

Supplementary Material for

Sex difference in developmental changes in visualized *Kiss1* neurons in newly generated *Kiss1-Cre* rats

Koki Yamada¹, Mayuko Nagae¹, Tetsuya Mano¹, Hitomi Tsuchida¹, Safiullah Hazim¹, Teppei Goto^{1,2},
Makoto Sanbo², Masumi Hirabayashi², Naoko Inoue¹, Yoshihisa Uenoyama¹, Hiroko Tsukamura¹

¹ Laboratory of Animal Reproduction, Graduate School of Bioagricultural Sciences, Nagoya University,
Nagoya, Aichi 464-8601, Japan

² Section of Mammalian Transgenesis, Center for Genetic Analysis of Behavior, National Institute for
Physiological Sciences, Okazaki, Aichi 444-8787, Japan

Materials and Methods

Animals. *Kiss1-Cre* knock-in rats were generated in this study, and *Cre*-dependent tdTomato reporter rats [LE-Tg(Gt(ROSA)26Sor-CAG-tdTomato)24Jfhy rats [1]] were supplied by the National BioResource Project - Rat (NBRP Rat No. 0734), Kyoto University (Kyoto, Japan), with support in part from the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Tokyo, Japan. Iar:Wistar-Imamichi (RGD ID: 125097496) rats were supplied by the Institute for Animal Reproduction (Kasumigaura, Japan), and *Kiss1*-floxed rats were generated in our laboratory [2]. The animals were maintained in a room with a 14:10 h light/dark cycle (lights on 05:00 h) at 22 ± 3 °C with free access to food (CE-2; CLEA Japan, Tokyo, Japan) and water. Female rats showing two consecutive regular estrous cycles, as determined by vaginal smears, were mated overnight with male rats on the day of proestrus. Pregnant females were identified based on the presence of vaginal plugs and housed individually. The day when newborn litter was found at noon was designated as postnatal day 0. Genotypes were analyzed by PCR using DNA obtained from newborn pups within 5 days. The primers used are listed in Supplementary Table S1. The litter size (8–10 pups) was adjusted by postnatal day 5 to minimize growth variation within and between litters. Pups were weaned on postnatal day 20 or 21. All surgical procedures were performed under anesthesia with a mixture of ketamine (26.7 mg/kg, Fujita Pharmaceutical, Tokyo, Japan)-xylazine (5.3 mg/kg, Bayer, Leverkusen, Germany), and isoflurane inhalation (1%–3% in air, Pfizer Japan, Tokyo, Japan). Care of the animals and all of the experimental procedures performed in the present study were reviewed and approved by the Animal Experiment Committees of Nagoya University and the National Institutes of Natural Sciences.

Gene targeting and generation of *Kiss1-Cre* knock-in rats. To generate *Kiss1-Cre* knock-in rats, a targeting vector was designed to replace the coding sequence of the *Kiss1* gene with *Cre* and neomycin-resistance genes via homologous recombination (Fig. 1A). Rat embryonic stem (ES) cells, WDB/Nips-

ES1 (Rat Genome Database identification: 10054010), were cultured and transfected with the targeting vector via electroporation, as described previously [3]. ES clones were selected in a neomycin-supplemented medium. The targeted ES clone was transfected with pCAGGS-FLPe to excise the neomycin-resistance gene. Targeted ES subclones were confirmed by PCR and Southern blot analyses (Supplementary Fig. S1, A and B). Primers used for ES selection are listed in Supplementary Table S1. A correctly targeted ES clone was used to generate germline chimeric rats. Targeted ES cells were microinjected into the blastocoelic cavity of *Prdm14* knockout blastocysts by crossing *Prdm14*^{HV/+} (RGD ID: 125097491) and *Prdm14*^{mut/+} (RGD ID: 41457452) rats [4]. These blastocysts were transferred to pseudopregnant female rats under isoflurane anesthesia (2%–3% in air). The resultant chimeric males were coupled with Crj:WI (RGD ID: 2312504) females (Charles River Laboratories Japan, Kanagawa, Japan). One of the two chimera rats produced germline pups with ES cell-derived genomes. *Kiss1-Cre* knock-in female rats were transported from the National Institute of Physiological Sciences to Nagoya University and were coupled with Iar:Wistar-Imamichi male rats for at least two generations.

Generation of *Kiss1-KpKO* rats. To generate *Kiss1-KpKO* rats, *Kiss1-Cre* rats were coupled with *Kiss1*-floxed rats. In the resultant *Kiss1-KpKO* rats, Cre recombinase was theoretically expressed with *Kiss1* because *Cre* was knocked in at the *Kiss1* locus, and Cre-mediated recombination would result in the removal of exons 2 and 3 of the *Kiss1* gene encoding the 52-amino acid rat kisspeptin in *Kiss1*-floxed allele.

Blood sampling for analyses of pulsatile LH release in the *Kiss1-KpKO* male rats. The adult (8 weeks of age) *Kiss1-KpKO* male rats and littermate *Cre* (-)/*Kiss1*-floxed control male rats were castrated, and their testes were weighed. Two weeks after the castration, the animals were subjected to blood sampling to determine LH pulses. A silicon cannula (inner diameter 0.5 mm and outer diameter 1.0 mm; Shin-Etsu Polymer, Tokyo, Japan) was inserted into the right atrium through the jugular vein 1 day before the onset

of blood sampling. Blood samples (100 μ L) were collected from freely moving conscious Cast rats every 6 min for 3 h (13:00 to 16:00 h), and plasma samples were collected after the centrifugation at 4 °C. An equivalent volume of rat red blood cells, obtained from donor rats and diluted with heparinized saline, was passed through the atrial catheter after each blood collection to maintain a constant hematocrit.

Brain and pituitary sampling from the *Kiss1*-KpKO male rats. After blood sampling, the animals were deeply anesthetized with sodium pentobarbital (40 mg/kg; Tokyo Chemical Industry, Tokyo, Japan) and intracardially perfused with 4% paraformaldehyde (PFA; Sigma-Aldrich Japan, Tokyo, Japan). The brains were immediately removed and postfixed in the same fixative overnight at 4 °C. The brains were then immersed in 30% sucrose in 0.05 M phosphate buffer until the brains sank at 4 °C. Frozen frontal brain sections containing the ARC (50- μ m thickness) were prepared using a cryostat (CM1800, Leica Biosystems, Wetzlar, Germany). Every fourth section of the hypothalamus was used for *in situ* hybridization to visualize *Kiss1* in the ARC. The pituitary was also collected and stored at -80 °C for analyses for *Lhb*, *Fshb*, and *Gnrhr* mRNA expression.

***Kiss1* in situ hybridization in the *Kiss1*-KpKO male rats.** To examine the expression of *Kiss1* mRNA in the ARC of *Kiss1*-KpKO rats, the brain sections from the adult *Kiss1*-KpKO male rats and *Cre(-)/Kiss1*-floxed control rats were hybridized overnight at 60 °C with 1 μ g/mL DIG-labeled *Kiss1* cRNA probe [5]. After hybridization, the DIG-labeled probe was detected using an alkaline phosphatase-conjugated anti-DIG antibody (1:500; Roche Diagnostics, Basel, Switzerland) and a chromogen solution (0.338 mg/mL 4-nitroblue tetrazolium chloride (NBT; Roche Diagnostics) and 0.175 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP; Roche Diagnostics)). The brain sections were cover-slipped with 90% glycerol in 0.05 M PB. *Kiss1* mRNA expression was observed under a light microscope (BX53; Olympus, Tokyo, Japan).

Radioimmunoassay (RIA) for LH and LH pulse parameter analysis. Plasma LH concentrations were determined by double-antibody RIA with a rat LH-RIA kit provided by the National Hormone and Peptide Program (NHPP). The lowest detectable level in the LH assay was 3.9 pg/tube, and the intra-assay coefficient of variation was 9.3% at 34 pg/tube. LH pulses were identified using the PULSAR computer program [6]. The mean LH concentration and frequency of LH pulses were calculated during the 3-h sampling period for each individual and then for the group.

qRT-PCR for analyses of pituitary gene expression. Total RNA was extracted from fixed hemipituitary tissues using the RNeasy FFPE kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. The cDNA from each sample was synthesized with oligo (deoxythymidine) primer at 37 °C by using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Gene expression levels were determined using QuantStudio3 (Applied Biosystems) with Thunderbird SYBR Green qPCR Mix (TOYOBO, Osaka, Japan) as described previously [7]. Forward and reverse primers for *Lhb* (encoding LH β -subunit) (NM_012858.2, NM_001033975.1), *Fshb* (encoding FSH β -subunit) (NM_001007597.2), *Gnrhr* (GnRH receptor) (NM_031038.3), and *Actb* (encoding β -actin) (NM_031144.3) are listed in Supplementary Table S1. The specificity of the amplification products was confirmed by dissociation curve analysis. The relative gene expression levels were normalized to *Actb*, and the fold changes between the adult *Kiss1*-KpKO male groups and the *Cre* (-) control groups were calculated using the $2^{-\Delta\Delta CT}$ method.

Generation of *Kiss1*-visualized rats. To generate *Kiss1*-visualized rats, *Kiss1-Cre* rats were coupled with *Cre*-dependent tdTomato reporter rats. In the resulting *Kiss1-Cre*: tdTomato rats, *Cre* recombinase was theoretically expressed with *Kiss1* because *Cre* was knocked into the *Kiss1* locus. *Cre*-mediated recombination results in the removal of the transcriptional stop cassette in the tdTomato reporter transgene and subsequent constitutive expression of tdTomato in potential kisspeptin neurons.

Gonadectomy and estrogen treatment of the *Kiss1*-visualized rats. To examine the effect of estrogen on *Kiss1* and tdTomato signaling, adult *Kiss1-Cre*: tdTomato female rats (8–16 weeks of age) with at least two consecutive 4-day estrous cycles and adult male rats (8–16 weeks of age) were gonadectomized for two weeks to serve as OVX and Cast rats, respectively. Some OVX or Cast rats were immediately implanted with subcutaneous Silastic tubing (1.57-mm inner diameter; 3.18-mm outer diameter; 25 mm in length; Dow Corning, Midland, MI) filled with E2 (Sigma-Aldrich, St Louis, MO, USA) dissolved in peanut oil (Sigma-Aldrich) at 20 µg/mL to produce a diestrus level of plasma E2 for 5 days. The E2 tubing was replaced with another tube containing E2 dissolved in peanut oil at 1000 µg/mL to produce a proestrus level of plasma E2 for 2 days to serve as OVX + E2 and Cast + E2 rats, respectively. This high E2 level induces an LH surge in OVX rats [8]. Brain samples from OVX rats with or without E2 treatment were double stained for *Kiss1* mRNA using *in situ* hybridization and for tdTomato using immunohistochemistry. Brain samples from OVX and Cast rats with or without E2 treatment were examined for tdTomato fluorescence signals.

Brain tissue preparation and sectioning from the *Kiss1*-visualized rats. To examine the developmental changes and sex differences in *Kiss1-Cre*-activated tdTomato signals, brains were obtained from gonad-intact female and male rats at 1 (neonatal), 7, and 19 days (only for the detection of *Kiss1* expression in the VMH), and 3 (prepubertal), 7 (postpubertal), and 10–13 weeks (adulthood) of age. After puberty, female brain samples were collected during diestrus from gonad-intact females, whose estrous cycles were monitored via daily vaginal smears.

The animals, except neonates (at postnatal days 1 and 7), were deeply anesthetized with sodium pentobarbital (40 mg/kg) and intracardially perfused with 0.05 M PBS and 4% PFA. Neonates were anesthetized on ice and intracardially perfused with 0.05 M PBS and 4% PFA. The brains were collected, postfixed in the same fixative overnight, and immersed in 30% sucrose in 0.05 M PBS at 4 °C for 3–4

days until the brains sank. Frozen frontal sections (neonates, 25- μ m thickness; others, 50- μ m thickness) containing the AVPV (from 0.60 mm anterior to -1.44 mm posterior to the bregma in adulthood) and the ARC, VMH, and MeA (from -1.44 to -4.56 mm posterior to the bregma in adulthood) were prepared using a cryostat (CM1800, Leica Biosystems). Every second AVPV section and every fourth ARC/VMH/MeA section (every second section in neonates) were used to analyze *Kiss1* mRNA expression, tdTomato fluorescent signals, and tdTomato immunoreactivity.

Double staining for *Kiss1* *in situ* hybridization and tdTomato immunohistochemistry in the *Kiss1*-visualized rats. To examine the co-expression of *Kiss1* mRNA and tdTomato protein in the AVPV and ARC of OVX and OVX+E2 rats, free-floating brain sections that included the AVPV and ARC were incubated with an anti-DsRed antibody (1:500; Takara Bio, Kusatsu, Japan, AB_10013483) overnight at 4 °C then incubated with a biotinylated anti-rabbit IgG antibody (1:200; Vector Laboratories, Burlingame, CA, USA) at room temperature for 2 h, as described previously [9]. The brain sections were fixed again with 10% formalin neutral buffer solution (Wako Chemicals, Osaka, Japan) for 10 min and rinsed with 0.05 M PBS containing 0.2% glycine. The sections were incubated with 1 μ g/mL digoxigenin (DIG)-labeled *Kiss1* cRNA probe (position 33–349, AY196983) overnight at 60 °C, as described previously [8]. The hybridized DIG-labeled probe was detected using a peroxidase (POD)-conjugated anti-DIG antibody (Roche Diagnostics) and the TSA Plus Fluorescein System (1:100; Akoya Biosciences, Marlborough, MA, USA). Sections were incubated with avidin-biotin complex (ABC) reagents (Vector Laboratories) for 1 h, followed by Alexa Fluor 594-conjugated streptavidin (1:500; Thermo Fisher Scientific, Waltham, MA, USA) for 2 h to detect the tdTomato protein.

***Kiss1* *in situ* hybridization in the *Kiss1*-visualized rats.** To examine the expression of *Kiss1* mRNA, brain sections obtained from adult gonadectomized females and males with/without E2 treatment were hybridized overnight at 60 °C with 1 μ g/mL fluorescein isothiocyanate (FITC)-labeled *Kiss1* cRNA probe.

The hybridized FITC-labeled probe was detected using a peroxidase (POD)-conjugated anti-FITC antibody (Roche Diagnostics) and the TSA Plus Fluorescein System (1:100).

To examine the temporal expression of *Kiss1* mRNA in the VMH, brain sections from gonad-intact male and female rats at postnatal day 19 were hybridized overnight at 60 °C with 1 µg/mL DIG-labeled *Kiss1* cRNA probe [5] because no *Kiss1* mRNA expression was detected in the VMH of male and female rats at 3 weeks of age (data not shown). After hybridization, the DIG-labeled probe was detected using an alkaline phosphatase-conjugated anti-DIG antibody (1:500; Roche Diagnostics) and a chromogen solution (0.338 mg/mL 4-nitroblue tetrazolium chloride (NBT; Roche Diagnostics) and 0.175 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP; Roche Diagnostics)). The sections were cover-slipped with 90% glycerol in 0.05 M PB. *Kiss1* mRNA expression was observed under a light microscope.

Quantitative analysis of the number of *Kiss1*-expressing and/or tdTomato-positive cells in the *Kiss1*-visualized rats. Brain sections were mounted on glass slides and cover-slipped with ProLong Diamond Antifade Mountant (Invitrogen, Carlsbad, CA, USA). *Kiss1* and tdTomato (fluorescence or immunoreactivity) signals in brain sections were observed under a fluorescence microscope with ApoTome optical sectioning (ZEISS, Jena, Germany). The total number of tdTomato fluorescence-positive cells in the AVPV, ARC, VMH, and MeA of male and female rats at 1 and 7 days of age was estimated by multiplying the data by two because the cells were counted in every second section. The total number of tdTomato fluorescence-positive cells in the AVPV of males and females at 3, 7, and 10–13 weeks of age was estimated by multiplying the data by two, because the cells were counted in every second section. The total number of fluorescence-positive cells in the ARC, VMH, and MeA at 3, 7, and 10–13 weeks of age was estimated by multiplying the data by four, because the cells were counted in every fourth section. The total number of *Kiss1*-expressing and tdTomato-IR cells in the AVPV of male and females at 8–16 weeks of age was estimated by multiplying the data by two, because the cells were counted in every second section. The total number of *Kiss1*-expressing and tdTomato-IR cells in the ARC,

VMH, and MeA at 8–16 weeks of age was estimated by multiplying the data by four, because the cells were counted in every fourth section. The percentage of tdTomato-IR- and *Kiss1*-expressing cells was calculated by dividing the number of *Kiss1*- and tdTomato-double-positive cells by the total number of *Kiss1*-expressing or tdTomato-IR cells in the AVPV and ARC.

The distribution and number of *Kiss1*-expressing and tdTomato fluorescence-positive cells in the AVPV were further analyzed because the cell distribution exhibited sexual dimorphism. *Kiss1*-expressing and tdTomato-IR cells in the AVPV of OVX + E2 rats and fluorescence-positive cells in the AVPV of OVX and Cast rats, with or without E2, were analyzed as described previously [10]. Briefly, the AVPV regions were divided into six layers in the mediolateral direction (every 50 μm). Cells in each layer were counted unilaterally, and the total number of cells was estimated by multiplying the cell counts by two because the cells were counted every second section.

Statistical analysis. Significant differences in the number of ARC *Kiss1*- expressing cells; LH pulse parameters; pituitary *Lhb*, *Fshb*, and *Gnrhr* mRNA levels; and testicular weights between the groups were determined using Welch's t-test. Significant differences in the number of *Kiss1*-expressing or tdTomato IR- or fluorescence-positive cells in the AVPV, ARC, MeA, and VMH between groups were determined using two-way ANOVA (E2 treatment and sex or age and sex as the main effects) or three-way ANOVA (E2 treatment, sex, and location in the AVPV as the main effects), followed by Tukey–Kramer's multiple comparisons test using js-STAR XR+ release 1.2.0 j (<http://www.kisnet.or.jp/nappa/software/star/index.htm>).

Supplementary Table

Table S1. Primer list

Primer	Sequence
<i>Kiss1-Cre</i> Forward for genotyping	5'-CCTTGTTTGGGGCTTATCCT-3'
<i>Kiss1-Cre</i> Reverse_1 for genotyping	5'-CTTTTCCGGGATGGTGTGTA-3'
<i>Kiss1-Cre</i> Reverse_2 for genotyping	5'-TTGCCCTGTTTCACTATCC-3'
tdTomato Forward for genotyping	5'-GCGAGGAGGTCATCAAAGAG-3'
tdTomato Reverse for genotyping	5'-GATGACGGCCATGTTGTTGT-3'
5' Forward for ES selection	5'-AGTGTGCTCCAAC TACCCAAGT-3'
5'/Cre Reverse for ES selection	5'-TGCATGATCTCCGGTATTGA-3'
3'/Cre Forward for ES selection	5'-AGCCGAAATTGCCAGGATCA-3'
3' Reverse for ES selection	5'-TTTCTCCGGGTCTTGTCATC-3'
Forward for making DNA probe for Southern blotting	5'-GTGTGTGTGTGTGTCTGCAT-3'
Reverse for making DNA probe for Southern blotting	5'-ACTGACCCTTGACCTGGATG-3'
<i>Lhb</i> Forward for qRT-PCR	5'-ATGAGTTCTGCCCAGTCTGC-3'
<i>Lhb</i> Reverse for qRT-PCR	5'-TGGGGAAGGTCACAGGTCAT-3'
<i>Fshb</i> Forward for qRT-PCR	5'-AGCTGTTGACTTACCTGGCC-3'
<i>Fshb</i> Reverse for qRT-PCR	5'-GGGTGTTTGGTCTAGCTGGG-3'
<i>Gnrhr</i> Forward for qRT-PCR	5'-CCAGCCTTCATGATGGTGGT-3'
<i>Gnrhr</i> Reverse for qRT-PCR	5'-GGGATGATGAACAGGCAGCT-3'
<i>Actb</i> Forward for qRT-PCR	5'-TGTCACCAACTGGGACGATA-3'
<i>Actb</i> Reverse for qRT-PCR	5'-GGGGTGTTGAAGGTCTCAA-3'

Supplementary Figures

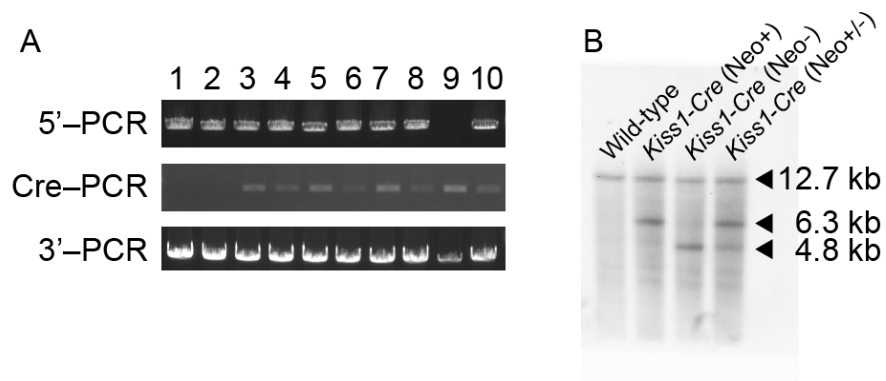


Fig. S1. Generation of *Kiss1-Cre* rats. (A) Screening of ES cell clones using polymerase chain reaction (PCR) and three sets of primers (5'-region, Cre-region, and 3'-region), as shown in Fig. 1A. (B) Southern blot analysis of BstXI-digested DNA using the probe on the 3'-region detected 12.7-, 6.3-, and 4.8-kb fragments in wild-type *Kiss1* allele, targeted *Kiss1-Cre* allele with Neo and *Kiss1-Cre* allele without Neo, respectively. The predicted sizes of the DNA fragments are shown by double arrows in Fig. 1A.

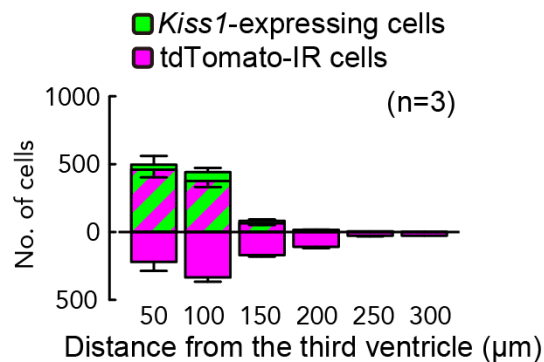


Fig. S2. Detailed analysis of the distribution of anteroventral periventricular nucleus (AVPV) *Kiss1*-expressing and tdTomato-immunoreactive (IR) cells in estradiol-17 β (E2)-treated ovariectomized (OVX) rats. (A) The numbers of *Kiss1*-expressing (green), tdTomato-IR (magenta), and double-positive cells in every 50- μ m layer (from medial to lateral) of the AVPV of OVX + E2 rats in adulthood. Sample size, n = 3.

Supplementary References

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