Supporting Information

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SI Materials and Methods

Animals and Tissue Preparation. C57BL/6 strain mice were purchased from Charles River Japan. The mice were kept under a lighting regimen of 14 h light/10 h darkness and were allowed free access to food and water. The vaginal smear was checked to determine the stage of the estrous cycle. Some animals were ovariectomized for 1 wk (OVX model), and some OVX mice were implanted s.c. with Silastic tubing (i.d., 1.5 mm; o.d., 3.0 mm; length, 18 mm) filled with estradiol-17β (E2) (Sigma) dissolved in peanut oil at 200 μg/mL to produce a preovulatory level of plasma E2 (OVX+E2 model). We confirmed that the E2 treatment induces luteinizing hormone (LH) surges that peaked at 18:00 h as shown in Fig. S4. Brain tissues were collected between 14:00 h and 16:00 h, before the expected peak of LH surges. Blood samples were collected by heart puncture ($n = 4$ or 5 for each time point). OVX and OVX+E2 mice were perfused with paraformaldehyde for *Kiss1* in situ hybridization. Coronal brain sections were subjected to in situ hybridization followed by cell sampling with laser-capture microdissection.

To prepare genomic DNA and total RNA, brains were removed immediately after decapitation. Coronal brain slices containing anteroventral periventricular nucleus (AVPV) at 1.5-mm thickness were obtained with a brain blocker (ASI Instruments), and the AVPV region was punched out using stainless steel tubing (i.d. 0.5 mm; Fine Science Tools). The arcuate nucleus (ARC) was dissected out with a microblade (Feather Safety Razor). All samples were frozen immediately in liquid nitrogen and stored at −80 °C until used for DNA/RNA extraction.

The present study was approved by the Committees on Animal Experiment of the Graduate School of Bioagricultural Science, Nagoya University, and by the National Institute for Basic Biology.

Cell Culture. Mouse hypothalamic N6 and N7 cell lines were purchased from Cellutions Biosystems Inc. The *Kiss1* gene has been demonstrated to be expressed in the N7 cell line but not in the N6 cell line. We confirmed *Kiss1* expression in N7 cell line under the following conditions: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; final extension, 72 °C for 10 min (Fig. S2). These cells were cultured in DMEM (Life Technologies) containing 10% (vol/vol) FBS (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C in 5% (vol/vol) CO_2. Both cell lines were cultured for 2 d and then for 2 additional d after the addition of trichostatin A (Sigma-Aldrich) at 200 nM and/or 5-aza-2′-deoxycytidine (Sigma-Aldrich) at 1 μM.

Trophoblast stem cells derived from C57BL/6 mice were grown in RPMI 1640 medium (Invitrogen) supplemented with 37.5 ng/mL FGF4 (PeproTech), 1.5 μg/mL heparin (Sigma-Aldrich), and 80% (vol/vol) mouse embryonic fibroblast cell-conditioned medium. Differentiation of trophoblast stem cells was induced by the medium without FGF4, heparin, and conditioned medium.

Determination of Transcription Start Sites of *Kiss1* in Hypothalamus. To determine the transcription start sites for *Kiss1* in the AVPV and ARC, 5′ RACE and 3′RACE PCR were conducted with the GeneRacer kit (Invitrogen) according to the manufacturer's instructions. cDNAs were prepared from RNA adaptor-ligated total RNA. Subsequent PCR amplification was performed using LA-Taq DNA polymerase (Takara) and the GeneRacer 5′ primer together with the gene-specific primer TTGCTCTCTGCATACCGCGATTCCT. Nested amplification with GeneRacer 5′-nested primer was conducted together with the gene-specific primer CTTTTCCCAGGCATTAACGAGTTCC. Detected PCR products were cloned and sequenced.

RT-PCR Analysis. To examine RNA expression, 1 μg total RNA isolated from cells and tissues with Isogen (Nippon Gene) was treated with DNase I (Invitrogen) and reverse transcribed using random hexamers and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was carried out using *Kiss1*-specific forward primers a–d (a: GTGGACTGAAGCATGATCTCC; b: CCCAAGCTGTGAATGTATCTGA; c: CGCTGTGTGTTCCAACTACC; d: GCTGCTGCTTCTCCTCTGTGT) and a common reverse primer e (GCATACCGCGATTCCTTTTC) or an *Actb*-specific primer set (TGTTACCAACTGGGACGACA and TCTCAGCTGTGGTGGTGAAG). Each PCR was performed under the following conditions: for *Kiss1*, 95 °C for 5 min; 36 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; final extension, 72 °C for 10 min; for *Actb*, 95 °C for 5 min; 25 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; final extension, 72 °C for 10 min.

Plasmid Construction. The 5′-flanking fragments of the *Kiss1* gene-containing sequences −5198 (A), −2630 (B), −1089 (C), or −180 (D) to +18 were isolated by genomic PCR using specific forward primers (A: GCTAGCGTTTTCTGACTGCCCCACAT; B: GGTACCGCAGCTGGTGACATCAAGAA; C: GGTACCCCTGCCCTTAAGGTGTGTGT; and D: CTTGCTAGCGCAGTCTTGGTGATTTCTGG) and a common reverse primer (GATATCTGGCTCCTGGGCTTACTCTA). The forward primers include the NheI or KpnI site at the 5′ end of the sequence, and the reverse primer includes the EcoRV site. Each PCR product was cloned into pGL4-Basic vector (Promega), a promoterless luciferase vector. The resultant plasmid constructs were designated as pGL4-A to pGL4-D (−5198 to +18, −2630 to +18, −1089 to +18, and −180 to +18) according to the respective positions of the fragments.

Transfection and Promoter Luciferase Assays. The hypothalamic N7 cells were replated on 24-well plates at 2.5×10^4 cells per well 12 h before transfection and were cultured under the conditions described above. All constructs (1 μg) were transfected into these cells using Lipofectamine Reagent 2000 (Invitrogen) with rat estrogen receptor α expression vector (pcDNA-rERα) and herpes simplex virus 1-thymidine kinase promoter-driven Renilla luciferase reporter vector (pRL-TK). Twenty-four hours after transfection, each cell was treated with 10 nM E2 dissolved in ethanol and was incubated further for 24 h. Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) 48 h after transfection according to the manufacturer's instructions. Promoter activity was normalized via Renilla luciferase activity. Each assay was performed in triplicate. Each value was determined by two assays.

Immunohistochemistry. Two types of transgenic (Tg) mice were ovariectomized, and some of them received an s.c. E2 implant to detect ARC and AVPV kisspeptin immunoreactivity, respectively. All animals then were given a lateral cerebroventricular injection of colchicine (10 μg/4 μL saline) 2 d before they were killed. Colchicine was injected to detect kisspeptin immunoreactivity in the AVPV by blocking axonal transport and accumulating kisspeptin peptides in the cell body. The animals were deeply anesthetized with sodium pentobarbital and perfused with 4% (wt/vol) paraformaldehyde in 0.05 M phosphate buffer, pH 7.4. The brains were removed immediately, postfixed in the same fixative overnight at 4 °C, and then immersed in PBS containing 30%

(wt/vol) sucrose at 4 °C. Frozen frontal sections (50 μm) were prepared with a cryostat. Free-floating sections were subjected to immunohistochemistry with anti-kisspeptin rabbit polyclonal antibodies (1:8,000) [C2 antibody raised against a C-terminal 12-aa sequence (amino acids 41–52) of rat kisspeptin conjugated with keyhole limpet hemocyanin] and anti-GFP chicken polyclonal antibodies (1:2,000) (ab1370; Abcam PLC). The sections then were incubated with Alexa 594-conjugated anti-rabbit IgG (1:800) (Invitrogen) and Alexa 488-conjugated anti-chicken IgG (1:800) (Abcam). Kisspeptin and GFP immunoreactivities were examined under a fluorescence microscope with an ApoTome (Carl Zeiss). No immunoreactivity was found in anti-kisspeptin polyclonal antibodies absorbed with 10 nmol Kp-10, a bioactive core sequence of kisspeptin (Fig. S5). The intensity of fluorescence was analyzed with Image J software (National Institutes of Health), which calculated the sum of the intensity of each pixel within a given microscopic field (referred to as the "integrated density of fluorescence"). The average of the integrated density of fluorescence values per section was calculated. Also, the number of kisspeptin- and GFP-expressing cells was counted in each section. The sum of the cell numbers in each nucleus was obtained and averaged for each group (Fig. S3). To avoid bias, digital images of the sections were coded, and the number of cells was counted by two independent persons.

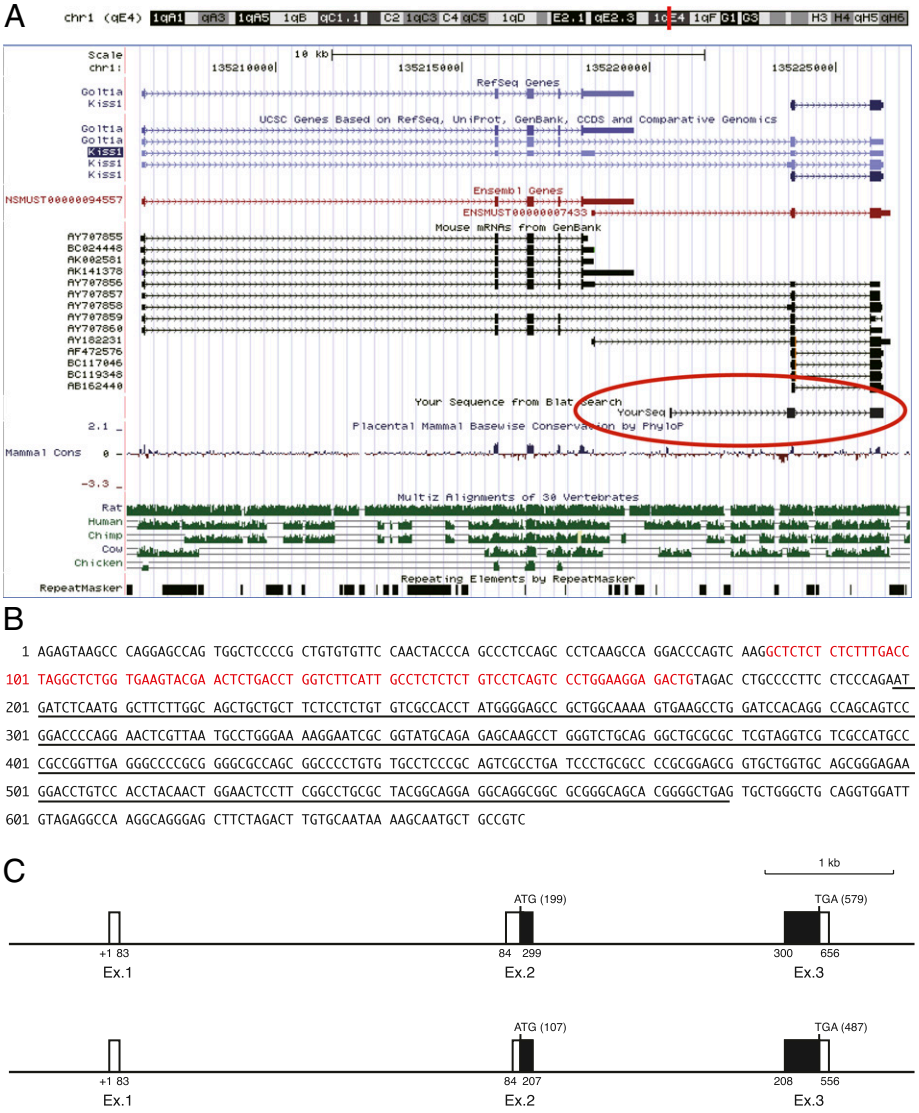

Fig. S1. Characterization of the *Kiss1* gene expressed in the mouse hypothalamus. (*A*) Information on the mouse *Kiss1* gene locus (chromosome 1: 135,206,001–135,227,000) from the present University of California, Santa Cruz database. The red circle shows the mRNA sequence we identified by RACE analysis using mouse brain tissue containing AVPV or ARC. (*B*) Full-length cDNA sequences of the *Kiss1* gene expressed within the AVPV and ARC. Numbers indicate nucleotide positions; the predicted ORF is underlined. Red characters indicate the sequence deleted in the smaller splice variant. Full lengths of major *Kiss1* cDNAs detected from the AVPV and ARC were 656 bp and 556 bp, respectively, including an ORF of 381 bp. (*C*) Genomic structure of mouse *Kiss1* gene. The ORF is indicated by the black box. The *Kiss1* gene spans >6 kb and is composed of three exons and two introns. *Kiss1* mRNAs transcribed in the AVPV and ARC originated from the same position, 3,336 bp upstream of the translation start site for kisspeptin.

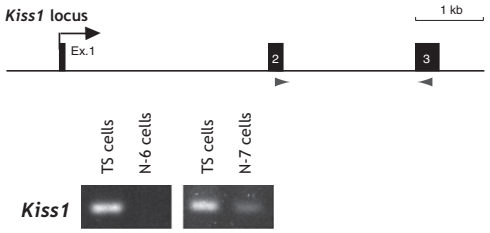

Fig. S2. *Kiss1* expression in N6 and N7 cell lines. (*Upper*) Schematic diagram of the *Kiss1* gene locus and location of the primers for detection. (*Lower*) RT-PCR analysis of *Kiss1* gene expression in the N6 and N7 cell lines.

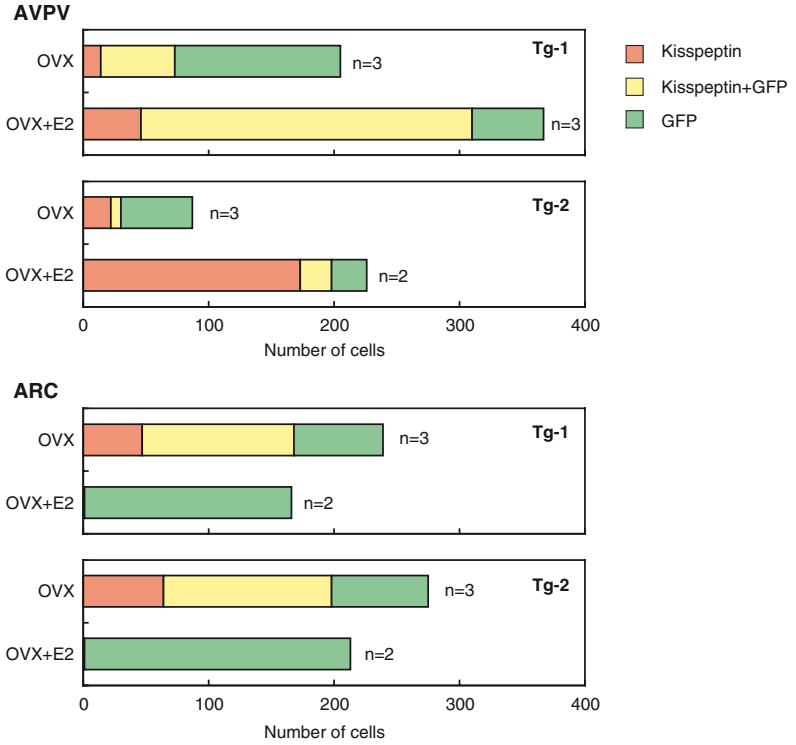

Fig. S3. Number of cells expressing kisspeptin alone (pink), both kisspeptin and GFP (yellow), or GFP alone (green) in the AVPV and ARC of Tg-1 mice (containing the entire sequence 22-kb upstream and 10- kb downstream from the *Kiss1* gene transcription start site) and Tg-2 mice, which contained the same sequence without the the 3′ region of the third exon. The numbers at the right of each bar indicate the number of animals used.

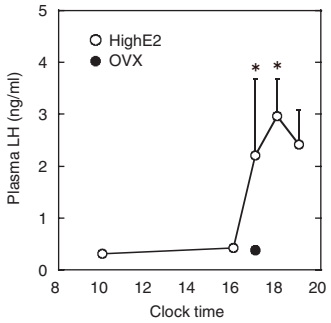

Fig. S4. Plasma LH concentrations in OVX+E2 mice. Blood samples were collected from OVX+E2 (HighE2) mice at 10:00 h and between 16:00–19:00 h. Some OVX mice (without E2 implantation) also were bled at 17:00 h. Values are means ± SEM, $*P < 0.05$.

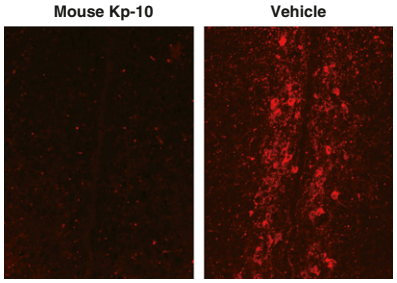

Fig. S5. The specificity of the C2 antibodies used for immunohistochemistry was confirmed by immunoneutralization. Antiserum was preabsorbed with 10 nmol of mouse kisspeptin C-terminal decapeptide (Kp-10) or vehicle and was subjected to immunohistochemistry using free-floating sections containing the mouse AVPV. Immunostaining by C2 antibodies was blocked completely by mouse Kp-10.