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ORIGINAL PAPER

The effects of gonadal steroid manipulation on the expression of *Kiss1* mRNA in rat arcuate nucleus during postnatal development

Ken Takumi · Norio Iijima · Kinuyo Iwata · Shimpei Higo · Hitoshi Ozawa

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Abstract Kisspeptins, encoded by *Kiss1* gene, play pivotal roles in the regulation of reproduction. Recently, several studies reported a sex difference in *Kiss1* expression in the arcuate nucleus (ARC) during the neonatal period. In this study, we investigated the effect of gonadal steroid manipulation on the sex difference in *Kiss1* expression in ARC of rats. At neonatal and prepubertal stages, females had a greater number of *Kiss1* neurons than the males. Gonadectomy at those stages resulted in significant increases in the *Kiss1* neuron number and the sex differences disappeared. We also confirmed the expression of estrogen receptor α in kisspeptin neurons in neonates. Altogether, our results indicate that ARC *Kiss1* expression is negatively regulated by gonadal steroids from early postnatal stages, and that the sex difference in ARC *Kiss1* expression is attributed to the difference in circulating gonadal steroid levels. We also found that neonatal estrogenization inhibits *Kiss1* expression and impairs negative feedback system.

Keywords Kisspeptin · Arcuate nucleus · Gonadal steroid · Negative feedback · Development

Introduction

Kisspeptin, a family of neuropeptides encoded by the *Kiss1* gene, has been shown to play important roles in the development and regulation of reproductive functions. The lack of kisspeptin signaling caused by an inactivating mutation of the kisspeptin receptor gene (*Kiss1r*) is associated with hypogonadotropic hypogonadism and impairment of pubertal maturation in humans [1, 2] and mice [1, 3]. Central or peripheral administration of kisspeptin induces a robust release of LH in various experimental animals [4–9] and in humans [10]. Such an LH release induced by kisspeptin can be blocked by GnRH receptor antagonist [4, 5, 9]. Kisspeptin immunoreactive fibers have been found to be closely associated with somata and axons of GnRH neurons [11–13], indicating that kisspeptin regulates the hypothalamic–pituitary–gonadal (HPG) axis by directly acting on GnRH neurons.

In rodent hypothalamus, neuronal populations synthesizing kisspeptin are known to reside in two discrete regions, the anteroventral periventricular nucleus and the periventricular nucleus continuum (AVPV/PeN) and arcuate nucleus (ARC). The majority of kisspeptin neurons in AVPV/PeN and ARC of adult rodents express estrogen receptor (ER) α [14–16] and the expressions of kisspeptin in both hypothalamic regions are regulated by circulating gonadal steroids, but the means of regulation differ between the regions. In AVPV/PeN, estradiol and testosterone stimulate the expression of *Kiss1* mRNA and kisspeptin protein, whereas in ARC, the same gonadal steroids inhibit their expression [15–18]. This indicates that kisspeptin neurons in AVPV/PeN and ARC are involved in positive and negative feedback regulation of gonadal steroids on GnRH release, respectively. In addition to such activational effects on the *Kiss1* expression during adulthood, gonadal steroids also have organizational effects on kisspeptin neurons during the critical period of brain sexual differentiation. Kisspeptin neurons in AVPV/PeN of rodents are sexually dimorphic: females possess more kisspeptin neurons than males from prepubertal stages to

K. Takumi (✉) · N. Iijima · K. Iwata · S. Higo · H. Ozawa
Department of Anatomy and Neurobiology, Graduate School of Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan
e-mail: takumiken@nms.ac.jp

adulthood [11, 17–20]. Although gonadectomy or steroid treatment in adulthood has no effect on this sex difference, neonatal exposure to estrogen or testosterone results in masculinization of *Kiss1* expression in AVPV/PeN of female rats [18, 19], and conversely, neonatal gonadectomy results in an elevation in *Kiss1* neurons in AVPV/PeN of male rats later in life [19]. These data indicate that the sex difference in AVPV/PeN kisspeptin neuron is attributed to the neonatal gonadal steroid milieu but not to the circulating gonadal steroid levels at the time of the experiments.

In contrast to AVPV/PeN, the expression of kisspeptin in rodent ARC has been reported to be comparable between both sexes at adulthood, regardless of the circulating gonadal steroid levels [18, 19]. However, in our previous study, we observed a clear sex difference in the number of kisspeptin neurons in ARC during the neonatal period [20], with female rats having a greater number of *Kiss1* mRNA expressing neurons compared to males. Other researchers have also reported similar sex differences in postnatal *Kiss1* mRNA expression by using autoradiographic in situ hybridization [21]. Moreover, Kauffman et al. [22] recently reported a sex difference in the response of ARC *Kiss1* neuron to gonadectomy only during the prepubertal period in mice. All together, these observations suggest the possibility that *Kiss1* expression and its regulation by gonadal steroids in ARC are also sexually dimorphic at early stages of postnatal development, but the details remain unclear.

In the present study, to clarify the effects of gonadal steroids on the sex difference in *Kiss1* expression in ARC during postnatal development, we analyzed the number of kisspeptin neurons in ARC of neonates, prepubertal, and adult rats which underwent gonadal steroid manipulation at neonatal and/or prepubertal stages.

Materials and methods

Animals and tissue preparations

Male and female Wistar rats were purchased from Saitama Experimental Animal Supply (Saitama, Japan) and bred in the vivarium of Nippon Medical School. All rats were kept under controlled condition of light (14 h light/day; lights on from 0600 hours) and temperature (24 ± 2 °C) with free access to standard rodent chow and water. The day of parturition was designated as postnatal day (PND) 0. All rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 0.9 % NaCl followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 (PB). Brains were immediately removed and immersed in the same fixative at 4 °C overnight, and then transferred to 20 % sucrose for cryoprotection. Serial coronal sections of the hypothalamus (from the organum vasculosum of the lamina terminalis to the mammillary body) were cut at a thickness of 30 μm on a cryostat (Leica CM3050, Heidelberg, Germany), thaw-mounted onto RNase-free APS-coated glass slides (Matsunami Glass, Osaka, Japan), air-dried and kept at −80 °C until used for in situ hybridization. All experimental procedures were conducted in accordance with the guidance on animal bio-ethics of Nippon Medical School, based on the guidelines issued by the US National Institute of Health for the humane treatment of experimental animals.

Experimental design; neonatal treatment and gonadectomy

Experiment 1: effects of neonatal gonadal steroid manipulation on the number of Kiss1 mRNA expressing neurons at PND7

Male rats were bilaterally GDX under hypothermic anesthesia at PND0 or left intact ($n = 5$ each group). Newborn female rats received a single subcutaneous injection of either estradiol benzoate (EB, 25 μg/50 μl sesame oil; Sigma-Aldrich, St. Louis, MO, USA) or 50 μl sesame oil ($n = 5$ each group). At PND7, all rats were euthanized and brains were collected and processed for in situ hybridization and double labeling fluorescent in situ hybridization and immunohistochemistry.

Experiment 2: effects of neonatal estrogenization and prepubertal gonadectomy on the number of Kiss1 mRNA expressing neurons at PND18

Newborn female rats received a single subcutaneous injection of either EB or vehicle, as in Experiment 1. At PND14, male and female rats were GDX under isoflurane anesthesia or left intact ($n = 6$ each group). All rats were euthanized at PND18 and brains were collected.

Experiment 3: effects of neonatal estrogenization on the number of Kiss1 mRNA expressing neurons in adulthood

Newborn female rats received a single injection of either EB or vehicle ($n = 5$ each group), as described above. The animals were weaned at PND21. Neonatal estrogenization has been known to impair normal estrus cyclicity [23, 24]. To remove the influence of the difference in endogenous gonadal steroid levels, all animals were GDX under isoflurane anesthesia at the age of 8 weeks, and brains were collected at 9 weeks.

In situ hybridization

Kiss1 mRNA expressing neurons were visualized by in situ hybridization as previously described [20]. Briefly, digoxigenin (DIG)-labeled antisense and sense RNA probes were synthesized from template cDNA for full-length rat *Kiss1* (GeneBank accession #AY196983) [25] by using DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Slides were washed in DEPC-treated 0.1 M phosphate-buffered saline (PBS) and incubated with Proteinase K (Invitrogen, Carlsbad, CA, USA) at 37 °C. After fixing again with 4 %PFA in PB and several washes in PBS, slides were incubated in 0.25 % acetic anhydride in 0.1 M triethanolamine for 10 min at room temperature (RT). Slides were incubated with 1× prehybridization solution (Sigma-Aldrich, Tokyo, Japan) containing 50 % formamide at RT, and then hybridized with DIG-labeled antisense or sense RNA probes diluted in 1× hybridization solution (Sigma-Aldrich) containing 50 % formamide and 10 % dextran sulfate for 16 h at 60 °C. After hybridization, slides were treated with RNase A (20 μg/ml; Roche Diagnostics) for 45 min at 37 °C and washed under conditions of increasing stringency. Visualization of the DIG labeling was achieved using anti-DIG fragments conjugated to alkaline phosphatase (AP) (1:500; Roche Diagnostics) with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate solution (Roche Diagnostics). DIG-labeled sense RNA probe was used as the negative control.

Every second section through AVPV/PeN and every fourth section through ARC from each animal were photographed with an AX80 microscope equipped with DP-70 (Olympus, Tokyo, Japan) and the images were exported as tiff format. *Kiss1* mRNA expressing cell bodies in AVPV/PeN and ARC were counted on a computer display and the sum of the cell number for each area was calculated. The analyzer was blind to the experimental groups.

Double labeling fluorescent in situ hybridization and immunohistochemistry

A series of slides of brain sections from oil-treated PND7 females ($n = 4$) and neonatally gonadectomized PND7 males ($n = 3$) were processed as described above and hybridized with DIG-labeled antisense RNA probe for *Kiss1* without Proteinase K treatment. After hybridization, slides were incubated with blocking buffer [4 % normal donkey serum and 1%BSA in 0.1 M Tris-buffered saline (TBS)] for 1 h at RT. Slides were incubated with a cocktail of anti-DIG fragments conjugated to AP (1:500; Roche Diagnostics) and rabbit anti-ERα antibody (1:200, sc-542; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted with blocking buffer overnight at 4 °C. After several washes in TBS, slides were incubated with Alexa Fluor 488 conjugate donkey anti-rabbit IgG antibody (1:200; Molecular Probes, USA) diluted with blocking buffer for 2 h at RT. Finally DIG-labeling was detected using 2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate (HNPP) and fast red TR (Roche Diagnostics). Confocal images were obtained using a confocal laser scanning microscope (LSM710; Carl Zeiss, Oberkochen, Germany). Scanning at each wavelength was performed individually. Pinhole diameter was optimized to 1.0 Airy disk. *Kiss1* mRNA expressing cells exhibiting ERα immunoreactivity were counted on a computer display and the amount of double labeled cells was calculated as a percentage of the number of *Kiss1* mRNA expressing neurons.

Statistical analysis

The mean number of *Kiss1* mRNA expressing cells and standard of error of mean in AVPV/PeN and ARC were calculated for the experimental group. Data were analyzed for statistically significant differences using *t* test, one-way ANOVA, and multiple comparisons with either Bonferroni or Games–Howell. Differences were considered statistically significant if the *p* value was <0.05.

Results

Experiment 1: effects of neonatal gonadal steroid manipulation on the number of *Kiss1* mRNA expressing neurons at PND7

In the hypothalamus of PND7 rats, *Kiss1* mRNA expressing neurons were found only in ARC and not in AVPV/PeN. In accord with our initial observation [20], the female rats that received a single oil injection at PND0 possessed a significantly greater number of ARC *Kiss1* neurons than intact males at PND7 (Fig. 1b). The number of ARC *Kiss1* neurons of neonatally oil-treated females was approximately fivefold greater than that of intact males (Fig. 1c). In males, gonadectomy at PND0 resulted in a significant increase in the number of *Kiss1* mRNA expressing neurons at PND7, which was comparable to that of oil-treated females. In females, a single bolus of EB at PND0 significantly decreased the number of *Kiss1* neurons at PND7. The number of ARC *Kiss1* neurons in EB-treated females was not significantly different from that in intact males (Fig. 1c). To confirm the expression of ERα in *Kiss1* neurons in ARC of neonates, we performed dual fluorescent in situ hybridization for *Kiss1* mRNA and immunohistochemistry for ERα. We observed clear ERα immunoreactivities in nuclei of neuronal cells in ARC of PND7 rats and 93 ± 0.9 and 94 ± 1.2 % of *Kiss1* mRNA expressing neurons showed ERα immunoreactivity in ARC

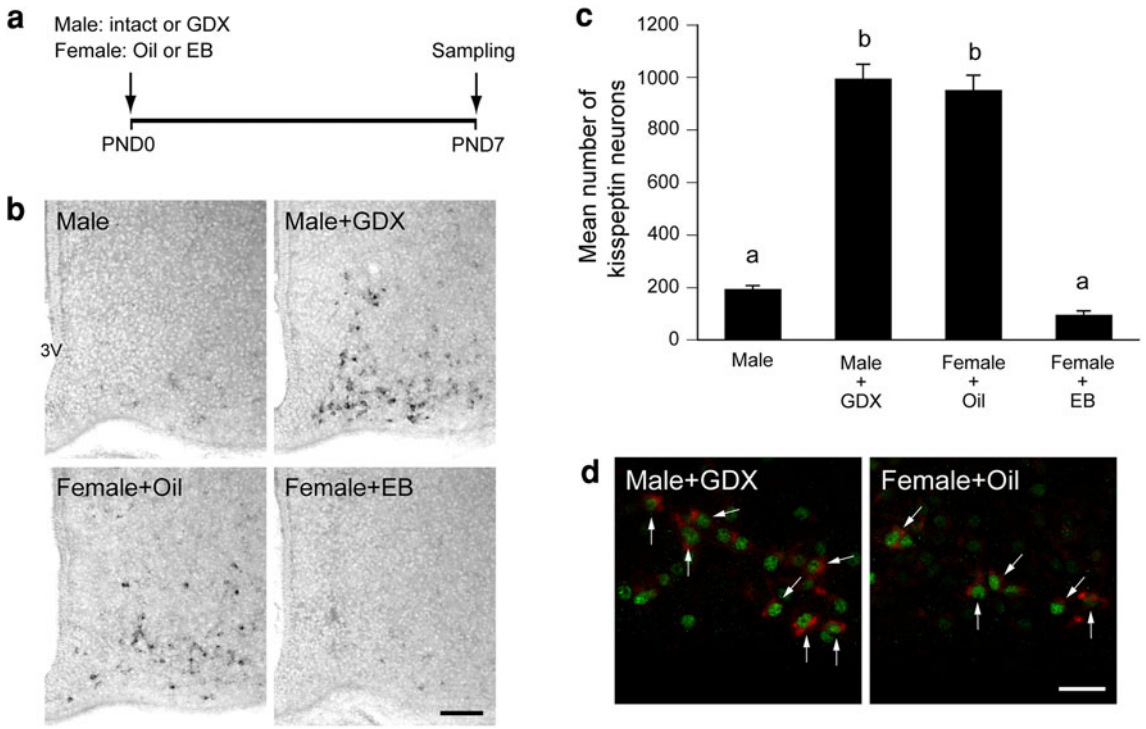

Fig. 1 Effects of neonatal gonadal steroid manipulation on *Kiss1* mRNA expression in arcuate nucleus (ARC) of neonatal rats. **a** Schematic representation of the experimental schedule of the experiment; *EB* estradiol benzoate, *GDX* gonadectomized, *PND* postnatal day. **b** Representative photographs of *Kiss1* mRNA expressing neurons in ARC of postnatal day (*PND*) 7 males that were gonadectomized (*GDX*) or left intact on PND0 and females that received oil or estradiol benzoate (*EB*) injection on PND0. *3V* third ventricle. *Scale bar* 100 μm. **c** Mean number of *Kiss1* mRNA expressing neurons in ARC of each experimental group. *Error bars* SEM. *Bars* labeled with *different letters* are significantly different from each other ($p < 0.05$). **d** Representative single optical sections showing *Kiss1* mRNA expressing neurons (*red*, *arrows*) which coexpressed ERα (*green*) in ARC of male rats that were GDX on PND0 and female that received oil injection at PND0. *Scale bar* 25 μm

of neonatally gonadectomized males and oil-treated females, respectively (Fig. 1d).

Experiment 2: effects of neonatal estrogenization and prepubertal gonadectomy on the number of *Kiss1* mRNA expressing neurons at PND18

In ARC of PND18 rats, as in PND7, neonatally oil-treated females possessed a significantly greater number of *Kiss1* neurons than males and EB-treated females. The number of *Kiss1* neurons in EB-treated females was significantly lower than in males and oil-treated females. Gonadectomy at PND14 significantly increased the number of *Kiss1* neurons in ARC of males and oil-treated females, but no significant change was found in EB-treated females. In GDX animals, although oil-treated females possessed larger number of *Kiss1* neurons than males, there was no significant difference between males and oil-treated females. EB-treated GDX females had a significantly lower number of *Kiss1* neurons than GDX males and oil-treated GDX females (Fig. 2b, c).

In AVPV/PeN of intact PND18 rats, oil-treated females possessed a significantly greater number of *Kiss1* neurons, compared to males. The number of *Kiss1* neurons in EB-treated females was significantly lower than those in males and in oil-treated females. Gonadectomy at PND14 resulted in significant decreases in the numbers of *Kiss1* neurons in AVPV/PeN of males and oil-treated females, but had no significant effects on EB-treated females. In GDX animals, oil-treated females possessed a significantly greater number of *Kiss1* neurons than males and EB-treated females (Fig. 3).

Experiment 3: effects of neonatal estrogenization on the number of *Kiss1* mRNA expressing neurons in adulthood

To determine whether neonatal estrogenization affects the expression of *Kiss1* mRNA in adulthood, we analyzed the number of *Kiss1* neurons of 9W females which were GDX at 8W to remove endogenous gonadal steroids. The neonatally EB-treated females possessed a significantly lower number of *Kiss1* neurons in ARC compared to oil-treated

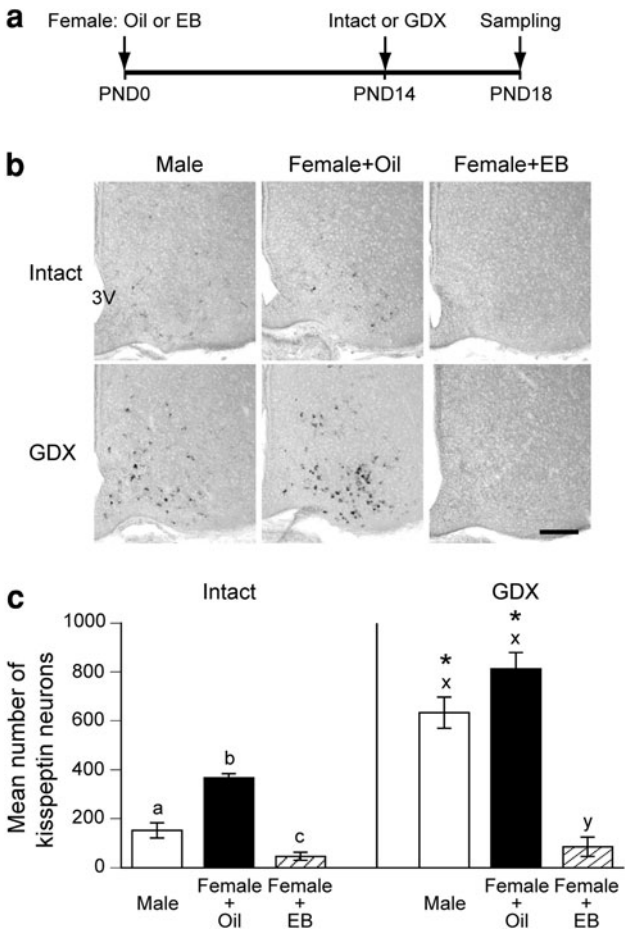

Fig. 2 Effects of neonatal estrogenization and prepubertal gonadectomy on *Kiss1* mRNA expression in ARC of prepubertal rats. **a** Schematic representation of the experimental schedule of the experiment; *EB* estradiol benzoate, *GDX* gonadectomized, *PND* postnatal day. **b** Representative photographs of *Kiss1* mRNA expressing neurons in ARC of PND18 males that were GDX or left intact on PND14 and females that received oil or EB injection on PND0 and were GDX or left intact on PND14. *3V* Third ventricle. *Scale bar* 150 μm. **c** Mean number of *Kiss1* mRNA expressing neurons in ARC of each experimental group. *Error bars* SEM. *Bars* labeled with *different letters* are significantly different from each other ($p < 0.05$). *Asterisks* indicate significant differences from corresponding intact group ($p < 0.05$)

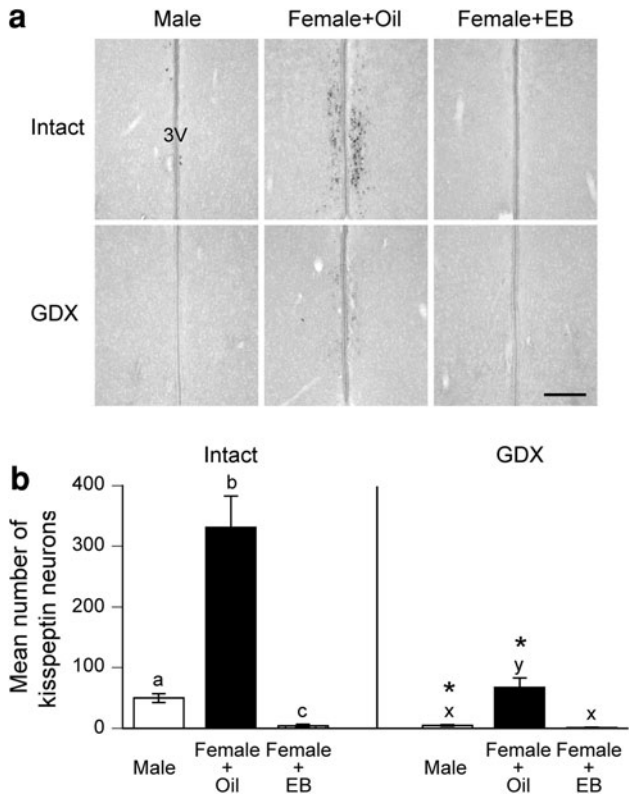

Fig. 3 Effects of neonatal estrogenization and prepubertal gonadectomy on *Kiss1* mRNA expression in anteroventral periventricular nucleus and periventricular nucleus continuum (AVPV/PeN) of prepubertal rats. **a** Representative photographs of *Kiss1* mRNA expressing neurons in AVPV/PeN of PND18 males that were GDX or left intact on PND14 and females that received oil or EB injection on PND0 and were GDX or left intact on PND14. *3V* Third ventricle. *Scale bar* 200 μm. **b** Mean number of *Kiss1* mRNA expressing neurons in AVPV/PeN of each experimental group. *Error bars* SEM. *Bars* labeled with *different letters* are significantly different from each other ($p < 0.05$). *Asterisks* indicate significant differences between intact and GDX groups ($p < 0.05$)

females (Fig. 4b, c). Similarly, the number of *Kiss1* mRNA expressing neurons in AVPV/PeN of EB-treated females was significantly lower than that of oil-treated females (Fig. 4d, e). Oil-treated females possessed 4- and 15-fold more *Kiss1* neurons than EB-treated females in ARC and AVPV/PeN, respectively.

Discussion

Kisspeptin neurons in the hypothalamus have been shown to be an important regulator of HPG axis in mammals. Despite detailed descriptions of the sex difference in kisspeptin expressions in AVPV/PeN and the role of gonadal steroids on its development, there are still few studies focused on the sex difference in ARC. Here, we examined the effect of the neonatal gonadal steroid milieu and gonadectomy on the sex difference in expressions of *Kiss1* mRNA in ARC during postnatal development.

We observed a clear sex difference in the number of *Kiss1* neurons in ARC at neonatal stage; control females possessed more *Kiss1* neurons than intact males at PND7. This is consistent with our previous reports and that of others [20, 21, 26]. During the neonatal period, the serum gonadotropin levels are known to be higher in female rats than in male rats [27, 28]. The sex difference in *Kiss1* expression in ARC of neonates might be responsible for this difference of circulating gonadotropin levels. It is well established that kisspeptin neurons in adult rodents express ERα and the *Kiss1* expression in ARC of adult rodents are inhibited by circulating gonadal steroids [15, 16, 18, 22]. In

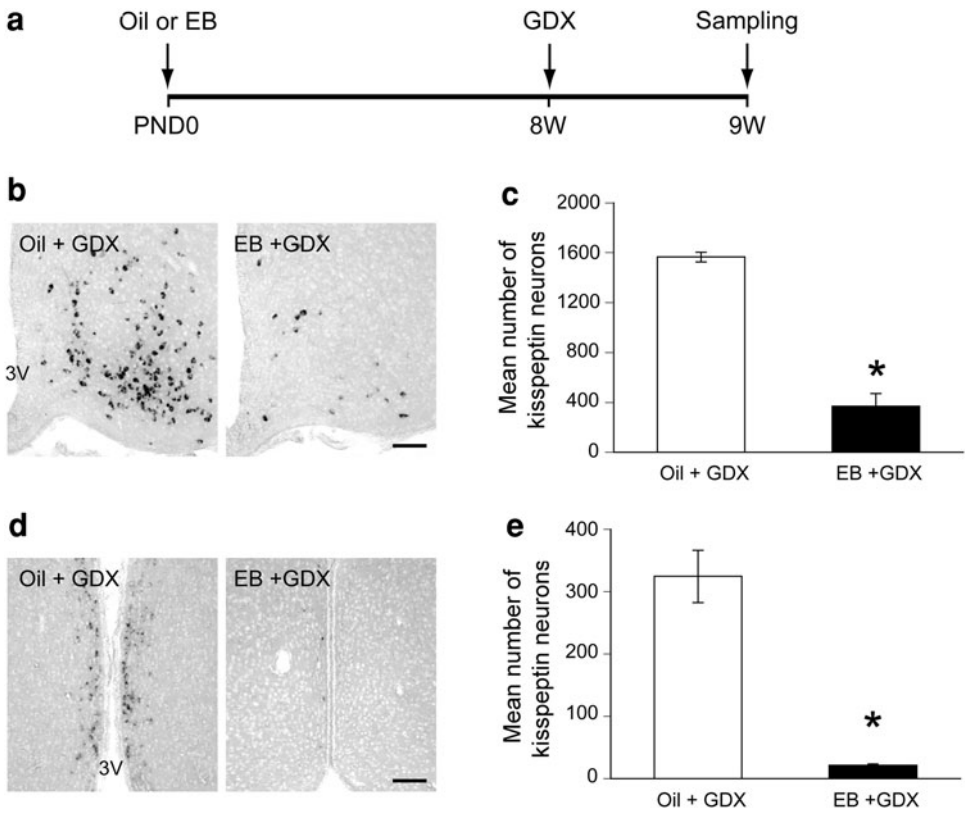

Fig. 4 Effects of neonatal estrogenization on *Kiss1* mRNA expression in adult female rats. **a** Schematic representation of the experimental schedule of the experiment; *EB* estradiol benzoate, *GDX* gonadectomized, *PND* postnatal day, *8W* 8 weeks, *9W* 9 weeks. **b** Representative photographs of *Kiss1* mRNA expressing neurons in ARC of 9W female rats that received oil or EB injection on PND0 and were GDX at 8W. *3V* third ventricle. *Scale bar* 100 μm. **c** Mean number of *Kiss1* mRNA expressing neurons in ARC. **d** Representative photographs of *Kiss1* mRNA expressing neurons in AVPV/PeN. *3V* third ventricle. *Scale bar* 100 μm. **e** Mean number of *Kiss1* mRNA expressing neurons in AVPV/PeN. *Asterisks* indicate significant differences between oil and EB groups ($p < 0.05$)

this study, we found that gonadectomy of newborn males resulted in a significant increase in *Kiss1* neurons in ARC at PND7; conversely, neonatal EB injection caused a significant decrease in female rats. In addition, we also confirmed the majority of kisspeptin neurons in ARC of both sexes coexpressed ERα during neonatal period. To our knowledge, this is the first study to show that neonate kisspeptin neurons in ARC coexpress ERα and are negatively regulated by gonadal steroids as in adulthood. We also found a sex difference in the number of *Kiss1* neurons in ARC of intact prepubertal rats. However, in GDX prepubertal animals, there is no significant difference between males and oil-treated females. All these results indicate that the sex difference in the number of *Kiss1* neurons in ARC of intact neonates and prepubertal rats can be attributed to an activational effect of gonadal steroids: the difference in the levels of circulating gonadal steroids between the sexes at the time of development, rather than the organizational effect of neonatal gonadal steroid milieu. This is in good accordance with the fact that the serum androgen level is higher in males than in females throughout postnatal development, whereas the estradiol level is comparable between sexes [28].

In prepubertal male mouse, *Kiss1* expression in ARC does not increase after gonadectomy, suggesting the presence of a gonadal steroid-independent inhibitory factor [22]. However, we demonstrated that *Kiss1* expression in ARC of prepubertal male rats was significantly increased after gonadectomy as in females. This is consistent with the changes in serum LH [29, 30] and *Kiss1* expression in the whole hypothalamus [31] after gonadectomy, indicating that, unlike in mice, gonadal steroid is a dominant inhibitory factor on *Kiss1* expression in ARC during prepubertal period in rats.

Neonatal estrogenization of females resulted in a significant decrease in *Kiss1* expression later in life. In this study, we found that prepubertal female rats which received a single bolus of EB at the day of birth had a significantly lower number of *Kiss1* neurons in both of ARC and AVPV/PeN. This is in good agreement with a previous report [31] which showed that neonatal estrogenization decreased *Kiss1* expression in the whole hypothalamus of both sexes at the prepubertal period. Moreover, we found that *Kiss1* neurons in ARC of EB-treated prepubertal females did not respond to the changes in gonadal steroid levels caused by gonadectomy, suggesting that negative feedback regulation on *Kiss1* expression is impaired in those rats. The effect of neonatal estrogenization was also present in adult animals: neonatally EB-treated females possessed a lower number of *Kiss1* neurons in both of ARC and AVPV/PeN than oil-treated females at 9 weeks. The decrease in *Kiss1* expression in neonatally estrogenized adult female rats are consistent with the data from neonatally estrogenized male rats [7], but inconsistent with a report in which female rats were estrogenized to mimic the testosterone surge in males and showed a similar

number of ARC *Kiss1* neurons as intact males in adulthood [19]. The doses of EB were the same but the time points of administration were different between this study (PND0) and the previous report (PND5), indicating that a relatively higher dose was administered in this study when it is standardized by body weight. However, it is intriguing that the effects of estrogenization in ARC is different between the two studies but not in AVPV/PeN, suggesting the possibility that *Kiss1* neurons in AVPV/PeN might be more vulnerable to gonadal steroids than those in ARC, and that the critical time window for gonadal steroids effect on ARC is set earlier than for AVPV/PeN.

The persistent inhibition of *Kiss1* in ARC by neonatal estrogenization also suggests the possibility that gonadal steroids have organizational effects on *Kiss1* neurons not only in AVPV/PeN but also in ARC of males, where testosterone surge occurs during the perinatal period. Some of the sexual dimorphisms in the brain have been shown to be established by apoptosis mediated by gonadal steroids during perinatal period [32]. Recently, Semaan et al. [33] reported that BAX, a pro-apoptotic protein, knockout mice had more *Kiss1* neurons in ARC than wild-type animals. Furthermore, within the BAX knockout mice, males possessed a greater number of ARC *Kiss1* neurons than females, suggesting that BAX-dependent apoptosis regulates the final number of *Kiss1* neurons in ARC and that a larger number of the programmed cell death occurs in ARC of male mice than females. It is possible that the decrease in *Kiss1* neurons in ARC of neonatally estrogenized female rats is the result of apoptosis caused by higher levels of estradiol in this study. More detailed studies are necessary to determine what mechanism underlies the decrease in *Kiss1* expression in ARC after estrogenization.

The sex difference in *Kiss1* expression in AVPV/PeN in the intact prepubertal rats and a significant decrease in the number of AVPV/PeN *Kiss1* neurons in neonatally estrogenized females are in good agreement with previous reports [19–21]. In prepubertal males and control females, *Kiss1* expression was significantly decreased after gonadectomy as in adult animals. This is consistent with the similar study performed in mouse [22] and indicates that *Kiss1* neurons in AVPV/PeN of rats are already capable of responding to the change in the gonadal steroid levels in the prepubertal period. As in ARC, there was no change in *Kiss1* expression in AVPV/PeN of estrogenized females after gonadectomy, but this seems to be a floor effect because the number of *Kiss1* neurons in intact EB-treated females was too small.

In summary, we demonstrated the sex-specific expression patterns of *Kiss1* in ARC of neonates and prepubertal rats and that they are regulated by circulating gonadal steroids, suggesting that the basis of negative feedback regulation of gonadal steroids on *Kiss1* expression in ARC is established early in development. We also showed that neonatal estrogenization inhibits *Kiss1* expression in both of ARC and AVPV/PeN of females, and it affects the negative feedback regulation on *Kiss1* gene expression in ARC.

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Conflict of interest The authors declare that they have no conflict of interest.

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