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—Original Article—

Central estrogen action sites involved in prepubertal restraint of pulsatile luteinizing hormone release in female rats

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Abstract. The present study aimed to determine estrogen feedback action sites to mediate prepubertal restraint of gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) release in female rats. Wistar-Imamichi strain rats were ovariectomized (OVX) and received a local estradiol-17β (estradiol) or cholesterol microimplant in several brain areas, such as the medial preoptic area (mPOA), paraventricular nucleus, ventromedial nucleus and arcuate nucleus (ARC), at 20 or 35 days of age. Six days after receiving the estradiol microimplant, animals were bled to detect LH pulses at 26 or 41 days of age, representing the pre- or postpubertal period, respectively. Estradiol microimplants in the mPOA or ARC, but not in other brain regions, suppressed LH pulses in prepubertal OVX rats. Apparent LH pulses were found in the postpubertal period in all animals bearing estradiol or cholesterol implants. It is unlikely that pubertal changes in responsiveness to estrogen are due to a change in estrogen receptor (ER) expression, because the number of ERα-immunoreactive cells and mRNA levels of *Esr1*, *Esr2* and *Gpr30* in the mPOA and ARC were comparable between the pre- and postpubertal periods. In addition, kisspeptin or GnRH injection overrode estradiol-dependent prepubertal LH suppression, suggesting that estrogen inhibits the kisspeptin-GnRH cascade during the prepubertal period. Thus, estrogen-responsive neurons located in the mPOA and ARC may play key roles in estrogen-dependent prepubertal restraint of GnRH/LH secretion in female rats.

Key words: Arcuate nucleus, Estrogen feedback action, GnRH, Medial preoptic area, Puberty

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In mammals, sexual maturation at puberty onset seems to be timed by an increase in pulsatile gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) secretion [1–5]. Previous studies showed that prepubertal restraint of GnRH/LH secretion seems to be controlled by gonad-dependent and gonad-independent mechanisms according to the species: plasma LH levels increased immediately after ovariectomy in prepubertal sheep [3] and rats [6], whereas plasma LH levels remained low even after ovariectomy in prepubertal rhesus monkeys [7, 8]. The precise mechanisms regulating the prepubertal restraint of and pubertal increase in GnRH/LH secretion remain very elusive. To date, the most plausible explanation has been that kisspeptin-GPR54 signaling controls the pubertal increase in GnRH/LH release, because loss-of-function mutations of *KISS1* or *GPR54* are responsible for pubertal failure in patients with hypogonadotropic hypogonadism [9–11]. These phenotypes were duplicated in *Kiss1* or *Gpr54* knockout mice [10, 12–16]. Indeed, *Kiss1* gene expression in the anteroventral periventricular nucleus and hypothalamic arcuate nucleus (ARC) in rodents is nearly absent in the prepubertal period and increases at the onset of puberty [17–19]. Since ARC *Kiss1* mRNA expression is strongly suppressed by estradiol-17β (estradiol) derived from immature ovaries in prepubertal female rats [19], ARC kisspeptin neurons are assumed to be a target of estrogen negative feedback action, which restrains GnRH/LH secretion in rodents during the prepubertal period.

The estrogen receptor α (ERα) seems to play a critical role in estrogen feedback action, because ERα knockout mice showed constant high plasma LH levels regardless of estrogen treatment, indicating a lack of estrogen-negative and estrogen-positive feedback actions [20]. Shivers *et al*. showed that tritiated estradiol did not concentrate in GnRH neurons in adult rats [21]. In addition, immunohistochemistry for ERα and *in situ* hybridization analysis for *Esr1* mRNA encoding ERα revealed the absence of ERα expression in rodent GnRH neurons [22, 23]. Thus, non-GnRH neurons expressing ERα, such as kisspeptin neurons [24–26], may play a critical role in prepubertal restraint of GnRH/LH secretion. In the rodent brain, ERα or *Esr1* mRNA expression is abundantly found in discrete areas of the hypothalamus, such as the medial preoptic area (mPOA), ventromedial nucleus (VMH) and ARC. In addition, *de novo* ERα expression in the paraventricular nucleus (PVN) and nucleus of the solitary tract was reported to be involved in fasting-induced LH suppression in rats [27, 28]. Nevertheless, the action site(s) of estrogen that suppressed GnRH/LH secretion during the prepubertal period remains poorly understood. Determination of the precise action site(s) of estrogen should provide clues for identifying target neurons involved in the estrogen-dependent restraint of GnRH/LH

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release in prepubertal females.

The present study aimed to determine which central action site(s) of estrogen is involved in the prepubertal restraint of GnRH/LH release and to clarify the pubertal changes in response to estrogen action in female rats. To this end, we examined if placement of estradiol microimplants into discrete brain areas, such as the mPOA, PVN, VMH and ARC, causes suppression of pulsatile LH secretion in OVX prepubertal and postpubertal female rats. We also determined the expression of ERs in the mPOA and ARC, because we found that estradiol microimplants in these two nuclei inhibited pulsatile LH release in prepubertal OVX animals.

Materials and Methods

Animals

Wistar-Imamichi strain rats were kept under a 14:10 h light/dark cycle (lights on at 0500 h) at 22 ± 2 C with free access to food (CE-2; CLEA Japan, Tokyo, Japan) and water. Female rats (8–10 weeks old of age) having at least two consecutive regular 4-day estrus cycles were mated with males overnight on the day of proestrus, and then the pregnant females were housed individually. The day on which a newborn litter was found at noon was designated postnatal day 0. The litter size was adjusted to eight on day 1 to minimize the growth variation within and between litters. The pups were weaned on day 20. Vaginal openings were checked every morning thereafter. After vaginal opening, vaginal smears were taken daily to determine the day of first estrus and estrous cyclicity. Since a preliminary study in female rats showed vaginal opening and first estrus at 29.6 ± 0.3 and 34.6 ± 0.9 days of age, respectively, days 26 and 41 were designated representative days for the pre- and postpubertal periods. Surgical procedures were performed under anesthesia with a ketamine-xylazine mixture.

Care of the animals and all of the experimental procedures used in these experiments were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University (Permit No. 2012031616).

Intracranial or subcutaneous estradiol implant

Rats were ovariectomized (OVX), and some of them received unilateral estradiol (Sigma-Aldrich, St. Louis, MO, USA) or cholesterol microimplants in the various hypothalamic regions in the pre- (day 20 of age) or postpubertal period (day 35 of age). The microimplants were made of a 1:1000 (w/w) mixture of estradiol and paraffin. Cholesterol was used instead of estradiol in controls. The paraffin mixture (approximately 0.05 mm^3), theoretically containing 33 ng estradiol or cholesterol, was then punched out with a 24-gauge stainless steel tubing (0.30 mm i.d.). The stainless steel tubing containing the paraffin mixture was then stereotaxically inserted in the discrete area of the hypothalamus. The paraffin mixture was immediately pushed out of the tubing and into the designated nucleus using a stainless steel wire (0.25 mm diameter). The stereotaxic coordinates (mm posterior and ventral to the bregma, and lateral to the midline) were as follows: mPOA, 0.8, 7.7, 0.4; PVN, 1.8, 7.4, 0.5; VMH, 2.8, 9.0, 0.8; and ARC, 2.8, 9.0, 0.5.

Some of the OVX animals were subcutaneously implanted with a Silastic tubing (1.57 mm i.d.; 3.18 mm o.d.; 8 mm in length for day 20 and 16 mm in length for day 35, Dow Corning, Midland, MI, USA) containing estradiol dissolved in peanut oil at 20 μg/ml to mimic the diestrous plasma estradiol level in ovary-intact female rats [19, 29].

Blood sampling

On days 26 or 41 of age, i.e., 6 days after the estradiol treatment in the pre- or postpubertal groups, blood samples (50 μl) were collected from the free-moving animals for 3 h at 6-min intervals, starting at 1300 h, through a silicone catheter (0.5 mm i.d., 1.0 mm o.d., Shin-Etsu Polymer, Tokyo, Japan) inserted into the right atrium through the jugular vein on the day before blood sampling. An equivalent volume of rat red blood cells, taken from donor rats and diluted with heparinized saline, was replaced through the cannula after each blood collection to keep the hematocrit constant. Plasma was separated by immediate centrifugation and stored at –20 C until assayed for LH.

Brain histology

After blood sampling, animals bearing the intracranial microimplants were perfused with saline followed by 10% formalin under deep anesthesia with sodium pentobarbital to confirm the placement of the microimplants. Coronal sections of the brain made with a cryostat were stained with thionin, and the sites of the microimplants were verified under a microscope according to a rat brain atlas [30]. Some animals bearing microimplants in an undesignated nucleus were eliminated from the further analysis.

Uteri collection to verify systemic leakage of estradiol

Uteri were collected immediately before perfusion and weighed. The wet weights of the uteri were not significantly different between intracranial estradiol- and cholesterol-implanted animals (data not shown), indicating no systemic leakage of estradiol from the intracranial microimplants.

Effect of intravenous kisspeptin or GnRH challenge on LH release in prepubertal rats

To test if the estrogen-dependent prepubertal suppression of LH release is due to the restraint of GnRH and/or kisspeptin release, subcutaneous estradiol-treated OVX rats at 26 days of age received four intravenous injections of the C-terminal decapeptide of rat kisspeptin (rKp-10, 10 nmol/kg body weight, n = 3) or GnRH (0.1 nmol/kg, n = 3) at 1, 1.5, 2 and 2.5 h after the onset of blood sampling. Blood samples (50 μl) were obtained every 6 min for 3 h (1300–1600 h).

LH assay

Plasma LH concentrations were determined by a double-antibody radioimmunoassay (RIA) using a rat LH RIA kit provided by the National Hormone and Peptide Program (Torrance, CA, USA) and were expressed in terms of the NIDDK rat LH-RP-3. The least detectable level was 7.8 pg/tube for 25 μl plasma samples. Intra- and inter-assay coefficients of variation were 8.0% and 13.8% at the level of 0.78 ng/ml, respectively.

Brain tissue sampling and immunohistochemistry

At 26 or 41 days of age, animals were deeply anesthetized with

pentobarbital and then perfused with phosphate-buffered saline followed by 4% paraformaldehyde. Serial frontal sections containing the entire mPOA or ARC (30 μm) were made on a cryostat, and every 8th section was immunostained with anti-ERα antibody (Upstate Biotechnology, Lake Placid, NY, USA) as previously described [31]. Briefly, brain tissue sections were incubated with the anti-ERα antibody (1:40,000) for 4 days at 4 C, followed by incubation in biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories, Burlingame, CA, USA) for 90 min and avidin biotin complex for 60 min. ERα immunoreactivities were visualized using 3,3'-diaminobenzidine as a chromogen.

Brain tissue sampling and quantitative RT-PCR for gene expression of Esr1 (ERα), Esr2 (ERβ) and Gpr30 (membrane estrogen receptor) in the mPOA and ARC

At 26 or 41 days of age, brain tissues were obtained from female rats after decapitation between 1300 and 1400 h. Since the present results suggested that the mPOA and ARC were estrogen action sites for prepubertal restraint of LH pulses, tissue samples containing the mPOA or ARC were obtained from 2-mm-thick brain tissue slices according to a rat brain atlas [30]. The anterior and posterior ends of tissue slices for the mPOA were approximately 0.48 mm anterior and 1.44 mm posterior to the bregma, respectively. The mPOA region was punched out with 18-gauge stainless steel tubing. The anterior and posterior ends of tissue slices for the ARC were approximately 1.80 mm and 4.20 mm posterior to the bregma, respectively. The ARC-median eminence (ARC-ME) region was dissected out with a microknife. Fifty-micrometer-thick coronal sections were made from the remaining brain tissues and stained with thionin to verify the brain regions dissected or punched out under a microscope according to a rat brain atlas [30].

Expression of *Esr1*, *Esr2* and *Gpr30* mRNA was determined by quantitative RT-PCR in the mPOA and ARC-ME regions. Real-time RT-PCR analysis was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Briefly, DNA-free total RNA was extracted from the tissues using ISOGEN (Nippon Gene, Tokyo, Japan) and DNase I (Invitrogen, Carlsbad, CA, USA). cDNA from each RNA sample was synthesized with a random primer using a High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. The following cycling protocol was used: 2 min at 50 C, 10 min at 95 C and 40 cycles amplification for 15 sec at 95 C and 1 min at 60 C. Predesigned primers and probes (TaqMan Gene Expression Assay, IDs: Rn00562166_m1 for *Esr1*, Rn00562610_m1 for *Esr2*, Rn005920911_s1 for *Gpr30* and Rn00667869_m1 for *Actb*) were purchased from Applied Biosystems.

Data analysis and statistical analysis

LH pulses were determined by the PULSAR computer program [32]. Each individual's mean LH concentrations and frequency and amplitude of LH pulses were calculated for the 3-h sampling period. Statistical differences in the LH pulse parameters and uterine weight were determined by a two-way (age and estradiol treatment as main effects) ANOVA (R version 3.1.1, http://www.R-project.org/). If the interaction was significant, simple main effects were determined.

Changes in the LH pulse frequency in each individual with an estradiol microimplant were calculated by subtracting the mean frequency of cholesterol-implanted controls from the frequency of each individual with an estradiol implant within each hypothalamic area.

The obvious ERα-immunoreactive cells in the mPOA and ARC and on their border were unilaterally counted twice by one of the authors under a microscope, and the average was calculated. The areas examined in the present study were identified based on a rat brain atlas [30]. The total numbers of sections used for quantification of ERα-immunoreactive cells were 4 and 8 for the mPOA and ARC, respectively.

The copy numbers of *Esr1*, *Esr2* and *Gpr30* transcripts were normalized to the ratio of the copy number of *Actb* transcripts for each sample. Statistical differences between age were determined by Student's *t*-test. Values were considered significant when $P < 0.05$.

Results

Placement of estradiol microimplants and their effects on LH pulse frequency in individuals

Figure 1 summarizes the placement of estradiol microimplants in the mPOA, ARC, PVN and VMH and changes in the number of LH pulses in each individual compared with the mean LH pulse frequency in the cholesterol-implanted controls. LH pulse frequency was decreased in all individuals with estradiol implants placed in the mPOA or ARC at 26 days of age compared with the cholesterol-implanted controls. On the other hand, no apparent change was found in animals with estradiol implant in the mPOA or ARC at 41 days of age or in those with estradiol implant in the PVN or VMH at 26 and 41 days of age. Cholesterol microimplants were placed in similar locations in each group (data not shown).

Effects of estradiol microimplants on pulsatile LH release in pre- and postpubertal OVX rats

Plasma LH profiles of representative OVX rats bearing estradiol or cholesterol implants are shown in Fig. 2. Regular LH pulses were found in rats with cholesterol microimplants in any of the hypothalamic regions at both 26 and 41 days of age. Estradiol microimplants into the mPOA or ARC caused partial suppression of pulsatile LH secretion at day 26. On the other hand, estradiol microimplants in the PVN or VMH had no apparent effect on LH secretion at 26 days of age. Pulsatile LH secretion was strongly suppressed at day 26 in OVX females with subcutaneous estradiol treatment. Regular LH pulses were found in OVX rats with estradiol microimplants in any of these hypothalamic regions or with subcutaneous treatment at 41 days of age.

Effects of estradiol microimplants on LH pulse parameters in pre- and postpubertal OVX rats

Three LH pulse parameters, i.e., mean LH concentration (Fig. 3A), frequency (Fig. 3B) and amplitude (Fig. 3C) of LH pulses, in pre- and postpubertal OVX rats treated with estradiol or cholesterol are summarized in Fig. 3.

The estradiol microimplants in the mPOA suppressed pulsatile LH secretion only in the prepubertal period. Specifically, two-way ANOVA revealed that estradiol microimplants into the mPOA (main effect) significantly suppressed LH pulse frequency ($F(1,17) = 11.9$, *, P

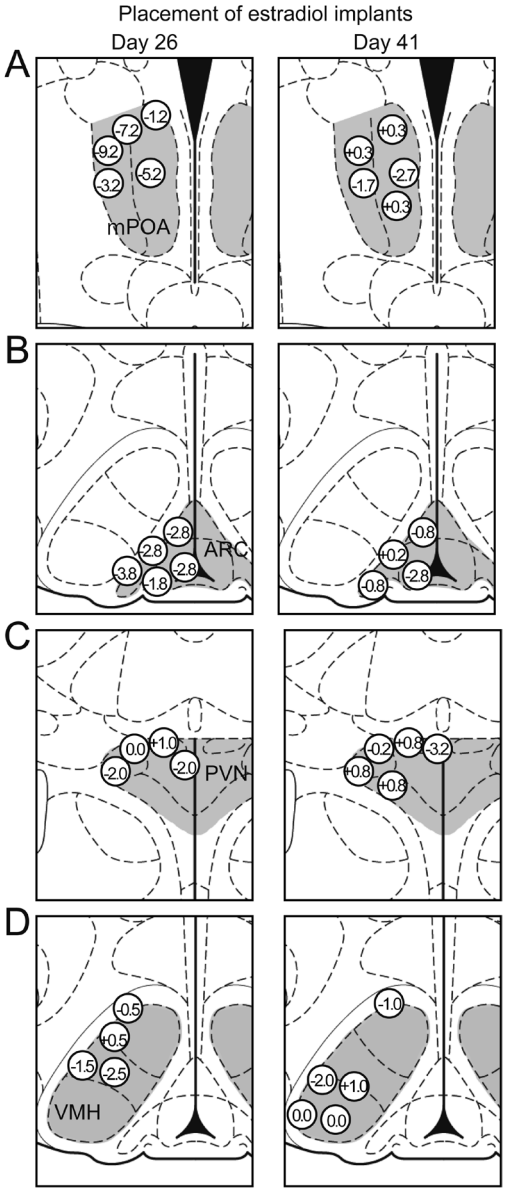

Fig. 1. Schematic drawings of the medial preoptic area (mPOA, A), hypothalamic arcuate nucleus (ARC, B), paraventricular nucleus (PVN, C) and ventromedial nucleus (VMH, D), illustrating the placement of estradiol microimplants in each animal sacrificed at 26 (left panels) and 41 (right panels) days of age. Encircled numbers indicate changes in the number of luteinizing hormone (LH) pulses during the 3-h sampling period induced by estradiol microimplants, compared with the mean LH pulse frequency in cholesterol-implanted controls.

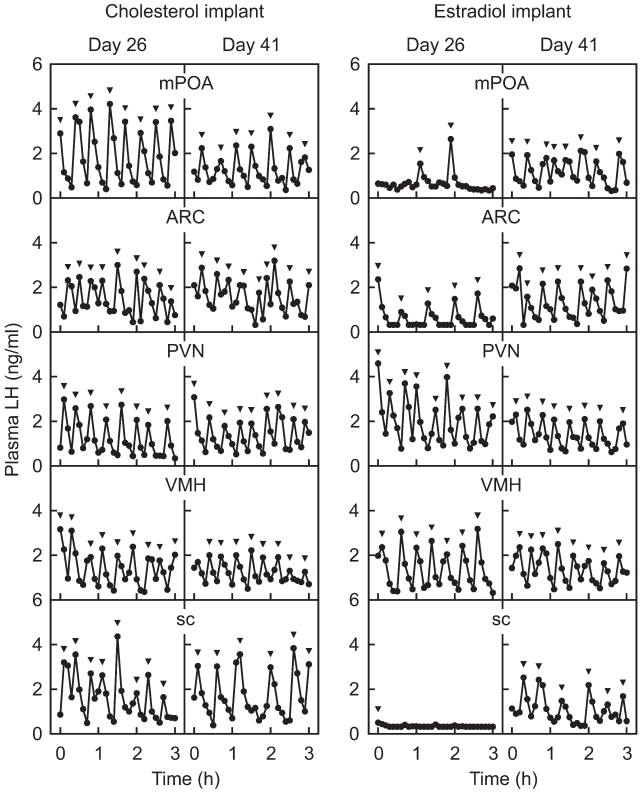

Fig. 2. Plasma LH profiles in representative cholesterol- (left panel) or estradiol-implanted (right panel) ovariectomized (OVX) rats at 26 (prepubertal period) and 41 (postpubertal period) days of age. Estradiol or cholesterol was implanted into the mPOA, ARC, PVN or VMH in OVX rats for 6 days. Some OVX animals received subcutaneous (sc) estradiol treatment for 6 days. Arrowheads indicate LH pulses identified with the PULSAR computer program.

< 0.05), and the main effect was qualified by significant interaction ($F(1,17) = 7.1$, $P < 0.05$). More specifically, estradiol-dependent suppression of LH pulse frequency was significant in the prepubertal period (†, $P < 0.05$, Fig. 3B), but not in the postpubertal period. Further, the frequency of LH pulses was lower in the prepubertal period than in the postpubertal period in rats bearing estradiol microimplants in the mPOA (§, $P < 0.05$, Fig. 3B). Also, estradiol microimplants in the mPOA failed to significantly change the mean LH concentrations and amplitude of LH pulses between the pre- and postpubertal periods.

The estradiol microimplants in the ARC suppressed pulsatile LH secretion in both the pre- and postpubertal periods. Specifically, two-way ANOVA revealed that ARC estradiol microimplants (main effect) significantly suppressed both mean LH concentrations ($F(1,14) = 4.7$, *, $P < 0.05$, Fig. 3A) and LH pulse frequency ($F(1,14) = 12.3$, *, $P < 0.05$, Fig. 3B). Further statistical analyses were omitted because the main effect of estradiol microimplants in the ARC on mean LH concentration or LH pulse frequency was not qualified by significant interaction.

On the other hand, the estradiol microimplants into the PVN or VMH failed to suppress pulsatile LH secretion in both the pre- and postpubertal periods, because no significant change was found in any pulse parameters in rats with PVN or VMH estradiol microimplants (Fig. 3).

The subcutaneous estradiol treatment suppressed pulsatile LH secretion in both the pre- and postpubertal periods. Specifically, two-way ANOVA revealed that subcutaneous estradiol treatment (main

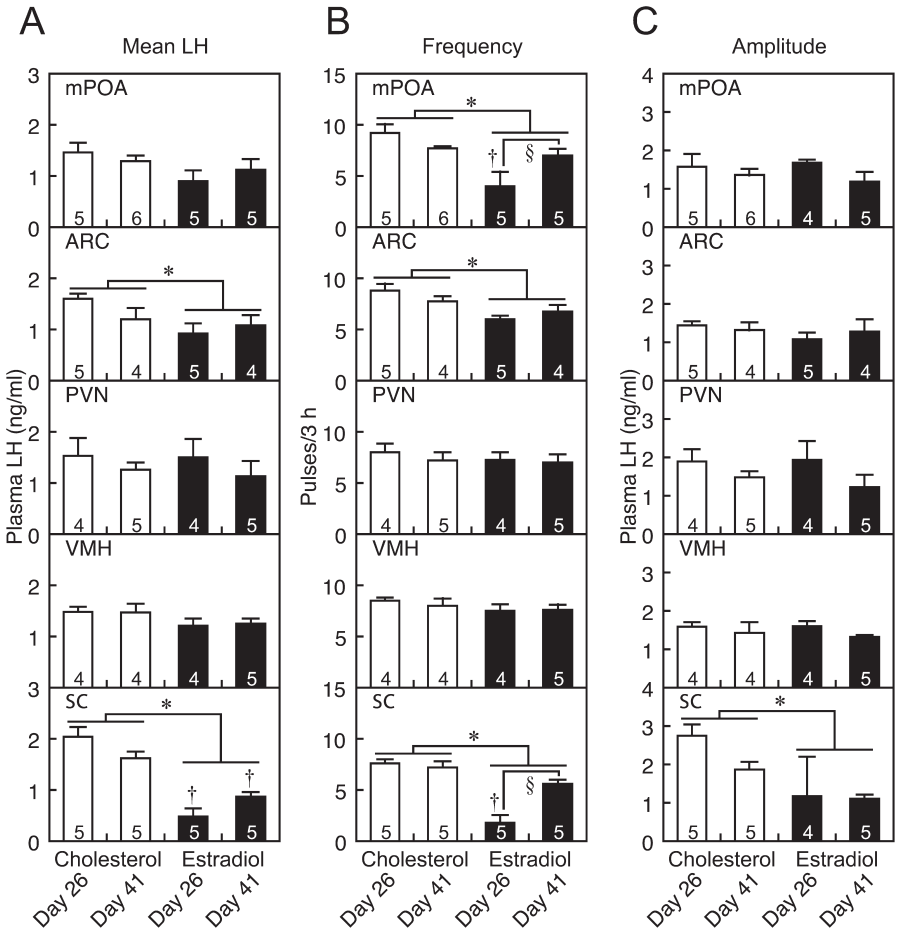

Fig. 3. Mean plasma LH concentrations (A) and the frequency (B) and amplitude (C) of LH pulses in cholesterol- (left panel) or estradiol-implanted (right panel) OVX rats at 26 (prepubertal period) and 41 (postpubertal period) days of age. Estradiol or cholesterol was implanted into the mPOA, ARC, PVN or VMH in OVX rats for 6 days. Some OVX animals received sc estradiol treatment for 6 days. Values are means ± SEM. Numbers in each column indicate the number of animals used. * Significant main effect of estradiol treatment ($P < 0.05$, two-way ANOVA). †Significant difference between cholesterol- and estradiol-implanted OVX rats within each period ($P < 0.05$, two-way ANOVA). §Significant difference between the pre- and postpubertal periods within estradiol-implanted OVX rats ($P < 0.05$, two-way ANOVA).

effect) significantly suppressed three LH pulse parameters (mean LH, $F(1,16) = 60.4$, *, $P < 0.05$, Fig. 3A; frequency, $F(1,16) = 45.6$, *, $P < 0.05$, Fig. 3B; amplitude, $F(1,15) = 6.2$, *, $P < 0.05$, Fig. 3C). The main effects of estradiol replacement on mean LH concentration and LH pulse frequency were qualified by significant interactions (mean LH, $F(1,16) = 7.3$, $P < 0.05$; frequency, $F(1,16) = 14.7$, $P < 0.05$). More specifically, subcutaneous estradiol treatment significantly suppressed mean LH concentrations in both the pre- and postpubertal periods (†, $P < 0.05$, Fig. 3A). Estradiol-dependent suppression of LH pulse frequency was found in the prepubertal period (†, $P < 0.05$, Fig. 3B), but not in the postpubertal period. The frequency of LH pulses was significantly lower in the prepubertal period than that in the postpubertal period in OVX rats with subcutaneous estradiol treatment (§, $P < 0.05$, Fig. 3B).

Expression of ERs in the mPOA and ARC of pre- and postpubertal OVX rats

A large number of ERα-immunoreactive cells were found at 26 and 41 days of age in the mPOA and ARC in pre- and postpubertal OVX rats (Fig. 4A). In both nuclei, no significant difference was found in the number of ERα-immunoreactive cells between 26 and 41 days of age (Fig. 4B). *Esr1, Esr2 and Gpr30* mRNA levels were quantitated in the mPOA and ARC-ME tissues, which are schematically illustrated as shaded areas in Fig. 5A. Dissected mPOA tissues included most of the medial preoptic nucleus and a part of the ventral limb of the diagonal band and median preoptic nucleus. Dissected ARC tissues included most of the ARC-ME region, a small portion of the VMH and the anterior part of the mammillary body nucleus. No significant difference in the expression of *Esr1*, *Esr2* and *Gpr30* was found between 26 and 41 days of age (Fig. 5B). The expression of *Esr1* was relatively higher than that of *Esr2* and *Gpr30* in both nuclei (Fig. 5B).

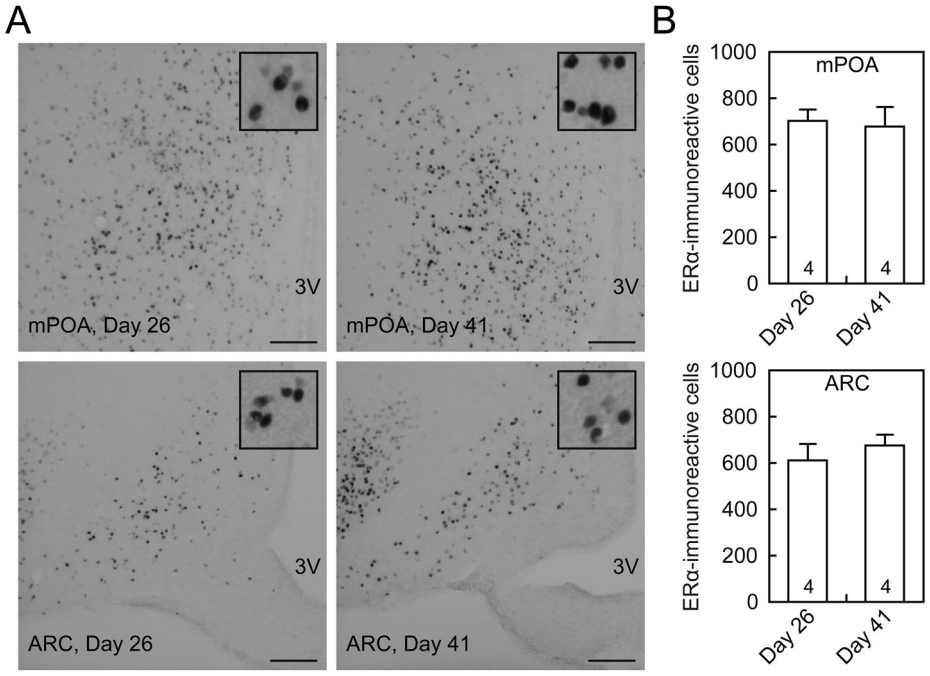

Fig. 4. Estrogen receptor α (ERα)-immunoreactive (ir) cells in OVX rats. A, Photomicrographs of ERα-ir cells in the mPOA and ARC at 26 and 41 days of age. Scale bars, 100 μm. B, Numbers of ERα-ir cells in the mPOA and ARC. Numbers of ERα-ir cells were not significantly different between 26 and 41 days of age. Values are mean ± SEM. Numbers in each column indicate the number of animals used.

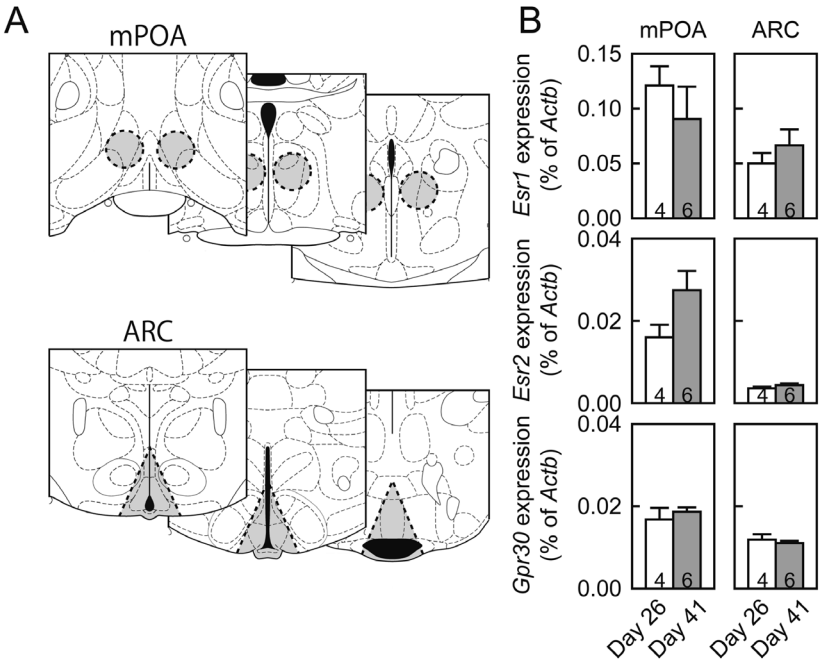

Fig. 5. Messenger RNA expression for three estrogen receptors in the mPOA and ARC in OVX rats. A, The schematic drawings show dissection or punch-out sites for the mPOA (upper panels) and ARC-median eminence (lower panels) regions (surrounded by dotted lines) from the anterior to posterior end. B, *Esr1*, *Esr2* and *Gpr30* mRNA expression. Values are normalized to the ratio to the copy number of *Actb* transcripts and presented as means ± SEM. Numbers in each column indicate the number of animals used.

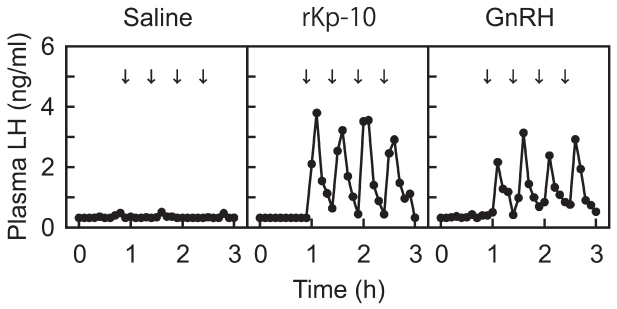

Fig. 6. Effects of the C-terminal decapeptide of rat kisspeptin (rKp-10, 10 nmol/kg body weight) and gonadotropin-releasing hormone (GnRH, 0.1 nmol/kg) on plasma LH levels in representative subcutaneous estradiol-treated OVX rats. Arrows indicate intravenous injection of rKp-10, GnRH or saline.

Effect of intravenous rKp-10 or GnRH challenge on LH release in prepubertal OVX rats subcutaneously treated with estradiol

Figure 6 shows the effects of rKp-10 or GnRH challenge on plasma LH levels in representative prepubertal (day 26) OVX rats with subcutaneous estradiol treatment. Four consecutive injections of rKp-10 or GnRH immediately increased plasma LH levels, while plasma LH levels remained low throughout the sampling period in the saline-injected controls. Every intravenous injection of rKp-10 or GnRH acutely increased the plasma LH level, which peaked within 12 min after injection and then declined to the basal level by 30 min after injection, mimicking spontaneous LH pulses in postpubertal rats.

Discussion

The present study suggests that at least two distinct hypothalamic regions, i.e., the mPOA and ARC, are action sites of estrogen for prepubertal restraint of LH release, because estradiol microimplants in the mPOA or ARC were capable of suppressing LH pulses in prepubertal OVX female rats. It also suggests that it is unlikely that estradiol implanted in the mPOA and ARC leaks into the circulation and acts on other sites, because the uterine weight of the rats with estradiol microimplants was comparable to that of cholesterol-treated controls. Indeed, estradiol microimplants in the PVN or VMH showed no suppressive effect on LH pulses in OVX rats in the prepubertal stage, suggesting that the effect of estradiol microimplants was largely limited within the precise brain area and that the mPOA and ARC are the estrogen action sites involved in the prepubertal suppression of LH pulses. Thus, in intact prepubertal female rats, circulating estrogens derived from the premature ovary may act on the mPOA and ARC, leading to the prepubertal restraint of GnRH/LH release. In addition, kisspeptin or GnRH challenge in the present study successfully stimulated LH secretion in prepubertal OVX rats with subcutaneous estradiol treatment, indicating that estrogen-dependent prepubertal restraint of LH release would be due to the suppression of kisspeptin and subsequent GnRH release. This notion is consistent with previous studies, which showed that changes in ARC *Kiss1*/kisspeptin expression are consistent with those in LH pulses in the peripubertal period and suggested that prepubertal LH suppression is mainly due to the suppression of kisspeptin expression/release and subsequently GnRH release [17, 19].

The present study also suggests a pubertal decrease in the responsiveness to estrogen in the mPOA, because mPOA estradiol microimplants failed to cause obvious suppression of LH pulse frequency in postpubertal animals. On the other hand, estradiol microimplants in the ARC constantly suppressed mean LH concentrations and LH pulse frequency in both the pre- and postpubertal periods. These results suggest that estrogen-responsive neurons in the mPOA and ARC play key roles in pubertal restraint of GnRH/LH secretion, whereas the ARC estrogen-responsive neurons mediate the negative feedback action of estrogen on GnRH/LH secretion in the postpubertal period. The current study showed that the number of ERα-expressing cells and the *Esr1*, *Esr2* or *Gpr30* expression in the mPOA and ARC were comparable between the pre- and postpubertal periods. Thus, it is unlikely that changes in responsiveness to estrogen negative feedback action during puberty on GnRH/LH suppression is simply caused by a change in expression of these ERs in the mPOA. It is possible that increases in stimulatory inputs or decreases in inhibitory inputs from the mPOA estrogen-responsive neurons to the GnRH pulse generator rather than a change in the expression of ERs would be involved in the different responses to estrogen between pre- and postpubertal female rats. Further, the current results obtained from OVX rats with subcutaneous estradiol treatment seem to be a summation of those obtained from the animals with mPOA or ARC estradiol microimplants in terms of two out of three LH pulse parameters (mean LH concentrations and LH pulse frequency). It cannot be ruled out that estrogen-responsive neurons in brain areas other than the mPOA and ARC are involved in the prepubertal restraint of LH secretion, because subcutaneous estradiol treatment profoundly suppressed LH pulses during the prepubertal period. Detailed analysis is needed to clarify these issues in the future.

The present study suggests that estrogen-responsive neurons located in the mPOA and ARC may play critical roles in the prepubertal restraint of kisspeptin and GnRH/LH secretion. The most plausible explanation for the current results using ARC estradiol microimplants is that ARC kisspeptin neurons could well serve as direct targets for estrogen negative feedback action for prepubertal restraint of GnRH/LH release in female rats. Indeed, ARC kisspeptin neurons exhibit ERα expression [24–26], and ARC *Kiss1* gene expression is nearly absent in the prepubertal period and increases at the onset of puberty [19]. In addition, ARC *Kiss1* expression was enhanced after ovariectomy and was strongly suppressed by estradiol replacement in prepubertal female rats [19]. Lomniczi *et al*. [33] showed that enrichment of histone H3 acetylation at the *Kiss1* promoter is associated with a pubertal increase in *Kiss1* expression in rats. ARC *Kiss1* expression is downregulated by estrogen-dependent histone deacetylation in adult mice [34]. Thus, prepubertal estrogen may decrease histone H3 acetylation at the ARC *Kiss1* promoter, resulting prepubertal suppression of *Kiss1* expression.

Interpretation of the current results for mPOA estradiol microimplants is very challenging. This is because a large number of neurons, such as GABAergic [35, 36], glutamatergic [37], dopaminergic [38], and several peptidergic [22, 38–42] neurons, have been reported to express ERα in the mPOA. The involvement of these neurons in prepubertal restraint of GnRH/LH secretion remains elusive. It

has been suggested that an increase in glutamatergic transmission precedes the pubertal decrease in GABAergic inhibitory input to the GnRH neurons [43]. In support of this, previous studies showed that an increase in hypothalamic GABA content, which is induced by a GABA-transaminase inhibitor, suppressed gonadotropin secretion and puberty onset in female rats [44] and that a GABAA receptor antagonist stimulated gonadotropin secretion in female rats [45]. On the other hand, it has also been suggested that an increase in GABAergic tone is involved in a prepubertal increase in GnRH secretion, because a GABAA receptor agonist stimulated gonadotropin secretion in prepubertal female rats [46, 47]. One possibility suggested by the current results using mPOA estradiol microimplants is that mPOA estrogen-responsive neurons, which may be GABAergic or other neurons, exert an inhibitory influence on ARC kisspeptin neuronal activity. If this is the case, ARC kisspeptin neuronal activity might be suppressed by estrogen both in direct and indirect manners. Further studies, e.g., projection of mPOA estrogen-responsive neurons into the ARC kisspeptin neurons, are required to clarify this issue.

In conclusion, the results obtained from the present study suggest that estrogen-responsive neurons located in the mPOA and ARC play key roles in estrogen-dependent prepubertal restraint of GnRH/LH secretion in female rats.

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