GnRH(1-5), a metabolite of gonadotropin-releasing hormone, enhances luteinizing hormone release via activation of kisspeptin neurons in female rats

Nahoko Ieda¹, Assadullah¹, Shiori Minabe¹, Kana Ikegami¹, Youki Watanabe¹, Yusuke Sugimoto¹, Arisa Sugimoto¹, Narumi Kawai¹, Hirotaka Ishii², Naoko Inoue¹, Yoshihisa Uenoyama¹ and Hiroko Tsukamura¹

¹Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan
²Department of Anatomy and Neurobiology, Nippon Medical School, Tokyo 113-8602, Japan

Abstract. Accumulating evidence suggests that kisspeptin neurons in the arcuate nucleus (ARC), which coexpress neurokinin B and dynorphin, are involved in gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) pulse generation, while the anteroventral periventricular nucleus (AVPV) kisspeptin neurons are responsible for GnRH/LH surge generation. The present study aims to examine whether GnRH(1-5), a GnRH metabolite, regulates LH release via kisspeptin neurons.

GnRH(1-5) was intracerebroventricularly injected to ovariectomized and estrogen-treated Wistar-Imamichi female rats. Immediately after the central GnRH(1-5) administration at 2 nmol, plasma LH concentration increased, resulting in significantly higher levels of the area under the curve and baseline of plasma LH concentrations compared to vehicle-injected controls. On the other hand, in Kiss1 knockout rats, GnRH(1-5) administration failed to affect LH secretion, suggesting that the facilitatory effect of GnRH(1-5) on LH release is mediated by kisspeptin neurons.

Double in situ hybridization (ISH) for Kiss1 and Gpr101, a GnRH(1-5) receptor gene, revealed that few Kiss1-expressing cells coexpress Gpr101 in both ARC and AVPV. On the other hand, double ISH for Gpr101 and Slc17a6, a glutamatergic marker gene, revealed that 29.2% of ARC Gpr101-expressing cells coexpress Slc17a6. Further, most of the AVPV and ARC Kiss1-expressing cells coexpress Grin1, a gene encoding a subunit of NMDA receptor. Taken together, these results suggest that the GnRH(1-5)-GPR101 signaling facilitates LH release via indirect activation of kisspeptin neurons and that glutamatergic neurons may mediate the signaling. This provides a new aspect of kisspeptin- and GnRH-neuronal communication with the presence of stimulation from GnRH to kisspeptin neurons in female rats.

Key words: GnRH metabolite, KNDy neurons, Kiss1-KO rats, G protein-coupled receptor
ognition that kisspeptin neurons are hub neurons that integrate and transfer upstream neuronal signaling to regulate GnRH/gonadotropin release.

A previous study suggested that the GnRH metabolite suppresses LH release at pituitary level, because a peripheral administration of an inhibitor for endopeptidase EP24.15, that cleaves GnRH, enhanced the LH secretion in rats [18]. The pentapeptide GnRH(1-5), a metabolite of GnRH, is considered to be generated by digestion of GnRH secreted from GnRH neurons in the hypothalamus, because the immunoreactivity of EP24.15 is detected in tanycytic fibers in close apposition to GnRH neuronal terminals in the median eminence [18]. A previous study showed that a central administration of GnRH(1-5) enhanced lordosis in rats, and GnRH receptor antagonist failed to attenuate the effect of the pentapeptide [19], suggesting that GnRH(1-5) is involved in regulating sexual behavior by acting its own receptor but not the conventional GnRH receptor. On the other hand, the possibility that the GnRH metabolite centrally regulates GnRH-LH release via hypothalamic kisspeptin neuronal activity has been yet to be clarified. The hypothalamic distribution of the GnRH metabolite receptor(s) is also unknown. Considering the important role of kisspeptin neurons to integrate the internal and external cues to regulate GnRH/LH secretion, we hypothesized that a central GnRH metabolite may directly or indirectly regulate kisspeptin release.

In the present study, thus, we examined the effect of a central administration of GnRH(1-5), a GnRH metabolite, on LH release in female rats to investigate the possibility that this metabolite regulates GnRH-LH release at the hypothalamic level. We also investigated if the central GnRH(1-5) challenge affects LH secretion in Kiss1-KO rats to confirm whether the metabolite is an upstream regulator for kisspeptin neurons but not for gonadotrophs. Further, we performed double in situ hybridization (ISH) for Gpr101, a GnRH(1-5) receptor gene [20], and Kiss1 in the hypothalamus of female rats to examine the distribution of a GnRH(1-5) receptor and determine if kisspeptin neurons could be a direct target of the GnRH metabolite. We also performed double ISH for Gpr101 and Slc17a6, a glutamatergic marker gene, in the ARC and Kiss1 and Grin1, gene of a subunit of NMDA receptor, in the ARC and AVPV of female rats to investigate the possibility that glutamatergic neurons may mediate the effect of GnRH metabolite on kisspeptin neurons and then LH release, because glutamates stimulate LH release by affecting kisspeptin neurons in female rats [6].

Materials and Methods

Animals and treatments

Wistar-Imamichi wild-type, Kiss1-tdTomato heterozygous and Kiss1 KO [6] female rats at 8-15 weeks of age, 180–290 g body weight were used. They were housed under controlled temperature (23°C ± 2°C) and light conditions (lights on, 0500–1900) with free access to standard chow (CE-2; CLEA Japan, Tokyo, Japan) and tap water. Vaginal smears of the wild-type and Kiss1-tdTomato heterozygous rats were checked daily and those showing two consecutive estrous cycles were used. The body weight of Kiss1 KO female rats was checked daily for at least one week instead of checking their estrous cycles, to familiarize the animals with handling, because these animals lack vaginal opening and estrous cycles. All surgeries were conducted under anesthesia using ketamine (5%)/xylazine (2%) (2:1 mixture, 80 μL/100 g body weight) with the additional use of isoflurane inhalation. All animal experiments were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Intracerebroventricular injection of GnRH(1-5) and rat kisspeptin-52

One week prior to the GnRH(1-5) injection, wild-type Wistar-Imamichi or Kiss1 KO female rats were ovarioctomized (OVX) and implanted with Silastic tubing filled with estradiol-17β (E2; 20 μg/mL, Sigma Aldrich, St. Louis, MO) (OVX + low E2) to produce a negative feedback level of plasma E2 levels at 35.8 pg/mL [21], and a stainless-steel guide cannula (22G; Plastics One, Roanoke, VA) was stereotaxically inserted into the third ventricle as described previously [22]. Briefly, the tip of the guide cannula was placed at 0.8 mm caudal to the bregma and 7.5 mm below the surface of the skull in its midline. GnRH(1-5) (Sigma-Aldrich) (0.4, 2 or 10 nmol/2 μL ultra-pure water), rat kisspeptin-52 (rKp-52; Peptide Institute, Osaka, Japan) (0.1 nmol/2 μL ultra-pure water) or vehicle was injected into the third ventricle through an inner cannula (28G; Plastics One) inserted to the guide cannula with a microsyringe pump (EICOM, Kyoto, Japan) at a flow rate of 1 μL/1 min. At the end of blood sampling, the animals were anesthetized and injected with 2 μL brilliant blue solution (3%) through the inner cannula to verify the cannula placement.

Blood sampling and LH assay

Blood samples from free-moving conscious rats were taken as described previously [22]. The first blood sample was taken through the silicon cannula inserted into the right atrium, immediately before the 0, 0.4, 2 or 10 nmol GnRH(1-5) icv injection, followed by continuous sam-
plunging at 6-min intervals for 3 hours. In four Kiss1 KO rats, 2 weeks after ovariectomy, 0.1 nmol rKp-52 was injected into the third ventricle 1.5 hours after the 2 nmol GnRH(1-5) injection. The order of the injection was reversed in two Kiss1 KO rats, namely 2 nmol GnRH(1-5) injection 1.5 hours after 0.1 nmol rKp-52 injection, to examine whether the rKp-52 priming would affect the effect of GnRH(1-5) on LH secretion.

LH concentrations in 50 μL of plasma samples were determined by a rat LH radioimmunoassay kit provided by the National Hormone and Peptide Program, and were expressed in terms of NIDDK rat LH RP-3 using anti-rat LH antibody (Lot number AFPC69701P and AFP240580). The minimal and the maximum detectable levels for 50 μL of plasma samples were 0.156 ng/mL and 20 ng/mL for Lot AFPC69701P and 0.078 ng/mL and 10 ng/mL for Lot AFP240580, respectively. Intra- and inter-assay coefficients of variation were 12.5% and 10.4%, respectively at 1.14 ng/mL. Baseline, amplitude and frequency of LH pulse were determined by the PULSAR computer program [23].

**Quantitative PCR for Gpr101**

The littermate wild-type of Kiss1 KO rats were OVX for 2 weeks, and the Kiss1 KO rats were also OVX for 2 weeks to undergo the same surgical procedure as the wild-type rats. Immediately after decapitation, the whole hypothalamus was dissected out and homogenized in 400 μL of ISOGEN (Wako Pure Chemical Industries, Osaka, Japan) to extract total RNA. Reverse transcription (RT) reaction was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Expression of Gpr101 was determined by quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems) and 7500 Real Time PCR System (Applied Biosystems). The cycling protocol used was: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The specificity of the amplification products was confirmed by dissociation curve analysis (60–95°C) after 40-cycle amplification. It was considered that a single DNA sequence was amplified during PCR when a distinct melting curve was detected with the sense probe. The numbers of Kiss1- and Gpr101-expressing cells and Kiss1- and Gpr101 mRNA expression were hybridized at 60°C overnight. Hybridized Kiss1 or Slc17a6 probe was detected using peroxidase-conjugated anti-fluorescein Fab fragment (Sigma Aldrich) and TSA Plus FITC Kit (Perkin Elmer, Waltham, MA), following the instructions from manufacturers. After inactivation of the peroxidase by incubating the brain sections in 0.1 N hydrochloric acid for 30 minutes, the Gpr101 or Grin1 probe was detected using anti-digoxigenin Fab fragment (Sigma Aldrich), TSA Plus Biotin Kit (Perkin Elmer) and DyLight 594 conjugated streptavidin (Thermo Fisher Scientific K.K., Yokohama, Japan). Brain sections were observed under a confocal fluorescence microscope (Carl Zeiss, Tokyo, Japan) and photographs of Gpr101- and Kiss1-expressing, Grin1- and Kiss1-expressing or Gpr101- and Slc17a6-expressing cells were obtained. Full-length sequence in coding region of Gpr101 was used as cRNA probe for hybridization. To detect Grin1 mRNA expression, two cRNA probes were applied as a mixture, which were synthesized using two pairs of primers. Specificity of cRNA probes for Gpr101 (GenBank accession number NM_001108258.1, nucleotide 224-1754), Kiss1 (GenBank accession number NM_053427.1, nucleotide 769-1678 and 1687-2609) and Grin1 (GenBank accession number NM_01270602.1 nucleotide 769-1678 and 1687-2609) was examined by comparing the signals between adjacent series of brain sections treated with anti-sense or sense probe. Gpr101, Slc17a6, and Grin1 expression was detected exclusively with the antisense probe, but no signal was detected with the sense probe. The numbers of Gpr101- and Kiss1-expressing cells and Kiss1- and Grin1-expressing cells were bilaterally counted at every 100 μm in the AVPV and at every 200 μm in the ARC. The number of Gpr101- and Slc17a6-expressing cells was unilaterally counted at every 200 μm in the ARC.

**Statistical analysis**

Statistical computing software R (https://www.r-project.org) was used for all analysis. The statistical significance of the differences between the 2 groups was...
compared using Student’s $t$-test, with $p < 0.05$ being significant. Comparison among multiple groups was performed using Kruskal-Wallis test followed by Dunn’s test with Benjamini-Hochberg adjustment, with $p < 0.05$ being significant, because the plasma LH concentration in response to the central GnRH(1-5) injection showed non-normal distribution.

### Results

**LH stimulation by central GnRH(1-5) administration in wild-type female rats**

Fig. 1A shows plasma LH profiles of two representative animals from each group; vehicle- ($n = 10$), 0.4 nmol GnRH(1-5)- ($n = 7$), 2 nmol GnRH(1-5)- ($n = 8$) and 10 nmol GnRH(1-5)-injected ($n = 4$) OVX + low $E_2$ rats. The mean ± SEM of the area under the curve (AUC) and baseline of plasma LH concentration and amplitude and frequency of LH pulses at respective dose are indicated in Fig. 1B. The AUC of plasma LH concentrations was significantly higher in 2 nmol GnRH(1-5)-injected group compared with the vehicle-injected controls (Fig. 1B) ($p < 0.05$, Kruskal-Wallis test followed by Dunn’s test with Benjamini-Hochberg adjustment). The baseline of plasma LH concentrations was significantly higher in 2 and 10 nmol GnRH(1-5)-injected group compared with the vehicle-injected controls ($p < 0.05$). The LH pulse amplitude tended to increase by 2 nmol GnRH(1-5), but there was no statistical difference in amplitude and frequency of the LH pulses between any groups.

**Failure of LH stimulation by central GnRH(1-5) challenge in Kiss1 KO rats**

The central challenge of 2 nmol GnRH(1-5) failed to affect LH secretion in the Kiss1 KO rats ($n = 4$), whereas 0.1 nmol rKp-52 following the GnRH(1-5) challenge immediately increased plasma LH levels in all animals as shown in the plasma LH profile in a representative Kiss1 KO female rat (Fig. 2A). Under kisspeptin-primed conditions ($n = 2$), 2 nmol GnRH(1-5) also failed to enhance

---

**Fig. 1** Effect of central GnRH(1-5) challenge on LH secretion in wild-type female rats. (A) Representative plasma LH profiles in OVX + low $E_2$ female rats bearing vehicle or GnRH(1-5) administrations. Vehicle ($n = 10$), 0.4 nmol GnRH(1-5) ($n = 7$), 2 nmol GnRH(1-5) ($n = 8$) or 10 nmol GnRH(1-5) ($n = 4$) was injected into the third ventricle of the animals at a flow rate of 1 μL/1 min after collecting the first blood sample, followed by continuous blood sampling at 6-minute intervals for 3 hours. Arrowheads are peaks of LH pulses detected by PULSAR Computer Program. (B) AUC and baseline of plasma LH concentration and amplitude and frequency of the LH pulses in OVX + low $E_2$ rats treated with vehicle, 0.4, 2 or 10 nmol GnRH(1-5). The bar plots and error bars indicates mean ± SEM of each parameter, respectively. Different alphabets indicate the medians being significantly different compared with each other ($p < 0.05$, Kruskal-Wallis test followed by Dunn’s test). AUC, area under the curve.
LH secretion in Kiss1 KO female rats. The AUC of plasma LH concentration after the injection of rKp-52 (totally n = 6) was significantly higher than that after GnRH(1-5) (Fig. 2B, p < 0.05 by Student’s t-test). As shown in Fig. 2C, there was no significant difference in the expression levels of Gpr101 in the whole hypothalamus between wild-type (n = 3) and Kiss1 KO female rats (n = 3; p = 0.80 by Student’s t-test).

Fig. 2 Failure of stimulation of LH secretion by GnRH(1-5) in Kiss1 KO rats. (A) Representative plasma LH profiles of Kiss1 KO rats injected with 2 nmol GnRH(1-5) and 0.1 nmol rKp-52 (n = 4). 2 nmol GnRH(1-5) was injected into the third ventricle of Kiss1 KO rats at a flow rate of 1 μL/1 min after collecting the first blood sample, followed by continuous blood sampling at 6-minute intervals. One and a half hours after the GnRH(1-5) administration, 0.1 nmol rKp-52 was injected to the third ventricle in the same manner, and blood sampling was continued for another 1.5 hours. Arrows indicate the timing of injection. (B) Statistical analysis of the effect of GnRH(1-5) on plasma LH concentration in Kiss1 KO rats. There was a significant difference (p < 0.05 by paired t-test; n = 4) between the mean plasma LH concentration after the injection of GnRH(1-5) and of rKp-52. (C) Gene expression of Gpr101 in the hypothalamus in wild-type (indicated in closed bars) and Kiss1 KO rats (indicated in white bars), determined by quantitative RT-PCR. Expression level of the target mRNA was normalized by Actb. No significant difference was found between the two genotypes in any gene expression (p = 0.80, Student’s t-test). AUC, area under the curve.

Distribution of Gpr101-expressing cells in the rat hypothalamus with double in situ hybridization for Kiss1 and Gpr101

Gpr101 expression was abundantly detected in the mediobasal hypothalamus mainly in the ARC in female rats. In addition, Gpr101 expression was detected in several other forebrain and hypothalamic nuclei, such as the ventromedial preoptic nucleus (VMPO), paraventricular nucleus (PVN), supraoptic nucleus and supraoptic recessus (SOR) (Fig. 3). On the other hand, very few Gpr101-expressing cells were detected in the AVPV. Little Gpr101 expression was detected in the extra-hypothalamic brain regions, except for the ventral part of the cortex, the parastrial nucleus and the amygdaloid nucleus.

Fig. 4A–4D show Kiss1- and Gpr101-expressing cells, visualized by double ISH, in the AVPV and ARC in representative OVX + low E2 wild-type female rats and quantitative analysis of the double ISH results. A large number of Gpr101-expressing cells were closely located around the ARC Kiss1-expressing cells (Fig. 4C), but Gpr101 signals were detected in few ARC Kiss1-expressing cells (0.13% ARC Kiss1-expressing neurons; 1.67 ± 0.67 neurons out of 1,276 ± 139 Kiss1-expressing neurons, n = 3) (Fig. 4D). Some Gpr101-positive cells were found in the AVPVs (Fig. 4A), but no co-localization of Gpr101 and Kiss1 was detected in the AVPV (Fig. 4B).

Expressions of Gpr101 in glutamatergic cells and of Grin1, a NMDA receptor gene, in Kiss1-expressing cells determined by double in situ hybridization for Gpr101/Slc17a6 and Kiss1/Grin1

Fig. 5A shows Gpr101 and Slc17a6, a marker gene of glutamatergic receptor, visualized by double ISH in the ARC in representative OVX + low E2 wild-type female rats. Slc17a6 expression was detected in 29.2%, nearly one third, of Gpr101-expressing cells in the ARC (328 ± 16 cells out of 1,096 ± 65 Gpr101-expressing cells, n = 4, Fig. 5B). Kiss1 and Grin1, a gene encoding subunit of NMDA receptor, were visualized by double ISH in the ARC in representative OVX + low E2 wild-type female rats (Fig. 5C). Majority (94.2%) of Kiss1-expressing cells coexpressed Grin1 in the ARC (930 ± 25 cells out of 987 ± 12 Kiss1-expressing cells, n = 3; Fig. 5D). Coexpression of Grin1 was also detected in majority of Kiss1-expressing cells in the AVPV (94.9%; 411 ± 30 cells expressing Grin1 out of 434 ± 36 Kiss1-expressing cells, n = 3, Fig. 5E and 5F).

Discussion

The present study demonstrated that GnRH(1-5), an N-terminal pentapeptide derived from GnRH, enhances
Fig. 3  Representative photographs of Gpr101-expressing brain regions in the hypothalamus. The signals of Gpr101 expression was detected in the hypothalamic nuclei such as the VMPO (Bregma 0.12 mm), PVN (Bregma –1.56 mm), ARC (Bregma –2.28 mm and –3.60 mm), and SOR (Bregma –2.28 mm). 3V, third ventricle; Och, optic chiasm; AVPV, anteroventral periventricular nucleus; VMPO, ventromedial preoptic nucleus; PVN, paraventricular nucleus; ARC, arcuate nucleus; SOR, supraoptic retrochiasm; scale bar, 1 mm.

Fig. 4  Kiss1- and Gpr101-expression in the AVPV and the ARC. (A) Representative photograph showing Kiss1- (green) and Gpr101- (magenta) expression in the AVPV. (B) Number of Kiss1-expressing cells in the AVPV. No coexpression of Gpr101 was detected in the AVPV. (C) Representative photograph of Kiss1- (green) and Gpr101- (magenta) expressing cells in the ARC. Inset shows a magnified cell body expressing both Kiss1 and Gpr101. (D) Number of Kiss1-expressing cells and Kiss1- and Gpr101-coexpressing cells in the ARC. Among the Kiss1-expressing cells in the ARC, 0.13% showed coexpression of Gpr101. 3V, third ventricle; scale bar, 100 μm.
GnRH/LH release through stimulation of the kisspeptin neurons in female rats, because central GnRH(1-5) administration increased LH release in wild-type rats, but failed to stimulate it in Kiss1 KO rats. To our knowledge, this is the first report to show that the GnRH metabolite stimulates gonadotropin release via affecting kisspeptin.
neurons. Interestingly, few ARC and AVPV kisspeptin neurons expressed Gpr101, a GnRH(1-5) receptor, suggesting the possibility that GnRH(1-5) indirectly activates kisspeptin neurons through interneurons. Glutamatergic neurons, at least in part, may mediate the facilitatory effect of GnRH(1-5) on kisspeptin neurons and then GnRH/LH release, because coexpression of Slc17a6 was evident in a part (nearly one third) of ARC Gpr101-expressing neurons and the majority of the kisspeptin neurons in the ARC and the AVPV coexpressed Grin1, a subunit of ionotropic glutamatergic receptor in female rats. These results suggest that GnRH(1-5) stimulates GnRH/LH release probably via affecting glutamatergic interneurons and consequently ARC and AVPV kisspeptin neurons. This notion is consistent with our previous study demonstrating that glutamatergic stimulation of LH release is mediated by kisspeptin neurons in female rats [6]. On the other hand, it is unlikely that the GnRH metabolite stimulates the anterior pituitary gonadotrophs or GnRH neurons, because GnRH(1-5) failed to stimulate LH secretion in Kiss1-KO rats in the present study. Besides, expression level of Gpr101 were comparable in Kiss1-KO rats to their wildtype littermates, suggesting that the responsiveness of LH secretion to GnRH(1-5) in the Kiss1-KO rats was not due to downregulated Gpr101 expression. Taken together, the stimulatory effect of the GnRH metabolite to kisspeptin neurons could be involved in the control of hypothalamic-pituitary-gonadal (HPG) axis, in addition to the classical, one-way directed signaling from kisspeptin to GnRH neurons.

It is speculated that the stimulatory effect of GnRH(1-5) on LH release via activating hypothalamic kisspeptin neurons could be involved in GnRH/LH surge generation rather than the pulse generation, because of the significant increase in the AUC and baseline levels of plasma LH but not in frequency and amplitude of LH pulses after the central GnRH(1-5) challenge. In this context, AVPV kisspeptin neurons might partly mediate the current GnRH metabolite challenge via glutamatergic interneurons, because the AVPV kisspeptin neurons, that are well accepted to be responsible for LH surge, coexpressed glutamate receptors. In addition to AVPV kisspeptin neurons, ARC kisspeptin neurons may mediate the effect of GnRH(1-5) challenge to enhance the surge-like increase in LH release. Involvement of ARC kisspeptin neurons and hypothalamic glutamatergic neurons in GnRH/LH surge generation has been suggested as follows: an increase in c-Fos expressions in the ARC kisspeptin neurons was shown prior to GnRH/LH surge in rats [24] and ewe [25, 26]; female rats with attenuated ARC kisspeptin neurons by neurotoxin showed limited magnitude of LH surge [27]; an increase in in vivo glutamate release prior to LH surge is evident in the medio-basal hypothalamus including the ARC in female rats [28]; glutamate strongly stimulated in vitro GnRH release from the ARC-median eminence fragment of female rats, but the stimulating effect was moderate with the fragment without the ARC [29]. In rodents, it is considered that majority of ARC kisspeptin neurons are glutamatergic ones as well, because nearly 90% of ARC kisspeptin neurons expressed glutamate transporter mRNA in mice [30]. Hence, it is also possible that kisspeptin neurons facilitate their own activity by glutamate in an autocrine/paracrine manner. The idea of involvement of GnRH(1-5) signaling in GnRH/LH surge generation would be supported by the previous studies indicating that GnRH concentration in the third ventricle is increased up to the nano molar scale during the GnRH surge in ewe [31]; EP24.15, an enzyme which cleaves full-length GnRH to GnRH(1-5), is expressed in some GnRH neuron terminals and tanyocytes in the median eminence, where the strongest immunoreactivity of EP24.15 is evident during the early proestrus period in rats [18]. Therefore, it is speculated that the concentration of GnRH(1-5) in the central spinal fluid may increase during the proestrus period in rats. Further study is required to measure the concentration of GnRH(1-5) in the central spinal fluid in which the metabolite is distinguished from the full length GnRH. It is considered that the surge-enhancing role of GnRH(1-5) is unique and not shared with the decapptide GnRH, because the full length GnRH is reported to show no effect on LH secretion when infused into the third ventricle of ewes [32].

The current central GnRH(1-5) administration at doses of 0.4 nmol to 2 nmol GnRH(1-5) increased LH secretion in a dose-dependent manner, while the response diminished at the highest dose (10 nmol) of GnRH(1-5) in the present study. Given the wide and abundant expression of Gpr101 observed in the hypothalamic and extra-hypothalamic regions in the present study, such as in the VMPO, PVN, SON, cortex, parastral nucleus and amygdaloid nucleus, it is plausible that the highest dose of GnRH(1-5) activated not only glutamatergic and kisspeptin neuronal pathway but also multiple other neurons, including inhibitory neuronal pathway that may affect kisspeptin-GnRH-LH secretion. Previous studies suggested that several neuropeptides, such as leptin and alpha-melanocyte-stimulating hormone (α-MSH), have a regulatory role of kisspeptin neurons, because those neuropeptide receptors are expressed in the ARC KNDy neurons and/or AVPV kisspeptin neurons in mice [30, 33] and sheep [34]. On the other hand, RFRP-3, a neuropeptide that belongs to RFamide peptide family and a mammalian ortholog of avian gonadotropin inhibitory hormone, is suggested to play an inhibitory role in GnRH-LH secretion [35] via direct modulation of kiss-
GnRH(1-5) receptor, expressed in the ARC glutamergic neurons, both stimulatory and inhibitory ones, are involved in regulation of LH release by GnRH(1-5)-GPR101 signaling in the rat hypothalamus.

In conclusion, the present study demonstrated that GnRH(1-5), a metabolite of GnRH, simulates LH release via affecting kisspeptin neurons and that GPR101, a GnRH(1-5) receptor, expressed in the ARC glutameric neurons may mediate the GnRH(1-5)-induced LH release via glutamergic receptors expressed in AVPV and ARC kisspeptin neurons in female rats. This study provides the first evidence for the presence of a reciprocal stimulatory pathway from GnRH to kisspeptin neurons in female rats, in addition to the one-directed classical signaling from kisspeptin to GnRH neurons.

Declaration of interest

The authors declare that there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgements

We respectfully acknowledge the contributions of our late colleague Dr. Kei-ichiro Maeda, who suddenly passed away during the preparation of this manuscript. His leadership, supervision and original ideas contributed greatly to this work. We thank the National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases and Dr AF Parlow for providing the LH assay kit. We also thank Drs GR Merriam and KW Wachter for the PULSAR computer program. Radioimmunoassay and pulse-analysis were performed at Radioisotope Research Center and Information Technology Center, Nagoya University, respectively. We thank Dr. Nicola Skoulding (Nagoya University) for editorial assistance. This work was supported in part by JSPS KAKENHI Grant Number 16K18783 (to Nle), 16H06742 (to YW), 18H03973; 19H03103 (to NIn) and 26252046; 18H03973; 18K19267 (to HT). This study was also supported in part by the following research grants to the late Kei-ichiro Maeda, PhD and DVM of The University of Tokyo: Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry; and Grants-in-Aid No. 24380157 from the JSPS.

References

1. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierino JS Jr, et al. (2003) The gpr54 gene as a regulator of puberty. N Engl J Med 349: 1614–1627.
2. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, et al. (2003) Hypogonadotropic hypogonadism due to loss of function of the kiss1-derived peptide receptor gpr54. Proc Natl Acad Sci U S A 100: 10972–10976.
3. Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, et al. (2003) The kiss-1 receptor gpr54 is essential for the development of the murine reproductive system. Biochem Biophys Res Commun 312: 1357–1363.
4. d’Anglemont de Tassigny X, Fagg LA, Dixon JP, Day K, Leitch HG, et al. (2007) Hypogonadotropic hypogonadism in mice lacking a functional kiss1 gene. Proc Natl Acad Sci USA 104: 10714–10719.
5. Lapatto R, Pallais JC, Zhang D, Chan YM, Mahan A, et al. (2007) Kiss1−/− mice exhibit more variable hypogonadism than gpr54−/− mice. Endocrinology 148: 4927–4936.
6. Uenoyma Y, Nakamura S, Hayakawa Y, Ikegami K, Watanabe Y, et al. (2015) Lack of pulse and surge modes and glutamatergic stimulation of luteinizing hormone release in Kiss1 knockout rats. J Neuroendocrinol 27: 187–197.
7. Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, et al. (2004) A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145: 4073–4077.
8. Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, et al. (2004) Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of kiss-1 mrna in the male rat. Neuroendocrinology 80: 264–272.
9. Plant TM, Ramaswamy S, Dipietro MJ (2006) Repetitive activation of hypothalamic g protein-coupled receptor 54 with intravenous pulses of kisspeptin in the juvenile monkey (macaca mulatta) elicits a sustained train of gonadotropin-releasing hormone discharges. Endocrinology 147: 1007–1013.
10. Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, et al. (2007) Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin a and neurokinin b. Endocrinology 148: 5752–5760.
11. Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, et al. (2009) Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/ neurokinin b neurons in the arcuate nucleus of the mouse.
Endocrine Journal Advance Publication

12. Ohkura S, Takase K, Matsuyama S, Mogi K, Ichimaru T, et al. (2009) Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. J Neuroendocrinol 21: 813–821.

13. Wakabayashi Y, Nakada T, Murata O, Ohkura S, Mogi K, et al. (2010) Neurokinin b and dynorphin a in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. J Neurosci 30: 3124–3132.

14. Goodman RL, Hileman SM, Nestor CC, Porter KL, Connors JM, et al. (2013) Kisspeptin, neurokinin b, and dynorphin act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. Endocrinology 154: 4259–4269.

15. Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA (2006) Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. J Neurosci 26: 6687–6694.

16. Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, et al. (2007) Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. J Reprod Dev 53: 367–378.

17. Clarkson J, d’Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbsion AE (2008) Kisspeptin-grpr54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. J Neurosci 28: 8691–8697.

18. Wu TJ, Pierotti AR, Jakubowski M, Sheward WJ, Glucksman MJ, et al. (1997) Endopeptidase ec 3.4.24.15 presence in the rat median eminence and hypophysial portal blood and its modulation of the luteinizing hormone surge. J Neuroendocrinol 9: 813–822.

19. Wu TJ, Glucksman MJ, Roberts JL, Mani SK (2006) Facilitation of lordosis in rats by a metabolite of luteinizing hormone releasing hormone. Endocrinology 147: 2544–2549.

20. Cho-Clark M, Larco DO, Semsarzadeh NN, Vasta F, Mani SK, et al. (2014) GnRH-(1–5) transactivates EGFR in Ishikawa human endometrial cells via an orphan G protein-coupled receptor. Mol Endocrinol 28: 80–98.

21. Cagampang FR, Maeda KI, Tsukamura H, Ohkura S, Ota K. (1991) Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile lH release in ovariectomized rats. J Endocrinol 129: 321–328.

22. Pheng V, Uenoyma Y, Homma T, Inamoto Y, Takase K, et al. (2009) Potencies of centrally- or peripherally-injected full-length kisspeptin or its c-terminal decapetide on LH release in intact male rats. J Reprod Dev 55: 378–382.

23. Merriam GR, Wachtcr KW (1982) Algorithms for the study of episodic hormone secretion. Am J Physiol 243: E310–E318.

24. Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyma Y, et al. (2005) Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 146: 4431–4436.

25. Smith JT, Qi P, Pereira A, Clarke IJ (2009) Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. Endocrinology 150: 5530–5538.

26. Merkley CM, Porter KL, Coolen LM, Hileman SM, Billings HJ, et al. (2012) Kiss1 (kisspeptin/neurokinin b/dynorphin) neurons are activated during both pulsatile and surge secretion of Lh in the ewe. Endocrinology 153: 5406–5414.

27. Helena CV, Toporikova N, Kalil B, Stathopoulos AM, Pogrebna VV, et al. (2015) Kiss1 neurons modulate the magnitude of the steroid-induced luteinizing hormone surges in ovariectomized rats. Endocrinology 156: 4200–4213.

28. Murahashi K, Nagatani S, Maeda KI, Tsukamura H (1998) Increase in in vivo glutamate release in the medio basal hypothalamus during progesterone-enhanced lH surge in estrogen-primed ovariectomized rats. J Reprod Dev 44: 135–140.

29. Murahashi K, Tsukahara S, Tsukamura H (2002) The arcuate nucleus mediates facilitating effect of estrogen on glutamate-induced in vitro GnRH release from nerve terminals of female rats. J Reprod Dev 48: 183–188.

30. Cravo RM, Margatho LO, Osborne-Lawrence S, Donato Jr, Atkin S, et al. (2011) Characterization of kiss1 neurons using transgenic mouse models. Neuroscience 173: 37–56.

31. Skinner DC, Caraty A, Malpaux B, Evans NP (1997) Simultaneous measurement of gonadotropin-releasing hormone in the third ventricular cerebrospinal fluid and hypothalamic portal blood of the ewe. Endocrinology 138: 4699–4704.

32. Skinner DC, Caraty A, Evans NP (1998) Does gonadotropin-releasing hormone in the cerebrospinal fluid modulate luteinizing hormone release? Neuroendocrinology 67: 37–44.

33. Smith JT, Acobido BV, Clifton DK, Steiner RA (2006) Kiss-1 neurones are direct targets for leptin in the ob/ob mouse. J Neuroendocrinol 18: 298–303.

34. Backholer K, Smith JT, Rao A, Pereira A, Iqbal J, et al. (2010) Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide y and proopiomelanocortin cells. Endocrinology 151: 2233–2243.

35. Pineda R, Garcia-Galiano D, Sanchez-Garrido MA, Romero M, Ruiz-Pino F, et al. (2010) Characterization of the potent gonadotropin-releasing activity of rf9, a selective antagonist of rf-amide-related peptides and neuropeptide ff receptors: physiological and pharmacological implications. Endocrinology 151: 1902–1913.

36. Poling MC, Quennell JH, Anderson GM, Kauffman AS (2013) Kisspeptin neurons do not directly signal to rfpe-3 neurones but rfpe-3 may directly modulate a subset of hypothalamic kisspeptin cells in mice. J Neuroendocrinol 25: 876–886.