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 E2). The mean LH concentrations and baseline levels of LH secretion in nor-BNI-injected, low E2-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 E2 treatment. These results suggest that Dyn is involved in the estrogen negative feedback regulation of pulsatile GnRH/LH release. The low E2 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
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-Iramichi 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 + low E$_2$ treatment was previously confirmed to have correct 3V cannula placement 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.

**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 as previously described [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 3 V 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].

**Semi-quantitative 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
was then expressed as a value relative to that of the software (version 1.45s; http://rsb.info.nih.gov/ij/). The intensity Oprk1 Pdyn and levels of C before reducing the temperature to 4°C for storage. The mRNA

30 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 1 min for Actb. 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; 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 semi-quantified 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 E2-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 E2 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 E2 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 E2**

Figure 1A shows LH profiles of representative OVX rats with a negative feedback level of E2 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 E2 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 E2 treatment. We found that Pdyn, Kiss1 and Tac2 mRNA-expressing cells were abundantly located in the ARC in both OVX + low E2 and OVX rats. There were no significant differences in the number of Pdyn mRNA-expressing cells in the ARC between the OVX + low E2 and OVX groups (Fig. 3B). Similarly, there were no significant differences between OVX + low E2 and OVX rats in the number of Kiss1- and Tac2-expressing cells.

**Effects of low E2 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 E2 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 E2 treatment on kisspeptin-immunoreactivity in the ARC**

Kisspeptin-immunoreactive cell bodies and fibers were dense in the ARC of low E2-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

### 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  | GTCCTCCTGTGTCAGTGAGGAC    | TAGGCCTTGGCCTGTTTCTCA     | 418               | NM_019374.3           |
| Oprk1 | GATGTCAATTGAAATCTCTTTCG   | CAGGATCATCAAGGGTGTAGCAG   | 138               | NM_017167.2           |
| Actb  | TGTCACCAACTGGGACGATA      | GGGGTGTGGAAGGTCTTCAAA     | 165               | NM_031144.3           |

**Gene**

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|---------------------------|---------------------------|-------------------|-----------------------|

2-treatment 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.

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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 immunoactivity 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.
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).
expressions in the nucleus. In terms of ERα binds to EREs in DNA to alter the transcription of genes [47].

activating protein 1 [46], whereas, in the classical genomic pathway, with DNA-bound transcription factors, such as specificity protein and release. This nonclassical estrogen action involves interactions of ERα action to control GnRH/LH secretion through ERα, a receptor subtype.

ARC kisspeptin was consequently increased. KNDy neurons. Further studies are warranted to address the precise roles of Dyn and NKB signaling in controlling GnRH/LH pulses in rats [49, 50]. These studies showed that central NKB receptors mediate the negative feedback effect on LH pulses via the current low E2 treatment without an accompanying effect on Kiss1 expression. 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 E2 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 E2 had no suppressive effect on ARC Kiss1 gene expression. On the other hand, a number of studies showed that a positive feedback level of E2 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 E2 treatment without an accompanying effect on Kiss1 expression implies that kisspeptin release from the ARC KNDy neurons was diminished by the low E2 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 E2 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 certain 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 O VX rats treated with a negative feedback level of E2. 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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