—Original Article—

Dynorphin-Kappa Opioid Receptor Signaling Partly Mediates Estrogen Negative Feedback Effect on LH Pulses in Female Rats

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Abstract. Accumulating evidence suggests that the arcuate nucleus (ARC) kisspeptin/neurokinin B (NKB)/dynorphin (KNDy) neurons play a role in estrogen negative feedback action on pulsatile gonadotropin-releasing hormone (GnRH)/ luteinizing hormone (LH) release. The present study aimed to determine if dynorphin (Dyn) is involved in estrogen negative feedback on pulsatile GnRH/LH release. The effect of the injection of nor-binaltorphimine (nor-BNI), a kappa-opioid receptor (KOR) antagonist, into the third cerebroventricle (3V) on LH pulses was determined in ovariectomized (OVX) adult female rats with/without replacement of negative feedback levels of estradiol (low E_2). The mean LH concentrations and baseline levels of LH secretion in nor-BNI-injected, low E_2-treated rats were significantly higher compared with vehicle-treated controls. On the other hand, the nor-BNI treatment failed to affect any LH pulse parameters in OVX rats without low E_2 treatment. These results suggest that Dyn is involved in the estrogen negative feedback regulation of pulsatile GnRH/LH release. The low E_2 treatment had no significant effect on the numbers of ARC *Pdyn* (Dyn gene)-, *Kiss1*- and *Tac2 (*NKB gene)-expressing cells. The treatment also did not affect mRNA levels of *Pdyn* and *Oprk1* (KOR gene) in the ARC-median eminence region, but significantly increased the ARC kisspeptin immunoreactivity. These findings suggest that the negative feedback level of estrogen suppresses kisspeptin release from the ARC KNDy neurons through an unknown mechanism without affecting the Dyn and KOR expressions in the ARC. Taken together, the present result suggests that Dyn-KOR signaling is a part of estrogen negative feedback action on GnRH/LH pulses by reducing the kisspeptin release in female rats.
Key words: Arcuate nucleus, GnRH, Kisspeptin, Neurokinin B

Animal reproduction is regulated by the hypothalamo-pituitary-gonadal axis. Two modes of gonadotropin-releasing hormone (GnRH) release, pulse and surge, are considered to regulate follicular maturation/steroidogenesis and ovulation, respectively, in female mammals [1–4]. The pulsatile GnRH/luteinizing hormone (LH) release is negatively regulated by circulating estrogen from the ovary [5] to fine-tune gonadotropin secretion for follicular maturation [6, 7]. The mechanism generating GnRH/LH pulses, called the GnRH pulse generator, has been suggested to be localized in the mediobasal hypothalamus (MBH) in rats and monkeys [8–10].

It has been well accepted that kisspeptin-GPR54 signaling plays a key role in controlling reproduction via stimulating GnRH release [2, 3, 11]. More specifically, a population of kisspeptin neurons located in the arcuate nucleus (ARC) has been considered to play an important role in GnRH/LH pulse generation in many mammalian species, including rats [12–14], mice [15], goats [16, 17], sheep [18, 19] and monkeys [20]. The ARC kisspeptin neurons are recently referred to as KNDy neurons [19] because they co-express neurokinin

B (NKB) and dynorphin (Dyn) [15, 17, 21]. Wakabayashi *et al.* [17] showed that central infusion of a Dyn antagonist or NKB increases the GnRH/LH pulse generator activity in ovariectomized (OVX) goats. Their results suggest that Dyn and NKB play an inhibitory and stimulatory role, respectively, in controlling GnRH pulses. It is speculated that kisspeptin is released in a pulsatile manner from the ARC KNDy neurons to stimulate GnRH pulses by acting on GnRH neuronal terminals at the median eminence (ME). This notion could be adapted in many mammalian species, such as mice [15], rats [13, 22] and sheep [18, 19].

Dynorphin exerts its effects mainly through the kappa-opioid receptor (KOR), a G-protein-coupled receptor. Dynorphin-KOR signaling is considered to be involved in the suppression of GnRH/ LH pulses, because central administration of nor-binaltorphimine (nor-BNI), a selective KOR antagonist, increases the amplitude and frequency of LH pulses during early pregnancy [23] and the mean LH level and LH pulse frequency during midpregnancy [24] in rats. Further, Goodman *et al.* [25] suggested that the central Dyn-KOR system mediates progesterone negative feedback on GnRH/LH pulses in ewes. LH pulses are strongly suppressed in prepubertal rats, in which both *Kiss1* (kisspeptin gene*)* expression and kisspeptin immunoreactivity in the ARC are suppressed in the presence of a negative feedback level of estrogen [26]. Since estrogen receptor (ER) α is located in ARC KNDy neurons [27–29], it is possible that

Dyn-KOR signaling mediates the estrogen negative feedback effect on GnRH/LH pulses in adult female rats.

The present study, therefore, aimed to determine if Dyn-KOR signaling mediates estrogen negative feedback on GnRH/LH release. We first examined the effects of central administration of a KOR antagonist on pulsatile LH release in OVX rats in the presence or absence of a negative feedback level of estradiol (E_2) [30]. We also determined the effects of this negative feedback level of E_2 on the gene expressions of *Pdyn* (Dyn gene), *Oprk1* (KOR gene), *Kiss1*, and *Tac2* (NKB gene) in the ARC-ME region to investigate if estrogen exerts its negative regulation of GnRH/LH pulses through the changes in these gene expressions. In addition, we examined kisspeptin immunoreactivity in the ARC in the presence or absence of a negative feedback level of E_2 to investigate if the current E_2 treatment affects the kisspeptin expression in this nucleus.

Materials and Methods

Animals

Adult female Wistar-Imamichi rats at 10–12 weeks of age (230–280 g BW) were used. They were maintained under a controlled environment (14 h light and 10 h darkness, lights on at 0500 h; 23 ± 3 C) with free access to food (CE2, Clea, Tokyo, Japan) and water. Vaginal smears were checked daily to determine estrous cyclicity, and females having at least two consecutive estrous cycles were used. Rats were bilaterally ovariectomized 2 weeks before the blood or brain sampling to serve as the OVX group. Some OVX rats immediately received subcutaneous Silastic implants (i.d., 1.57 mm; o.d., 3.18 mm; 25 mm in length; Dow Corning, Midland, MI, USA) filled with E_2 (20 µg/ml peanut oil) for 1 week to serve as the OVX + low E_2 group. The low E_2 treatment was previously confirmed to produce a plasma E_2 level of 35.8 pg/ml and to produce a negative feedback effect on LH pulses but not to induce LH surges [30]. All surgeries were performed under ketamine/xylazine anesthesia and aseptic conditions. All rats were injected with antibiotics (Mycillin Sl; Meiji Seika, Tokyo, Japan) after any surgery. All experiments were conducted in accordance with the guidelines of the Committee of Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University, Japan.

Brain surgery

Some OVX and OVX + low E_2 rats were stereotaxically implanted with a stainless-steel guide cannula (22 G, Plastics One, Roanoke, VA, USA) for drug administration into the third cerebroventricle (3V) with its tip 0.8 mm posterior and 7.5 mm ventral to the bregma at the midline according to the rat brain atlas [31]. The rats were allowed a one-week recovery period prior to blood sampling.

Drug administration and blood sampling

To examine the effect of blockade of central KOR on pulsatile LH release, nor-BNI (Sigma-Aldrich, St. Louis, MO, USA), a selective KOR antagonist [23, 32, 33], was infused into the 3V at a dose of 20 µg/head. The dose of nor-BNI was chosen according to a previous study, in which central nor-BNI treatment increased LH pulse frequency and mean LH levels during midpregnancy in rats [24]. Nor-BNI (10 µg/µl) was dissolved in ultrapure water (UPW) and administered into the 3 V of OVX rats with/without low E_2 treatment at a flow rate of 1 µl/min for 2 min using a microsyringe pump (Eicom, Kyoto, Japan) through an inner cannula (28 G, Plastics One), which was inserted into the guide cannula. The drug was administered just after the first blood sampling at 1300 h. Control rats were infused with an equivalent volume of UPW in the same manner. Blood samples (100 µl) were collected every 6 min for 3 h from free-moving conscious rats via a silicone cannula (i.d., 0.5 mm; o.d., 1.0 mm; Shin-Etsu Polymer, Tokyo, Japan) that was 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 prepared in heparinized saline was replaced through the same atrial cannula after each blood collection. Plasma was separated by centrifugation and stored at –20 C until LH assay. At the end of the experiment, the animals were anesthetized and infused with 3% brilliant blue dye solution at the same flow rate as drug administration to check if the drug was infused into the 3 V. The brain was removed, and cannula placement and blue dye staining in the brain were visually verified. All animals used in the present study were confirmed to have correct 3V cannula placement and drug administration.

Radioimmunoassay (RIA) for LH

Plasma LH concentrations were measured using a rat LH RIA kit provided by the National Hormone and Peptide Program. The concentrations were expressed in terms of NIDDK rat LH RP-3. The least detectable LH concentration was 0.16 ng/ml for 50 µl of plasma, and the intra- and interassay co-efficient of variations were 8.81% at 2.60 ng/ml and 7.68% at 2.76 ng/ml, respectively.

In situ hybridization (ISH) for Pdyn, Kiss1 and Tac2

To detect *Pdyn*, *Kiss1* and *Tac2* mRNA expressions in the brain, we performed non-radioactive free-floating single ISH with rat *Pdyn*-, *Kiss1*- or *Tac2*-specific digoxigenin (DIG)-labeled probes in coronal sections (50-µm thickness) of the hypothalamus taken from OVX rats with/without low E_2 treatment as previously described [27]. DIG-labeled antisense cRNA probes for rat *Pdyn* (position 315-731; GenBank accession no. NM_019374), *Kiss1* (position 33-348; GenBank accession no. AY196983) and *Tac2* (position 180-483; GenBank accession no. NM_019162) were synthesized by *in vitro* transcription from the cDNA clones. Overnight hybridization with DIG-labeled cRNA probes was executed at 60 C. Hybridized probes were detected using an alkaline phosphatase-conjugated anti-DIG Fab fragment (Roche Diagnostics, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (Roche Diagnostics). Numbers of *Pdyn*-, *Kiss1*- and *Tac2*-expressing cells were counted under a light microscope, and the sum of the cell number in the ARC (11 sections) was obtained. The ARC area was identified according to the rat brain atlas [31].

Semiquantitative analysis of mRNA expressions of Pdyn and Oprk1 in the ARC-ME region by RT-PCR

The ARC-ME tissue of OVX rats with/without low E_2 was dissected out from the brain according to rat brain atlas as previously described [31, 34]. Total RNA was extracted from the ARC-ME tissue using ISOGEN reagents (Nippon Gene, Tokyo, Japan) according to the

Table 1.   Primer set sequences for RT-PCR used in this study

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Product size (bp)	GeneBank accession ID
Pdyn	GTTCCCTGTGTGCAGTGAGGAC	TAGCGTTTGGCCTGTTTTCTCA	418	NM_019374.3
Oprk1	GATGTCATTGAATGCTCCTTGC	CAGGATCATCAGGGTGTAGCAG	138	NM_017167.2
Actb	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTTCAAA	165	NM_031144.3

manufacturer's instructions. A total of 500 ng of RNA was used for reverse transcription using MultiScribe Reverse Transcriptase enzyme (Applied Biosystems, Foster City, CA, USA) to synthesize the cDNA. All primer sequences are described in Table 1. *Pdyn* and *Oprk1* were analyzed by RT-PCR. β-Actin (*Actb*) was used as the internal control. The RT-PCR for the mRNAs for *Pdyn, Oprk1* and *Actb* was performed using AmpliTaq Gold DNA Polymerase (Applied Biosystems) under the following conditions: 95 C for 5 min and 35 cycles of 94 C for 30 sec, 62 C for 1 min and 72 C for 1 min for *Pdyn*; 95 C for 5 min and 35 cycles of 94 C for 30 sec, 64 C for 1 min and 72 C for 1 min for *Oprk1*; and 95 C for 5 min and 30 cycles of 94 C for 30 sec, 60 C for 1 min and 72 C for 1 min for *Actb*. The final cycle was followed by a 10 min extension step at 72 C before reducing the temperature to 4 C for storage. The mRNA levels of *Pdyn* and *Oprk1* were semiquantified using the ImageJ software (version 1.45s; http://rsb.info.nih.gov/ij/). The intensity was then expressed as a value relative to that of the *Actb* amplicon.

Immunohistochemistry

Ovariectomized or low E_2-treated OVX rats were perfused with 4% paraformaldehyde at 1400 h, and their brains were quickly removed. Frozen coronal sections (50 μm) were obtained using a cryostat. Every sixth section through the ARC (6 sections, from 2.3 to 4.1 mm posterior to the bregma) from each rat was stained with mouse monoclonal anti-rat kisspeptin antibody (no. 254 outstretched in Takeda Pharmaceutical, Osaka, Japan), the cross-reactivity of which was described previously [26]. Brain tissue sections from each rat were incubated with the anti-rat kisspeptin antibody (1:50,000) for 24 h at room temperature, followed by incubation with Alexa Fluor 488-conjugated donkey antimouse IgG (1:800; Molecular Probes, Eugene, OR, USA). Then the sections were mounted with an antifade reagent (FluoroGuard; Bio-Rad, Hercules, CA, USA). Fluorescence images were obtained on an ApoTome microscope (ApoTome; Carl Zeiss, Jena, Germany). Six digital photomicrographs of each ARC per rat were processed by the ImageJ analysis software. The ARC area was outlined on the gray-scale image and processed for density measurement. Nonspecific background density points were eliminated using the same threshold for each rat.

Statistical analysis

LH pulses were identified using the PULSAR computer program [35] as previously described [36]. The statistical differences in LH pulse parameters were determined by the Student's *t*-test between nor-BNI-treated rats and vehicle-treated controls within OVX + low E_2 and OVX groups. The statistical differences in the mRNA expressions of *Pdyn* and *Oprk1* relative to *Actb* and kisspeptin immunoreactive areas between the OVX + low E_2 and OVX groups

were also determined by the Student's *t*-test.

Results

Effect of nor-BNI injection into 3V on pulsatile LH release in OVX rats with/without low E_2

Figure 1A shows LH profiles of representative OVX rats with a negative feedback level of E_2 that received a 3V injection of a KOR antagonist, nor-BNI, or vehicle. The mean LH level was significantly ($P < 0.05$, Student's *t*-test) higher in nor-BNI-injected animals compared with that in vehicle-treated controls. The mean baseline level of LH pulses was significantly ($P < 0.05$) higher in the animals treated with nor-BNI compared with that in controls (Fig. 1B), but the frequency and amplitude of LH pulses did not significantly differ between these two groups.

Figure 2A shows the representative profiles of LH release in OVX individuals treated with nor-BNI or vehicle. There were no significant differences in any LH pulse parameters between nor-BNI- and vehicle treated-OVX rats (Fig. 2B).

Effects of low E_2 treatment on numbers of Pdyn, Kiss1 and Tac2 mRNA-expressing cells in the ARC

Figure 3A shows the *Pdyn, Kiss1* and *Tac2* mRNA expressions in the ARC of representative OVX rats with or without a negative feedback level of E_2 treatment. We found that *Pdyn, Kiss1* and *Tac2* mRNA-expressing cells were abundantly located in the ARC in both OVX + low E_2 and OVX rats. There were no significant differences in the number of *Pdyn* mRNA-expressing cells in the ARC between the OVX + low E_2 and OVX groups (Fig. 3B). Similarly, there were no significant differences between OVX + low E_2 and OVX rats in the number of *Kiss1*- and *Tac2*-expressing cells.

Effects of low E_2 treatment on mRNA expressions of Pdyn and Oprk1 in the ARC-ME region

Representative images of mRNA expressions of *Pdyn* and *Oprk1* in the ARC-ME region of OVX + low E_2 and OVX rats are shown in Fig. 4A. Semiquantitative measurements revealed no significant differences in *Pdyn* and *Oprk1* mRNA expression levels between these two experimental groups (Fig. 4B).

Effects of low E_2 treatment on kisspeptin-immunoreactivity in the ARC

Kisspeptin-immunoreactive cell bodies and fibers were dense in the ARC of low E_2 -treated OVX rats (Fig. 5A), whereas few kisspeptin immunoreactivities were observed in OVX rats (Fig. 5A). ImageJ analysis showed that the kisspeptin-immunoreactive area in the ARC was significantly ($P < 0.05$, Student's *t*-test) higher
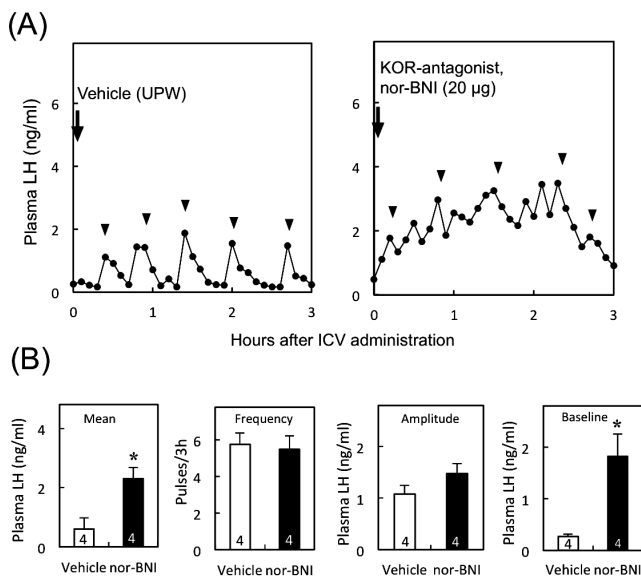
Fig. 1. Effect of central administration of KOR antagonist on pulsatile LH release in OVX rats treated with a negative feedback level of E_2 (low E_2). Profiles of LH release in representative OVX + E_2 rats treated with nor-BNI, a KOR antagonist, or vehicle (A). Nor-BNI or vehicle (UPW) was injected into the 3V immediately after the start of blood sampling (arrows). Blood samples were collected for 3 h at 6-min intervals. Arrowheads indicate the peaks of LH pulses identified by the PULSAR computer program. Mean plasma LH concentrations and frequency, amplitude and baseline levels of LH pulses in low E_2-treated OVX rats (B). Values are means ± SEM. The numbers in each column represent the numbers of animals used in each group. * P < 0.05 vs. the vehicle-treated controls (Student's t-test).
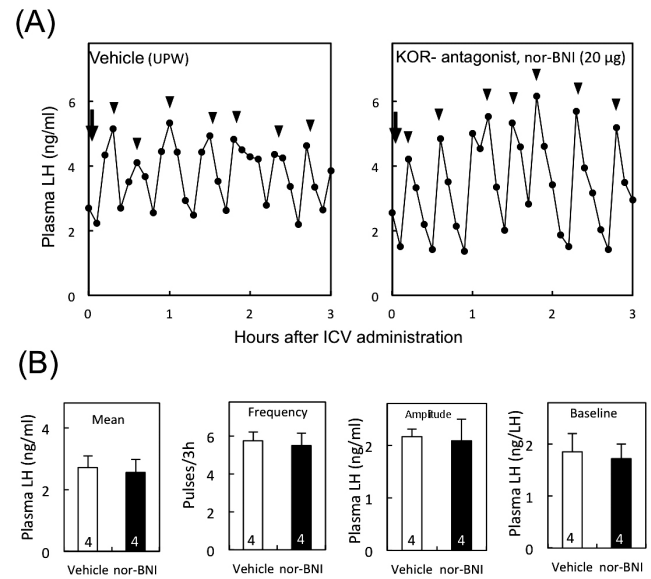
Fig. 2. Effect of central administration of KOR antagonist on pulsatile LH release in OVX rats without E_2 treatment. Profiles of LH release in representative OVX rats treated with nor-BNI or vehicle (arrows) (A). Arrowheads indicate the peaks of LH pulses. Mean plasma LH concentrations and frequency, amplitude and baseline levels of LH pulses in OVX rats (B). The numbers in each column represent the numbers of animals used in each group. See Fig. 1 for details.

in the OVX + low E_2 rats compared with the OVX rats (Fig. 5B).

Discussion

The present study demonstrates that Dyn-KOR signaling is involved in the estrogen negative feedback action on GnRH/LH release in adult female rats, because central nor-BNI administration significantly increased LH release only in the presence of a negative feedback level of E_2 in OVX rats. The Dyn-KOR signaling may be involved in the suppression of basal LH release but not in suppression of the GnRH/LH pulse generation system, because baseline levels of LH pulses were significantly increased but LH pulse frequency and amplitude were not affected by nor-BNI administration. Interestingly, the current low E_2 treatment did not affect the number of *Pdyn*-expressing cells and gene expression levels of *Pdyn* and *Oprk1* gene in the ARC. These results suggest that the negative feedback level of E_2 alters Dyn-KOR tone in brain areas other than the ARC. The present E_2 treatment increased kisspeptin immunoreactivity in the ARC KNDy neurons without affecting *Kiss1* mRNA expressions, suggesting that the current E_2 treatment inhibited the release of kisspeptin from the ARC kisspeptin neurons. ARC KNDy neurons are considered to play a role in GnRH pulse generation, and kisspeptin is a direct stimulator for GnRH release in several mammals [15, 17, 19, 22]. Thus, the present study suggests that Dyn-KOR signaling in other brain areas

than the ARC KNDy neurons is partly involved in estrogen negative feedback actions on GnRH/LH pulses via inhibition of kisspeptin release from KNDy neurons.

Involvement of KOR in suppression of LH release is consistent with previous studies that demonstrated a stimulatory effect of KOR antagonists on GnRH/LH secretion [17, 23–25]. In E_2-treated ewes, WIN 44,441-3, a KOR antagonist [37], increased LH pulse amplitude [38]. In addition, nor-BNI also reversed the inhibitory effect of progesterone on pulsatile LH secretion in the ewe by increasing the mean LH level and pulse frequency, suggesting that the Dyn-KOR signaling plays a major role in mediating progesterone negative feedback [25]. Central administration of nor-BNI increased LH pulse frequency and mean LH levels in pregnant rats, in which LH pulses are strongly suppressed [23, 24]. The LH pulse suppression during pregnancy could be more dependent on Dyn-KOR signaling than the suppression by estrogen negative feedback, because our results showed that nor-BNI increased the mean and baseline levels of LH pulses in low E_2-treated rats without affecting LH pulse frequency and amplitude.

The present study showed that the nor-BNI treatment failed to alter LH pulses in OVX rats, suggesting that Dyn-KOR signaling is not activated in the absence of E_2. On the other hand, central administration of nor-BNI increased the frequency of MUA volleys in OVX goats [17], suggesting that Dyn-KOR signaling plays a role in suppressing KNDy neuronal activity and then GnRH/LH pulses even in the absence of estrogen. The inconsistency between their results and ours may be due to the species difference in the role of Dyn-KOR in controlling GnRH release.
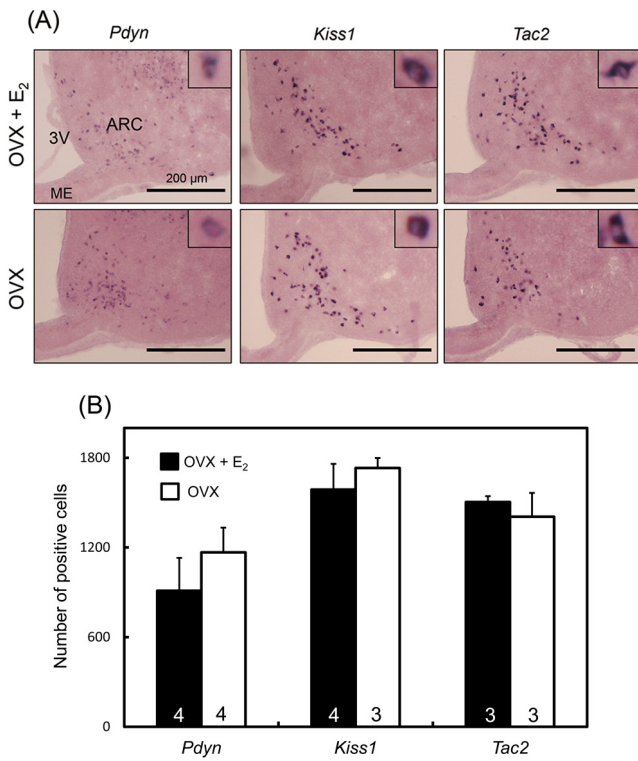
(A)

(B)

Fig. 3. *Pdyn, Kiss1* and *Tac2* mRNA expressions in the ARC in OVX + low E_2 and OVX rats determined by *in situ* hybridization. The upper and lower panels show the *Pdyn, kiss1* and *Tac2* mRNA expressions in representative rats in the OVX + low E_2 and OVX groups, respectively (A). Insets show the sections at higher magnification. ARC, arcuate nucleus; ME, median eminence; 3V, third ventricle. The numbers of *Pdyn, Kiss1* or *Tac2* mRNA-positive cells in the ARC of OVX + low E_2 and OVX groups (B). Values are means ± SEM. The numbers in each column represent the numbers of animals used in each group.
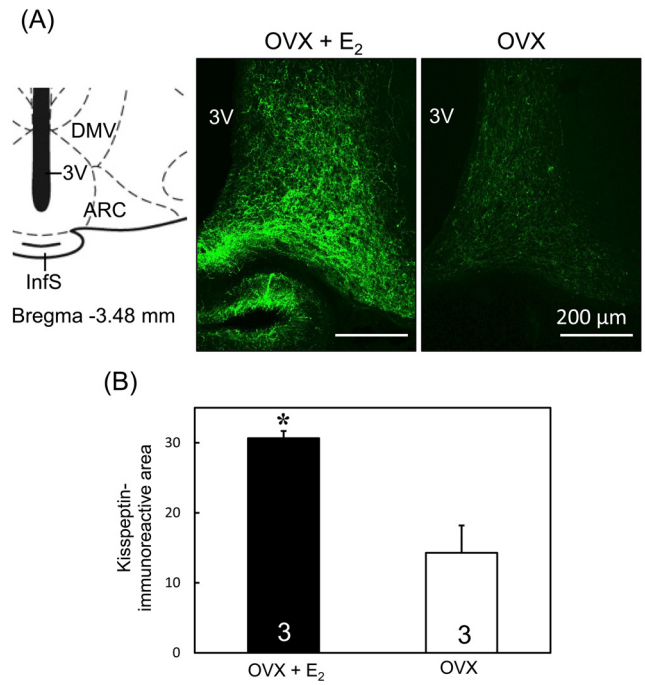
(A)

(B)

Fig. 5. Kisspeptin immunoreactivities in the ARC of OVX + low E_2 and OVX rats. Schematic illustration and photomicrographs of coronal sections of rat brains including the ARC (A). Schematic drawing of the ARC according to Paxinos and Watson [31] (left). The value in the drawing indicates the distance (mm) to the bregma. Photomicrographs (right) show kisspeptin immunoreactivity (green) in representative animals in the ARC of OVX + low E_2 and OVX groups. ARC, arcuate nucleus; DMV, ventral dorsomedial hypothalamic nucleus; InfS, infundibular stem; 3V, third ventricle. Scale bars: 200 μm. Semiquantitative analysis of kisspeptin immunoreactivity in the ARC of OVX + low E_2 and OVX rats was determined using the ImageJ software (B). Values are means ± SEM. The numbers in each column represent the numbers of animals used in each group.
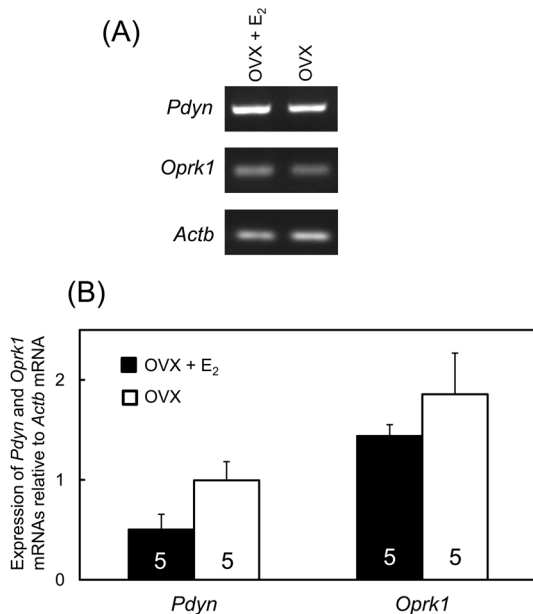
The present study demonstrated that the current E_2 treatment did not affect the numbers of *Pdyn-, Kiss1-* and *Tac2*-expressing cells as well as *Pdyn* and *Oprk1* expressions in the ARC-ME region in OVX rats. These results suggest that the ARC KNDy neurons may not be an estrogen action site that exerts a negative feedback effect on LH pulses via Dyn-KOR signaling. Dyn neurons are abundantly distributed in several regions in the rat brain, such as the hypothalamus, medulla-pons, midbrain and spinal cord [39]. In the rat hypothalamus, Dyn expressions are obvious in the paraventricular nucleus (PVN)

(A)

(B)

Fig. 4. *Pdyn* and *Oprk1* mRNA expressions in the ARC-ME region of OVX + low E_2 and OVX rats. Representative images of mRNA expressions of *Pdyn, Oprk1* and *Actb* in the ARC-ME region of the OVX + low E_2 and OVX groups (A). mRNA levels of *Pdyn* and *Oprk1* were determined semiquantitatively by RT-PCR with the primers shown in Table 1 (B). The analysis was carried out using the ImageJ software of the National Institutes of Health. Values are indicated as the expression relative to *Actb*, as an internal control. Values are means ± SEM. The numbers in each column represent the numbers of animals used in each group.

[40, 41], MBH and preoptic area (POA) [42]. Therefore, it is possible that some brain areas other than the ARC could be estrogen action sites that mediate the negative feedback effect on LH pulses via Dyn-KOR signaling in rats. Further studies are required to address this issue in more detail. The current low E_2 treatment increased ARC kisspeptin immunoreactivity without affecting *Kiss1* gene expressions in the nucleus. In terms of *Kiss1* gene expression, the present result is consistent with our previous reports, which showed that the negative feedback level of E_2 had no suppressive effect on ARC *Kiss1* gene expression. On the other hand, a number of studies showed that a positive feedback level of E_2 suppresses both Kiss1 and kisspeptin expressions in the ARC of female rodents [15, 27–29]. The increased ARC kisspeptin immunoreactivity caused by the current low E_2 treatment without an accompanying effect on *Kiss1* expression implies that kisspeptin release from the ARC KNDy neurons was diminished by the low E_2 treatment and that ARC kisspeptin was consequently increased.

Previous studies revealed that estrogen mainly exerts its feedback action to control GnRH/LH secretion through ERα, a receptor subtype in the brain [43–45]. This estrogen negative feedback action on LH secretion is considered to be mediated by nonclassical ERα action instead of classical ERα action [43, 45]. These studies suggest that estrogen response element (ERE)-independent ERα is sufficient to convey a major portion of estrogen negative feedback action on LH release. This nonclassical estrogen action involves interactions of ERα with DNA-bound transcription factors, such as specificity protein and activating protein 1 [46], whereas, in the classical genomic pathway, ERα binds to EREs in DNA to alter the transcription of genes [47]. Thus, it is conceivable that our negative feedback level of E_2 acts through the nonclassical ERα pathway without affecting the gene expressions of *Pdyn, Kiss1, Tac2* and *Oprk1* in the ARC-ME and may suppress the kisspeptin release from KNDy neurons. It is also possible that a membrane receptor, such as GPR30 [48], is involved in the estrogen negative feedback regulation of LH secretion. This mechanism should be investigated in greater detail in future studies.

Interestingly, recent studies showed that central NKB receptors rather than KOR would be involved in the suppression of GnRH/LH pulses in rats [49, 50]. These studies showed that central administration of NK3 receptor agonist increases LH pulse intervals, while both Dyn agonist and antagonist had no effect on LH pulses in diestrous rats and OVX rats treated with a negative feedback level of E_2. More recently, O'Byrne and colleagues showed that KOR in the ARC mediates LH pulse suppression induced by the activation of NKB receptors within the ARC in rats [50]. These findings suggest that roles of Dyn and NKB signaling in controlling GnRH/LH pulses are still controversial.

In conclusion, the present study suggests that Dyn-KOR signaling at least partly mediates estrogen negative feedback on GnRH/LH release by reducing kisspeptin release from the ARC KNDy neurons in female rats. The estrogen action sites that enhance the Dyn-KOR signaling would be in other brain regions than the ARC KNDy neurons. Further studies are warranted to address the precise mechanism involved in the estrogen negative feedback on GnRH/LH release via alteration of Dyn-KOR signaling.

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