all sections from the AVPV and ARC had approximately the same numbers of positive cells in each brain half.

ISH

Nonradioactive ISH was used for KiSS1 mRNA-positive cell detection. Slides were washed with PBS and treated with 1 μg/ml proteinase K (Takara Bio Inc.), followed by a brief 1% PFA immersion. After washing in PBS, slides were incubated with 0.25% acetic anhydride in 0.1 M triethanolamine and then prehybridized with hybridization buffer for 30 min. After prehybridization, slides were hybridized with 0.5 μg/ml of digoxigenin-labeled antisense RNA probe (sequence position 3-358, GenBank accession No. AY196983.1; Genostaff, Japan) overnight at 60°C. A sense RNA probe was used as a negative control. Following hybridization, the slides were washed with 4× SSC (Roche Applied Science)/50% formamide, 2× SSC, and 1× SSC. Between SSC washes, slides were briefly immersed in a 20 μg/ml RNase working solution at 37°C. After washing, slides were blocked with 2% bovine serum albumin (Sigma) in ISH buffer-1 and incubated with anti-digoxigenin antibody (1:1000; Roche Applied Science, Mannheim, Germany) in ISH buffer-1. Following washing with ISH buffer-1, the slides were immersed in ISH buffer-3 (100 mM Tris-HCl, pH9.5, 100 mM NaCl, and 50 mM $MgCl_2$) and then incubated with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution (Roche Applied Science) in ISH buffer-3 for 2 h.

Hormone Assays

Serum samples obtained from decapitation were stored at −80°C before assay. The serum concentrations of follicle-stimulating hormone (FSH) and LH were determined using double-antibody radioimmunoassays and 125I-labeled radioligands. National Institute of Diabetes and Digestive and Kidney Disease radioimmunoassay kits were used for rat FSH and rat LH with anti-rat FSH-S-11 and anti-rat LH-S-11 (National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD), as described previously [27]. The intra- and interassay coefficients of variation were 4.01% and 16.71% for FSH and 3.27% and 17.74% for LH, respectively.

Investigation of Histopathology and Immunohistochemistry at VO

To investigate treatment-related histopathological changes in the female reproductive organs at VO, four out of 30 rats in each group were used. Because acceleration of VO was observed in PND 10 and 14 groups, animals in these groups were necropsied on the next day of respective days of VO under isoflurane anesthesia, and the ovaries, uteri, and vaginas were resected from each animal. In the control, PND 0 and 5 groups without acceleration of VO, animals were necropsied at similar ages (in days) before VO to compare age-matched histology of these tissues. All the organs were fixed in 10% neutral buffered formalin and routinely processed and stained with HE for histopathological examination. For immunohistochemistry (IHC), serial sections of vaginas were subjected to IHC for ERα. Anti-ERα antibody (1:1000, sc-542, MC-20; Santa Cruz Biotechnology Inc.) was used as a primary antibody, and the sections were visualized with 3,3′-diaminobenzidine using the Vectastain Elite ABC kit (Vector Laboratories Inc.). The percentage of ERα-positive cells in vagina was assessed with reference to previous report [12]. The percentage was judged separately in epithelial and stromal cells and scored as follows: 0, negative; 1, slightly positive (<20%); 2, partly positive (20%–50%); 3, positive more than half (50%–80%); and 4, mostly positive (>80%). The average score was calculated by observation of 10 randomly selected areas at 200-fold magnification.

TABLE 1. Mean days and body weight of vaginal opening in rats exposed to 17α-ethynylestradiol (EE) at various postnatal days.

Groups	n	Vaginal opening days (PND)[a]	Body weight (g)[a]
Sesame oil			
Control	30	29.3 ± 2.2	79.2 ± 9.2
EE 20 μg/kg			
PND0	30	30.4 ± 2.1	83.6 ± 9.7
PND5	30	28.2 ± 1.6	74.8 ± 13.5*
PND10	30	24.5 ± 1.3**	58.2 ± 6.2**
PND14	30	24.7 ± 1.1**	57.1 ± 4.1**

[a]Values are presented as mean ± SD.
*/**Significantly different from the control group (*$P < 0.05$ and **$P < 0.01$ by Dunnett test).

Statistical Analysis

Following Bartlett's test, variances in data for the day of VO, body and organ weight, hormone level, real-time PCR, number of KiSS1-positive cells and score of ERα expression were compared with those for the control group by one-way analysis of variance or the Kruskal–Wallis test. When statistically significant differences were detected, the Dunnett multiple comparison test was employed for comparisons between the control group and the EE-exposed groups. The percentages of normal estrous cycling were compared by the Fisher exact test. In these tests, the level of significance was set at 0.05.

RESULTS

Clinical Observation, Body Weight, and VO

No deaths or abnormal clinical findings were observed during the experiment period. No treatment-related changes were detected in body weight. Mean day of VO and body weight on the day of VO are shown in Table 1. As compared to the control group, the mean day of VO was significantly earlier in the PND 10 and 14 groups ($P < 0.001$ in both groups vs. control group), and mean body weight on the day of VO was also significantly decreased in these two groups ($P < 0.001$ in both groups vs. control group).

Long-Term Observation of Estrous Cycle

The sequential changes in the percentage of animals showing normal estrous cycle in each group are summarized in Figure 1. In the control group, almost all the animals showed 4- or 5-day cycles until 29 wk of age. Thereafter, the number of animals presenting abnormal cycles gradually increased, and the percentage of animals with normal cycles decreased to 43.8% at 40 wk of age. In contrast, the percentages in the PND 0, 5, and 10 groups gradually decreased starting from an earlier age; the first statistically significant decreases in percentages were detected at 17, 19, and 19 wk of age in the PND 0, 5, and 10 groups, respectively. Regarding the differences among the EE-exposed groups, the PND0 group showed the most rapid decrease, and statistical significant differences were observed through Wk 21–29 versus PND5 and/or PND10 groups. PND 5 and 10 groups showed nearly the same pace of decline and statistical significant differences versus PND14 group were detected in several time points until Wk 40. The percentage in the PND14 group was comparable to that of the control group until 25 wk of age and then showed a gradual decrease slightly earlier than the control group, but statistical significance was not observed between these two groups. Slight prolongation of the estrous cycle (such as 6-day cycle) frequently observed at the beginning of the cycle disruption was considered as abnormal

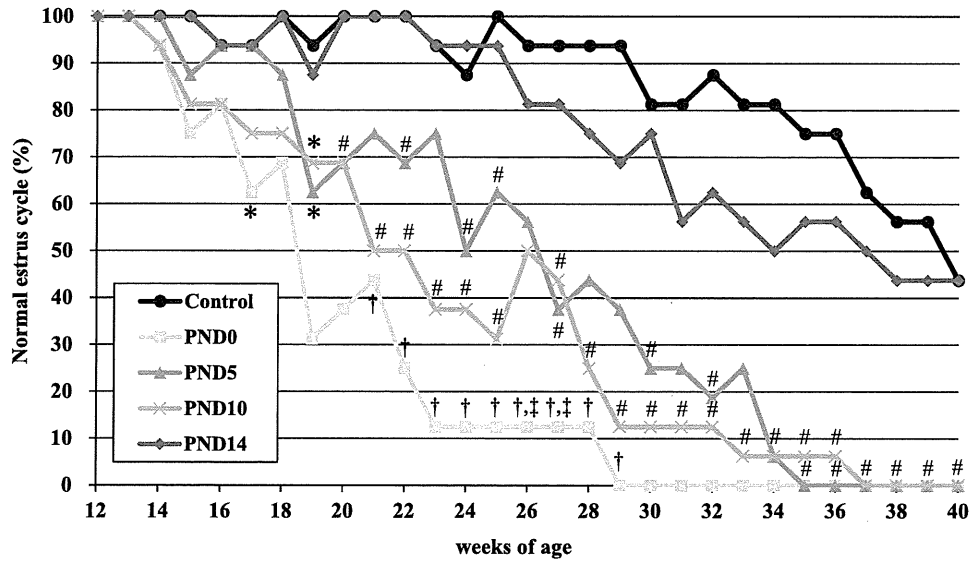

FIG. 1. Sequential changes in the percentage of animals maintaining a normal estrous cycle until 40 wk of age (n = 16/group). The vertical line indicates the percentage of animals that maintained a normal estrous cycle in each group, and the horizontal line indicates weeks of age. Symbols indicate significant differences (*$P < 0.05$ thereafter until Wk 40 without Wk 20–23 in PND5 group vs. control group; #$P < 0.05$ vs. PND14 group; †$P < 0.05$ vs. PND5 group; ‡$P < 0.05$ vs. PND10 group by Fisher exact test).

cycle in the present study, and thus recoveries of the percentage were occasionally observed throughout the experimental period. These animals finally turned out to present persistent estrus or diestrus in most cases.

Quantitative Real-Time PCR for KiSS1 and Related Genes

KiSS1 mRNA expression in the anterior and posterior hypothalamus (i.e., AVPV and ARC, respectively) at the time of the LH surge is presented in Figure 2. Relative expression levels of KiSS1 in the AVPV (mean ± SD) were 1.00 ± 0.22, 0.54 ± 0.06, 0.61 ± 0.13, 0.76 ± 0.12, and 0.83 ± 0.12 in the control, PND 0, 5, 10, and 14 groups, respectively. In the AVPV, the relative expression levels of KiSS1 mRNA were significantly decreased in the PND 0, 5, and 10 groups compared to those in the control group ($P < 0.001$, < 0.001, and $= 0.047$ vs. control group, respectively), and the decrease was inversely correlated with age at exposure. Although no statistical significance was found, the KiSS1 mRNA expression level in the PND14 group was also slightly decreased compared to that of the control group ($P = 0.20$ vs. control group). Relative expression levels of KiSS1 in the ARC (mean ± SD) were 1.00 ± 0.17, 0.84 ± 0.19, 0.73 ± 0.19, 0.94 ± 0.12, and 0.99 ± 0.09 in the control, PND 0, 5, 10, and 14 groups, respectively. In the ARC, a significant decrease in KiSS1 mRNA expression was detected in the PND5 group ($P = 0.049$ vs. control group), but no change was observed in any other group. The mRNA expression of KiSS1-related genes (GnRH1 and KiSS1R) were also analyzed in the present study. Although a significant decrease in KiSS1 mRNA expression in the AVPV was detected in the PND 0, 5, and 10 groups, mRNA expression of GnRH1 and KiSS1R was not significantly different between the EE-exposed and control groups in the AVPV (data not shown).

ISH for KiSS1 in the AVPV and ARC

Representative images of KiSS1-positive cells in the AVPV and ARC are presented in Figures 3 and 4, respectively. Clear blue-violet-positive reactions for KiSS1 mRNA were detected around the third ventricle (3V). The number of KiSS1-positive cells in the AVPV (mean ± SD) were 164.0 ± 35.6, 90.4 ± 17.1, 116.8 ± 14.8, 123.8 ± 15.1, and 156.3 ± 11.0 in the control, PND 0, 5, 10, and 14 groups, respectively. In the PND0 group, visible reactions for KiSS1-positive cells were clearly diminished in number in the AVPV, and the total number of KiSS1-positive cells (half brain) in this group was significantly decreased compared to that of the control group ($P < 0.001$ vs. control group). Expression of KiSS1 mRNA was also diminished in PND5 and 10 groups; the total number of KiSS1-positive cells was also significantly decreased in these groups ($P = 0.009$ and $= 0.037$ vs. control group, respectively). In the PND14 group, no significant difference was found in total number of KiSS1-positive cells when compared to that of the control group. Similar to the results obtained from real-time PCR and long-term estrous cycle analysis, the decrease in the number of KiSS1-positive cells detected by ISH also was inversely correlated with age at exposure. The number of KiSS1-positive cells in the ARC (mean ± SD) were 208.6 ± 48.7, 156.8 ± 32.8, 159.6 ± 68.7, 224.6 ± 21.6, and 214.2 ± 58.0 in the control, PND 0, 5, 10, and 14 groups, respectively. In the ARC, slight decreases in the number of KiSS1-positive cells were observed in the PND 0 and 5 groups, but no statistical significance was detected in these groups ($P = 0.30$ and $= 0.34$ vs. control group, respectively).

Serum Concentration of LH and FSH

Serum concentrations of LH and FSH at the peak time of the LH surge are shown in Figure 5. All the blood samples were collected by decapitation during the interval 1600–1700, corresponding with the peak time of the LH surge under our laboratory conditions [11]. Serum LH concentrations (mean ± SEM) were 11.64 ± 3.87, 5.56 ± 2.56, 6.73 ± 2.07, 2.70 ± 0.95, and 10.32 ± 2.51 ng/ml in the control, PND 0, 5, 10, and 14 groups, respectively. The peak concentration of LH showed a decreasing trend in the PND 0, 5, and 10 groups, however, statistical significances were not detected ($P = 0.30$, $= 0.48$, and $= 0.07$ vs. control group, respectively). On the other hand, the peak LH concentration in the PND14 group was

comparable to that of the control group ($P = 0.99$ vs. control group). FSH concentrations (mean ± SEM) were 19.25 ± 2.40, 13.36 ± 2.35, 20.44 ± 1.94, 14.23 ± 3.48, and 28.80 ± 1.68 ng/ml in the control, PND 0, 5, 10, and 14 groups, respectively. Concentration of FSH in the PND14 group showed an increasing trend ($P = 0.06$ vs. control group), but statistically significant changes were not observed including other experimental groups.

Pathology of the Female Reproductive Tract at 40 wk of Age

The weights of uteri and ovaries at 40 wk of age are presented in Table 2, and representative histologies of ovaries at the same ages are presented in Figure 6. Both the absolute and relative weights of ovaries at 40 wk of age were significantly decreased in the PND 0, 5, and 10 groups compared to those of the control group ($P = 0.012$, < 0.001, and $= 0.005$ in the absolute weight and $= 0.020$, $= 0.007$, and $= 0.018$ in the relative weight vs. control group, respectively). Corresponding to the decrease in ovarian weight, most ovaries in these groups presented moderate to severe atrophy, increased number of follicular cyst, and decreased number of corpora lutea (CL) in the ovary. In contrast, most animals in the control and PND14 groups had large, solid CL and small- to medium-sized antral follicles, meaning that a number of animals maintained estrous cycles until this age in these groups. In other organs examined, there was no significant histopathological finding.

Histopathological and Immunohistochemical Investigation of VO

Representative images of ovarian histology and vaginal IHC in the control group (without acceleration of VO) and PND14 group (with acceleration of VO) as well as the scores of ERα expression are presented in Figure 7. The animals were necropsied on PND24–PND27 (the day after the day of VO) in the PND10 and PND14 groups and on PND25 or PND27 (before VO; two rats on each day) in the control and PND0 and PND5 groups. In the PND10 and PND14 groups, formation of new CL (evidence for the first ovulation) was not found in the ovary, and no other histological changes were detected in the ovaries, uteri, and vaginas. For further investigation, we performed IHC for ERα in vaginas. The scores of ERα expression in the vaginal epithelium (mean ± SD) were 3.88 ± 0.10, 3.90 ± 0.14, 3.93 ± 0.10, 3.93 ± 0.10, and 3.90 ± 0.12 and in the stroma were 2.78 ± 0.30, 2.80 ± 0.18, 2.78 ± 0.10, 2.53 ± 0.26, and 2.70 ± 0.16 in the control, PND 0, 5, 10, and 14 groups, respectively. No statistical significance was detected and the scores of ERα expression were comparable in all groups regardless of VO status.

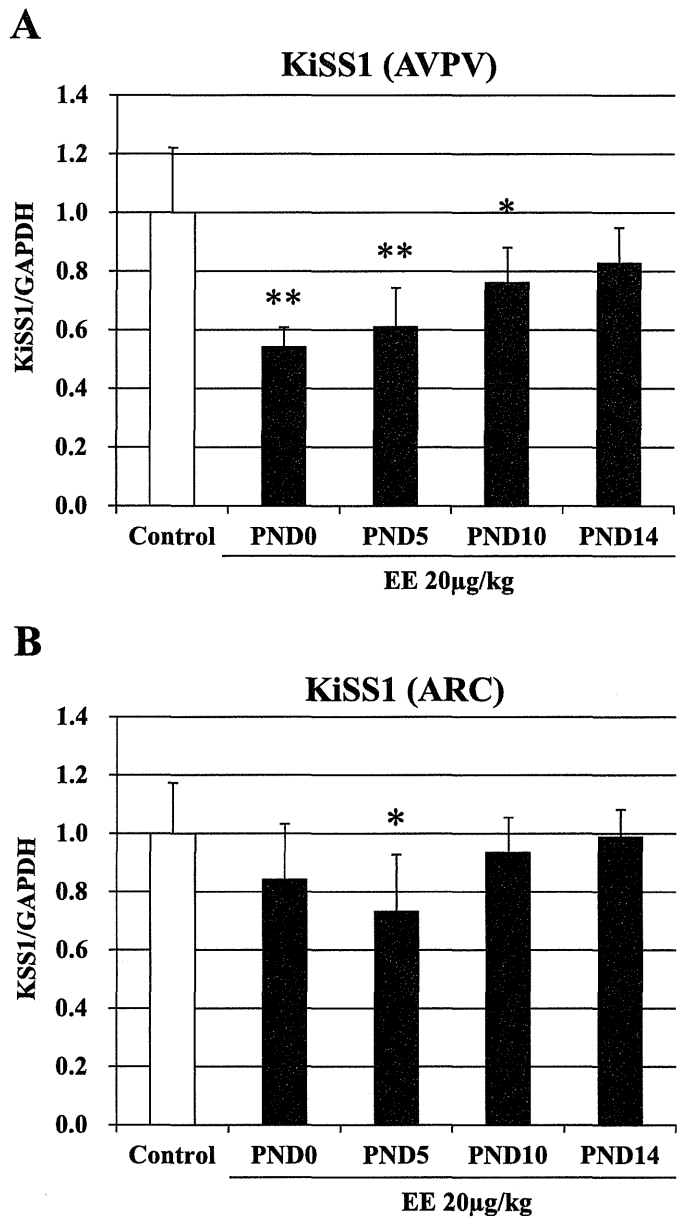

FIG. 2. KiSS1 mRNA expression in the AVPV (**A**) and ARC (**B**) at the time of LH surge. All data are presented as mean ± SD (n = 5/group). Expression levels in each group are normalized to GAPDH levels, and the level of the control group is adjusted to 1.0. Symbols indicate significant differences from the control group (*$P < 0.05$ and **$P < 0.01$ by Dunnett test).

DISCUSSION

In the present study, postnatal exposure to EE at 20 μg/kg at PND 0, 5, and 10 decreased KiSS1mRNA expression and the number of KiSS1-positive cells at 11 wk of age, indicating that the critical window of sensitivity to EE for the development of delayed effects is maintained up to PND10 in rats. Hypothalamic kisspeptin neurons are now widely recognized as central regulators of various female reproductive functions, and the AVPV is known to play critical roles in the regulation of estrous cycling [14, 28]. Therefore, the down-regulation of KiSS1 mRNA in the AVPV in the present study means that there is a reduction in neurotransmission in the HPG axis at the time of the LH surge, suggesting attenuated reactivity of kisspeptin neurons to exogenous estrogenic treatment, such as LH surge priming. In fact, in association with the decrease in KiSS1 mRNA expression, an attenuating trend of the LH surge was also observed in these groups, indicating that diminished expression of KiSS1 mRNA leads to the reduction in LH release in the pituitary. Although the results of LH concentration lack the statistical support due to the small sample size in the present study, similar results were confirmed in a previous study using lager number of animals and time points [11]. These serial alterations in KiSS1 expression and LH surge in the PND 0, 5, and 10 groups indicate that EE exposure at these ages could influence the development of kisspeptin neurons, consequently causing delayed effects at later ages. Consistent with the groups with diminished KiSS1 mRNA expression and LH surges, the PND 0, 5, and 10 groups showed early onset of

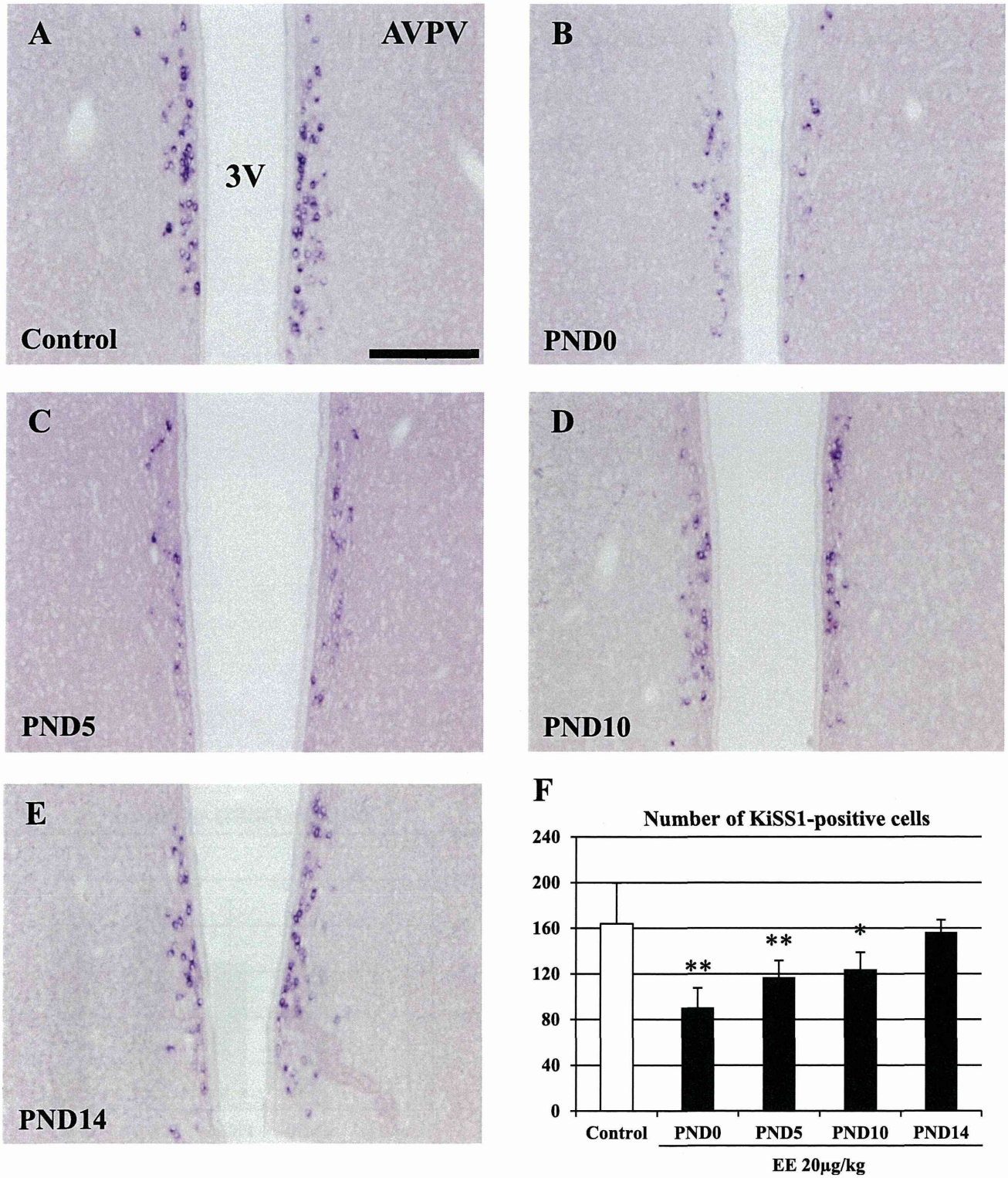

FIG. 3. Representative images of KiSS1 mRNA-positive cells in the AVPV of control (**A**), PND0 (**B**), PND5 (**C**), PND10 (**D**), and PND14 (**E**) groups, and total number of positive cells (**F**). All the brain samples were collected during the interval 1600–1700. All the data are presented as mean ± SD (n = 4–5/group). 3V: third ventricle. Bar = 200 μm. Symbols indicate significant differences from the control group (*$P < 0.05$ and **$P < 0.01$ by Dunnett test).

age-matched abnormal estrous cycling, which was reported to be a sensitive and established indicator of delayed effects [8]. Atrophic ovary accompanying increased follicular cyst formation and decreased CL in these groups are considered to reflect long-term cessation of the estrous cycle. It is notable that all of these alterations were observed in the same PND 0, 5, and 10 groups, once again suggesting that the developing hypothalamus maintains sensitivity to exogenous estrogen exposure until late postnatal ages.

Another important finding in the present study is an age-dependent decrease in sensitivity to postnatal estrogen exposure. In the PND0 groups, early onset of abnormal estrous cycling started at 17 wk of age, and this result is mostly consistent with our previous study [8]. The PND 5 and 10

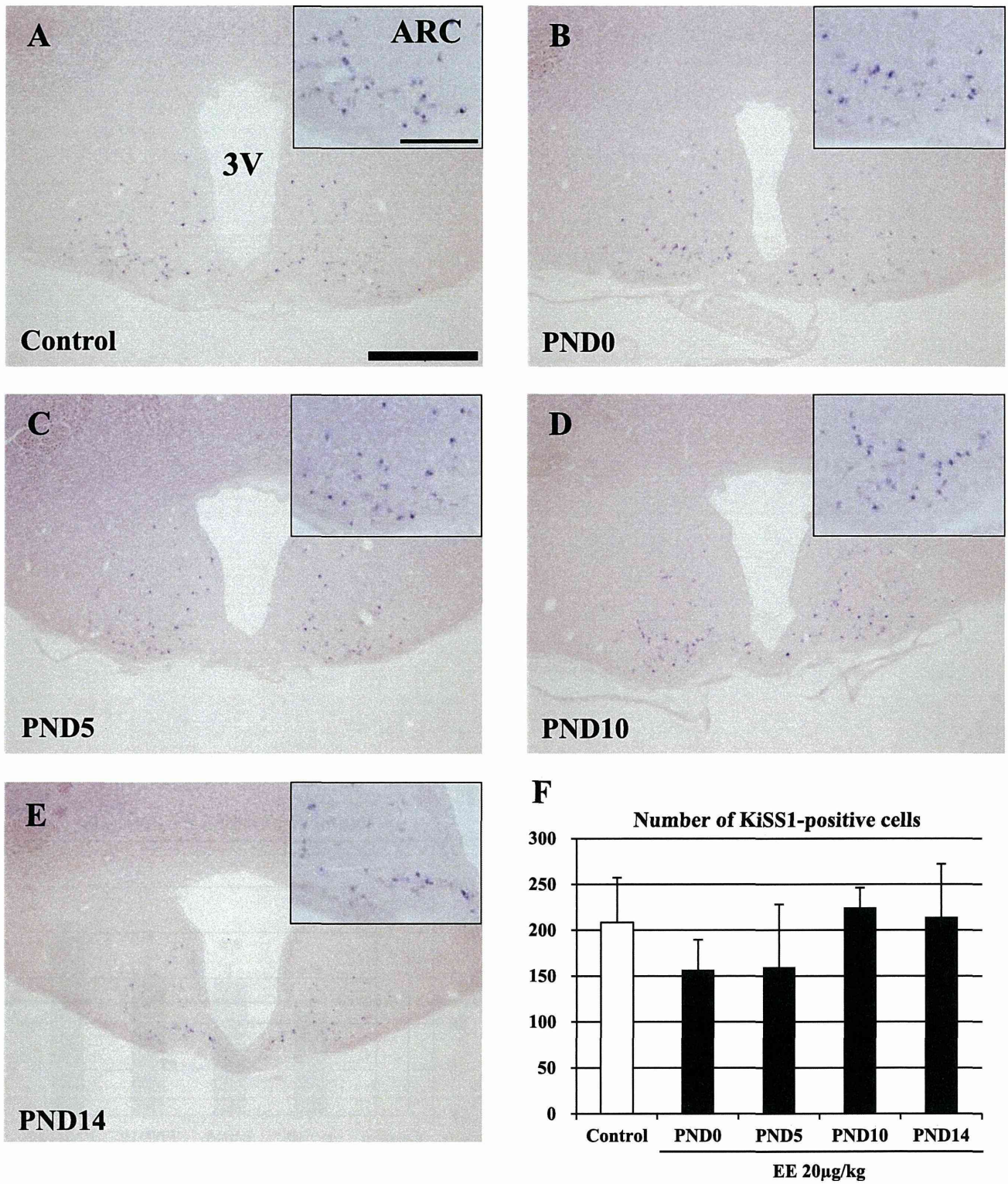

FIG. 4. Representative images of KiSS1 mRNA-positive cells in the ARC of the control (**A**), PND0 (**B**), PND5 (**C**), PND10 (**D**), and PND14 (**E**) groups, and total number of positive cells (**F**). The insets in **A**–**E** represent a higher magnification. All the brain samples were collected during the interval 1600–1700. All the data are presented as mean ± SD (n = 5/group). 3V: third ventricle. Bars = 500 μm (low magnification) or 200 μm (high magnification).

groups also showed early onset of abnormal estrous cycling starting at 19 wk of age, but the timing and pace of decline was clearly delayed compared to that of the PND0 group. In addition, diminishment of KiSS1 expression in the AVPV was also inversely correlated with age at exposure in both the real-time PCR and ISH analyses. These results clearly indicate that sensitivity of the neonatal brain to postnatal estrogen exposure could change with developmental stage and that the neonatal brain is more vulnerable at earlier developmental stage. On the other hand, a similar pattern of decline in estrous cycle in the PND 5 and 10 groups suggests that the sensitivity may not differ so much between these ages. Considering the slight

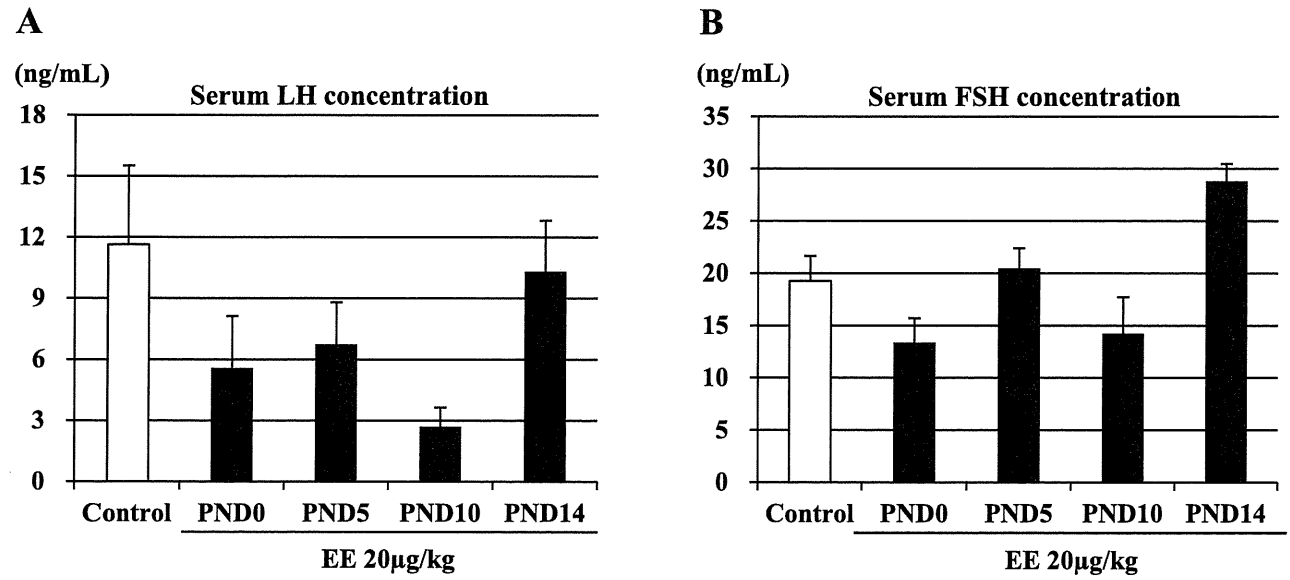

FIG. 5. Serum concentration of LH (**A**) and FSH (**B**) at the time of LH surge. All the data are presented as mean ± SEM (n = 5/group).

earlier cessation of estrous cycling and slight decrease in KiSS1 expression in the PND14 group, it could be said that the critical window for delayed effects closes gradually along with progression of brain development during late postnatal ages.

Delayed effects are a growing concern because they might be overlooked in existing reproductive or developmental toxicity studies due to the limited observation period of these studies [8]. Therefore, it is toxicologically important to elucidate the mechanism of delayed effects, including identification of the critical window of sensitivity for the delayed effects. The perinatal period is well-known to be a crucial hormone-sensitive period during which the hypothalamus undergoes sexual differentiation [29], and it has been generally estimated to be consistent with approximately late embryonic to early postnatal periods in rats [1, 22]. In detail, the critical period for CNS sexual differentiation has been classically considered to be 18–27 days after conception in rats [24, 25]. Therefore, if the gestation period is 21 days, Embryonic Day 18 to PND5 would be assumed to be the critical period of CNS sexual differentiation in rats. However, the results obtained from the present study indicate that neonatal brains maintain sensitivity to estrogens at least until PND10 and are still vulnerable to exogenous estrogens during late postnatal ages in rats. This period corresponds approximately to the third trimester of human gestation, when the hypothalamus is still undergoing sexual differentiation in humans [30, 31]. Maternal exposure to DES in human is widely known to cause serious adverse reproductive outcome such as increased risk of vaginal clear cell adenocarcinomas and various structural abnormalities of the genital tract in young women who are the so-called DES daughters [32, 33]. In many epidemiological researches of DES based on clinical data, Swan [33] reported that vaginal adenosis, a noncancerous abnormalities often seen in DES-exposed women, was seen in 85% of those who had been exposed to DES by 14 wk gestation but in none of those first exposed after 22 wk (second trimester) of gestation. These results indicates the higher sensitivity of embryo/fetus to exogenous estrogens in the earlier period of gestation; however, our results in the present study also provides new insight about the potential risk of later-exposed estrogens to induce invisible influences such as development impairment of kisspeptin neuron. Though it is difficult to directly extrapolate the result in rats to human, at least our results may suggest that developing brains in human also have the possibility of maintaining sensitivity to estrogens longer than the expected period.

Regarding the endpoint of the critical window, very few reports have directly investigated and discussed the precise endpoint. Cruz and colleagues [34] reported that single exposure to 10 mg/kg EV in rats at PND 1, 7, or 14 caused permanent cessation of the estrus cycle and morphological changes in the ovary with multiple cystic structures, but exposure at PND21 and PND30 caused only a transient cessation, suggesting that the hormone-sensitive period in postnatal rats spanned the neonatal-infantile period. Regarding sensitivity at PND14, another publication from the same group reported that a single

TABLE 2. Mean weights of uterus and ovary in rats necropsied at 40 wk of age.[a]

Groups	Control	EE 20 μg/kg			
		PND0	PND5	PND10	PND14
Uterus					
Absolute (g)	0.91 ± 0.23	0.95 ± 0.35	1.00 ± 0.19	1.12 ± 0.38	0.94 ± 0.22
Relative (g/100 g BW)	0.28 ± 0.09	0.29 ± 0.10	0.32 ± 0.06	0.36 ± 0.16	0.28 ± 0.08
Ovary					
Absolute (g)	0.080 ± 0.018	0.062 ± 0.014*	0.057 ± 0.012**	0.060 ± 0.020**	0.074 ± 0.021
Relative (g/100 g BW)	0.024 ± 0.005	0.019 ± 0.004*	0.018 ± 0.004**	0.019 ± 0.007*	0.022 ± 0.007

[a]Values are presented as mean ± SD. BW, body weight.
*/**Significantly different from the control group (*$P < 0.05$ and **$P < 0.01$ by Dunnett test).

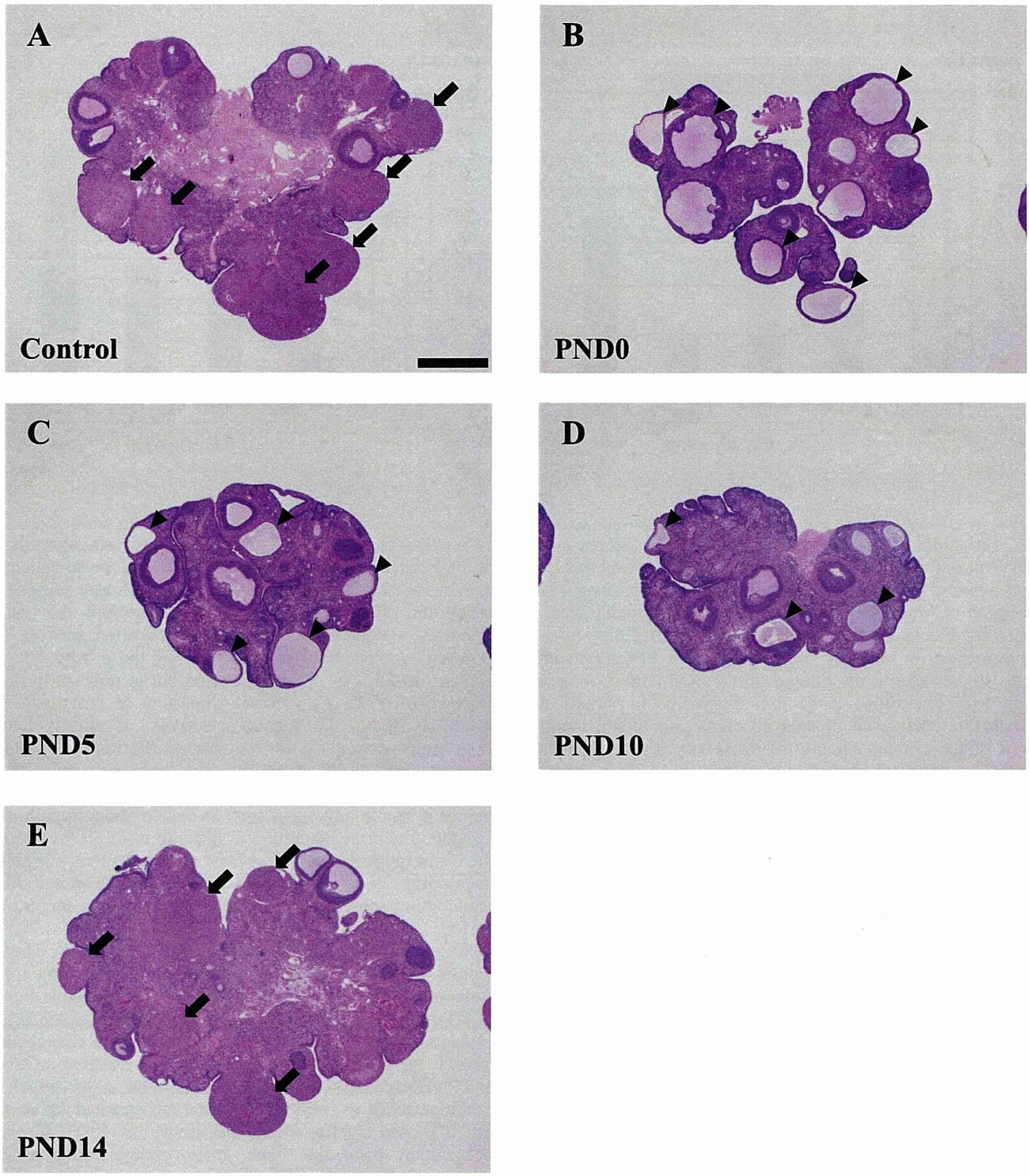

FIG. 6. Ovarian histology of the control (**A**), PND0 (**B**), PND5 (**C**), PND10 (**D**), and PND14 (**E**) groups at 40 wk of age. Graphics indicate corpora lutea (CL) (arrows) or follicular cysts (arrowheads). HE stain. Bar = 1 mm.

intramuscular injection of 2 mg EV at PND14 also induced decreased cycle frequency at PND70–PND90 in rats [23]. These previous findings indicate that the critical window in postnatal rats is still open at PND14; however, alteration of the estrous cycle and KiSS1 expression in the PND14 group was not apparent in the present study. Considering the extremely high dose of EV utilized in these studies, manifestation of the effects caused by the exposure at PND14 may require a much higher dose, and other experimental factors, such as chemicals, strains of rats, and timing of analysis, may also affect the results of the investigation. Conversely, the seemingly contradictory results for PND14 may indicate that sensitivity to estrogen exposure at PND14 is very weak but still maintained, and also support the hypothesis that the critical window for delayed effects closes gradually during the late postnatal ages with PND14 being a provisional endpoint for the window.

In the present study, we also found that postnatal exposure to EE at PND10 and PND14 could significantly accelerate VO.

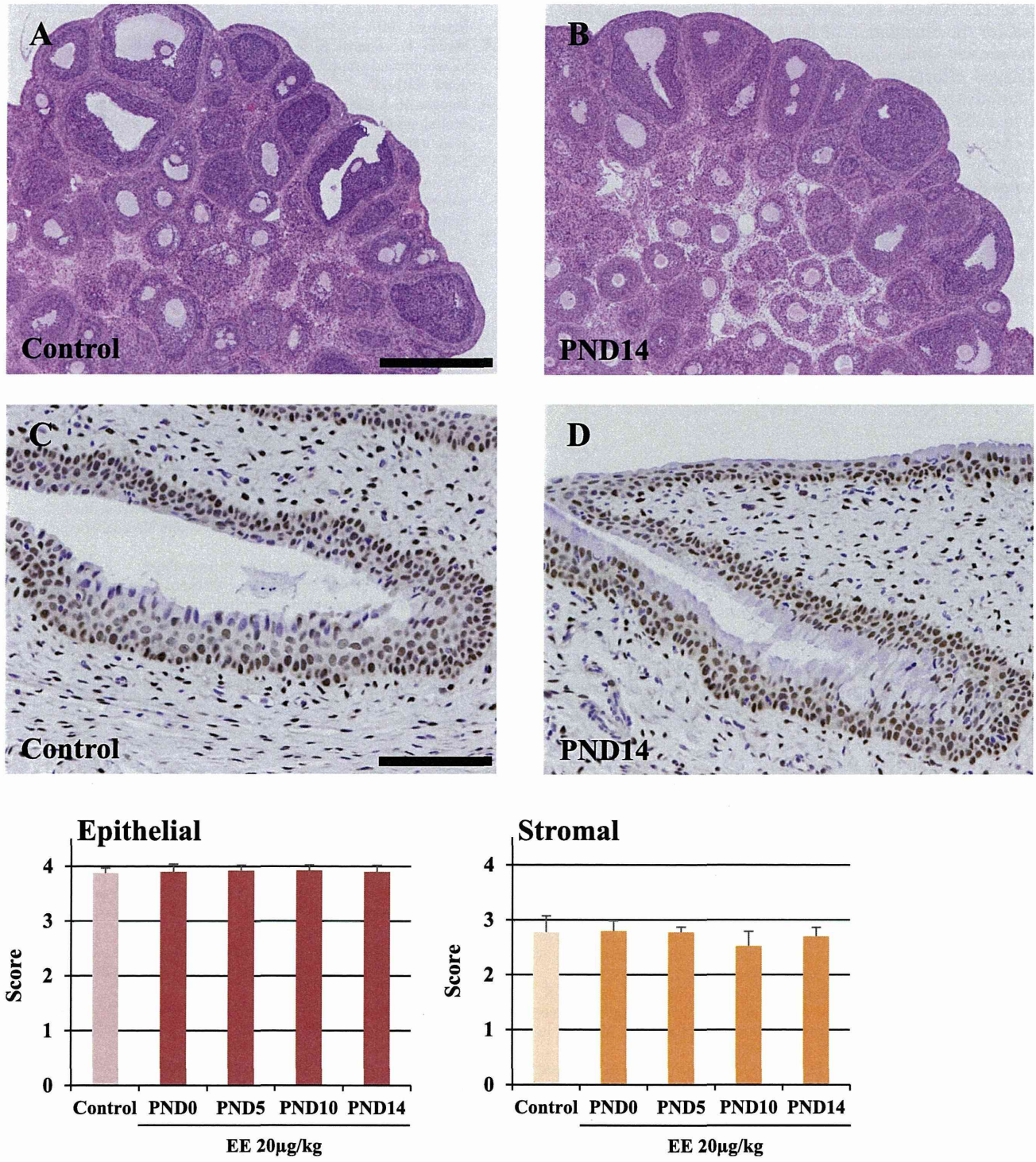

FIG. 7. Ovarian histology and vaginal IHC for ERα in the control group before VO (**A** and **C**) and PND14 group after VO (**B** and **D**). Animals in PND14 group were necropsied on the next day of the day of accelerated VO. Lower graphs show the scores of ERα expression in the epithelial and stromal cells in each group. Score: 0, negative; 1, slightly positive (<20%); 2, partly positive (20%–50%); 3, positive more than half (50%–80%); 4, mostly positive (>80%). HE stain (**A** and **B**). Bars = 500 μm (**A** and **B**) or 100 μm (**C** and **D**).

Early VO in response to perinatal exposure to higher levels of estrogenic compounds has been described in several articles [23, 28, 35, 36]. In these reports, various hypotheses of the mechanisms for the acceleration of VO are discussed, but the precise mechanism is still undetermined [36]. Lack of first ovulation on the day following premature VO in the present study suggests that the acceleration was not caused by the effect of postnatally exposed EE on the HPG axis, but instead was caused by the direct action of EE on the vagina. Enhancement of the sensitivity of the vagina to endogenous estrogen, which is responsible for natural VO, might be plausible, but the mechanism remains undetermined due to lack of supportive data on ERα expression in the vagina in the present study. Considering that acceleration of VO was observed only in the PND10 and PND14 groups, the late postnatal period might be more sensitive to the enhancing

effect of EE than the neonatal period. Further investigation is needed for the elucidation of the true mechanism.

The present study clarifies the critical window of sensitivity for delayed effects induced by a single postnatal exposure to EE. Diminished KiSS1 expression, decreased number of KiSS-positive cells in the AVPV, and an attenuating trend of LH surge were observed at 11 wk of age in animals postnatally exposed to 20 μg/kg of EE at PND 0, 5, and 10. A corresponding early onset of abnormal estrous cycling was also observed in the same groups, but these serial changes were not evident in the animals exposed to EE at PND14. These results indicate that PND0–PND10 is the critical window of susceptibility for delayed effects in rat neonates, and PND14 is presumed to be a provisional endpoint of this window.

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Prior attenuation of KiSS1/GPR54 signaling in the anteroventral periventricular nucleus is a trigger for the delayed effect induced by neonatal exposure to 17alpha-ethynylestradiol in female rats

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ABSTRACT

Neonatal exposure to 17alpha-ethynylestradiol (EE) causes delayed effect, a late-occurring irreversible damage to reproductive functions characterized by the early onset of age-matched abnormal estrous cycling. To clarify the involvement of a hypothalamic key cycling regulator KiSS1/GPR54 in the delayed effect, we investigated artificially induced LH surges and KiSS1 mRNA expression in the anteroventral periventricular nucleus (AVPV) of cycling young adult rats neonatally exposed to EE, and compared these parameters to those in about 5 months old middle-aged rats. KiSS1 mRNA expression, the number of KiSS1-positive cells and KiSS1/ERα co-expressing cells in the AVPV decreased in both EE-exposed and middle-aged rats. The peak area and levels of LH surge dose-dependently decreased in EE-exposed rats, and reduction was more evident in middle-aged rats. These results indicate that the prior attenuation of KiSS1 and consequent depression of LH surges plays a key role in the onset of abnormal estrous cycling in the delayed effect.

1. Introduction

Exposure to the endocrine disrupting chemicals (EDCs) contained in man-made chemicals such as pesticides, plasticizers and drug medicines, has been a major concern for human health because EDCs possess exogenous estrogenic activity and interfere with normal physiological systems and hormone balance, potentially induce various adverse effects on hormones regulating reproductive functions in human being and wildlife [1,2]. It is widely known that prenatal and neonatal exposure to estrogens can cause irreversible, complex damage to the hypothalamus–pituitary–gonadal (HPG) axis and disrupt the programming of endocrine signaling pathways during early developmental stages in mammals [3,4]. For example, neonatal exposure to large amounts of various estrogenic compounds during critical periods of development have a serious influence on the sex differentiation of the brain, causing various irreversible abnormalities, such as masculinized sexual behavior, lower gonadotropin levels in puberty, malformation of the reproductive tract, and cessation of cyclic ovulation [5–7]. These effects are known as "defeminization" or "masculinization" of female sexual behavior and reproductive functions and these abnormalities are usually manifested during the pre- or peri-pubertal periods.

On the other hand, shorter and lower-dose neonatal exposure to estrogenic compounds like EDCs also causes multiple adverse

Abbreviations: AVPV, anteroventral periventricular nucleus; ARC, arcuate nucleus; EB, estradiol benzoate; EDCs, endocrine disrupting chemicals; EE, 17alpha-ethynylestradiol; FSH, follicle-stimulating hormone; HPG, hypothalamus–pituitary–gonadal; IHC, immunohistochemistry; ISH, in situ hybridization; LH, lutenizing hormone; OVX, ovariectomy; PND, postnatal day; P4, progesterone; 3V, third ventricle.

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effects on female reproductive functions, but the timing of appearance and type of effects are reported to be different from that observed in "musculinization". For instance, neonatal or perinatal exposure to chemicals such as *p*-t-octylphenol, methoxychlor or a synthetic estrogen, diethylstilbestrol, are reported to induce early onset of abnormal estrous cycle, advanced reproductive senescence and increased uterine carcinogenic risk in young adult or aging rat [8–10]. In these animals, reproductive tracts are known to normally develop and normal estrous cycles were also observed after the vaginal opening; however, the early onset of age-matched abnormal estrous cycle become apparent in young adult age, and increased carcinogenic risk due to the chronic increase of estrogen/progesterone ratio were observed later in life [9–11]. These late-occurring adverse effects are recognized as "delayed effects" or "delayed reproductive dysfunction" in the previous reports [10–13], and could be defined as various reproductive dysfunctions having the following features; 1) be induced by the perinatal exposure to relatively low-dose of estrogenic compounds, and 2) manifest after the peripubertal period in association with the early cessation of estrous cycle. Disruption of the hypothalamic cycle regulating center in early developmental stages has been considered as one of the major mechanism of the delayed effect; however, the precise mechanism underlying the delayed effect remains unknown [10,11]. Moreover, the potential of compounds like EDCs to induce the delayed effect might be overlooked in short-term bioassay and existing developmental toxicity studies, because the delayed effect usually becomes apparent only after the general observation period of most authorized reproductive toxicity studies. Therefore, investigation and elucidation of the mechanism and exploration of the early indicator of delayed effect are toxicologically important for the risk assessment of these estrogenic compounds.

Kisspeptin, the endogenous ligand of the G-protein-coupled receptor GPR54 (KiSS1R) previously called metastin [14,15], is now recognized as playing critical roles in various female reproductive functions and in the regulation of estrous cycling [16,17]. Kisspeptin is a neuropeptide encoded by the KiSS1 gene and regulates GnRH/LH secretion in the HPG axis [17,18]. In the rat brain, the population of Kisspeptin neurons is located mainly in two specific areas of the hypothalamus: the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC). Interestingly, each area is considered to play a site-specific role: the AVPV triggers the surge in secretion of GnRH, leading to ovulation in the normal estrous cycle, and the ARC induces GnRH pulsatile secretion, which is responsible for follicular development [17–19]. A number of recent studies focused on kisspeptin-GPR54 have revealed that this neurotransmission pathway is the target of estrogen feedback systems in the HPG axis, i.e., activation of kisspeptin neurons is regulated by ovarian estrogens and receives positive feedback in the AVPV or negative feedback in the ARC via estrogen receptors expressed on kisspeptin neurons themselves [20–22]. Regarding cyclic ovulation control, activated kisspeptin neurons secrete kisspeptin in the AVPV and consequently activate GnRH secretion, which induces the transient secretion of large amounts of LH from the pituitary (LH surge) and leads to cyclic ovulation in the normal estrous cycle [23–25]. Since these reproductive functions are unique to female animals, the number of kisspeptin neurons in the AVPV is known to be markedly sexually dimorphic [26,27].

Previously, we reported that neonatal exposure to 17alpha-ethynylestradiol (EE) causes an age- and dose-dependent delayed effect, characterized by an increased incidence of early onset of abnormal cycling from 10 to 22 weeks of age [11]. This abnormal estrous cycling is mainly persistent estrus, which is similar to the age-related persistent estrus observed in the middle-aged female rat. Since no abnormalities in ovarian follicles were detected in these animals, disruption of the HPG axis, especially of the luteinizing hormone (LH) surge and hypothalamic KiSS1/GPR54 signaling are predicted. In fact, there are a few reports that show a significant decrease in KiSS1 mRNA expression and kisspeptin-immunoreactivity in the hypothalamic kisspeptin neurons in middle-aged rats [28,29] and in animals neonatally exposed to various estrogenic compounds [30–32]. However, the selected dose in these reports are extremely high, and sufficient to cause the "masculinization" of the female brain; therefore, the contribution of kisspeptin to the occurrence of delayed effects induced by low-dose neonatal exposure to estrogenic compounds remains unclear.

In the present study, we investigate the artificial LH surge and KiSS1 mRNA expression in the anterior/posterior hypothalamus of neonatal EE-exposed and middle-aged ovariectomy (OVX) rats to elucidate the functional changes of kisspeptin neuron in the delayed effect and in middle-aged rats, and to explore the potential of changes in kisspeptin neurons as early toxicological indicators of the delayed effect.

2. Materials and methods

2.1. Animals

Pregnant Crj:Donryu rats maintained in house were prepared for Experiment 1 ($n=40$) and Experiment 2 ($n=28$). This strain is known to show regular 4-days cycle after puberty [8]. The rats were housed individually in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature, 24 ± 1 °C; relative humidity, 55 ± 5%; 12-h light/dark cycle: light on, 5:00–17:00; light off, 17:00–5:00) with a basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and tap water available ad libitum. CRF-1 is a standard diet that includes soy protein and is known to contain a relatively low level of estrogens. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences (Japan).

2.2. Chemicals

EE (CAS No. 57-63-6) with purity >98% was purchased from Sigma (St. Louis, MO, USA). EE was stirred in a small amount of sesame oil overnight, then used after dilution. EE was selected because of its rapid excretion and lower binding affinity for α-fetoprotein in neonatal blood, so as to limit the exposure time to the neonatal period [33].

2.3. Experiment 1

Dams were assigned to 5 groups: control, EE 0.02 μg/kg (EE0.02), EE 0.2 μg/kg (EE0.2), EE 20 μg/kg (EE20), and middle-age (6–10 dams/group). The pups of the control and EE groups received a single subcutaneous injection of sesame oil (control) or EE (0.02, 0.2, 20 μg/kg of BW) within 24 h after birth. EE 0.02 μg/kg was selected as the non-inducing and EE 0.2 and 20 μg/kg were selected as the inducing doses of the delayed effect, with reference to a previous report [11]. Litters were culled randomly to preserve 10 pups with a female predominance on postnatal day (PND) 3. On PND 21, the offspring were weaned, and 31–40 female pups per group were housed 3–4 per cage and maintained until 10 weeks of age. Starting on PND 23, we checked the vaginal opening every day. After that, all animals were observed for estrous cycle by vaginal smear for 5 consecutive days every week throughout the experiment. A decision on the cycle pattern was made with every 5-day observation. Regular 4- or 5-day cycles were deemed normal cycles, and other patterns were judged to be abnormal estrous cycles. In particular, animals showing proestrus or estrus continuously for 5 days were designated as having persistent estrus. Observations of clinical signs, body weight, and mortality were made throughout the experimental period. At 10 weeks of age, each rat confirmed as