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—Original Article—

Conditional kisspeptin neuron-specific *Kiss1* knockout with newly generated *Kiss1*-floxed and *Kiss1*-Cre mice replicates a hypogonadal phenotype of global *Kiss1* knockout mice

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Abstract. The present study aimed to evaluate whether novel conditional kisspeptin neuron-specific *Kiss1* knockout (KO) mice utilizing the Cre-loxP system could recapitulate the infertility of global *Kiss1* KO models, thereby providing further evidence for the fundamental role of hypothalamic kisspeptin neurons in regulating mammalian reproduction. We generated *Kiss1*-floxed mice and hypothalamic kisspeptin neuron-specific *Cre*-expressing transgenic mice and then crossed these two lines. The conditional *Kiss1* KO mice showed pubertal failure along with a suppression of gonadotropin secretion and ovarian atrophy. These results indicate that newly-created hypothalamic *Kiss1* KO mice obtained by the Cre-loxP system recapitulated the infertility of global *Kiss1* KO models, suggesting that hypothalamic kisspeptin, but not peripheral kisspeptin, is critical for reproduction. Importantly, these *Kiss1*-floxed mice are now available and will be a valuable tool for detailed analyses of roles of each population of kisspeptin neurons in the brain and peripheral kisspeptin-producing cells by the spatiotemporal-specific manipulation of Cre expression.

Key words: Cre/loxP system, Gonadotropin, Kisspeptin, Pubertal failure

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It has been well established that kisspeptin (encoded by *KISS1/Kiss1*)-GPR54 (a kisspeptin receptor encoded by *GPR54/Gpr54*) signaling plays a critical role in the central mechanism controlling reproductive function in mammals including primates [1–3], and rodents [4–7]. Loss-of-function mutations in *KISS1* or *GPR54* in humans resulted in hypogonadotropic hypogonadism manifested by pubertal failure [1–3]. Similarly, global *Kiss1* or *Gpr54* knockout (KO) mice showed pubertal failure and gonadal atrophy [4–6]. In addition, *Kiss1* KO rats showed a lack of both pulse and surge modes of gonadotropin secretion [7]. The most plausible interpretation is that kisspeptin-GPR54 signaling in the hypothalamus is fundamental for controlling reproductive function via direct activation of gonadotropin-releasing hormone (GnRH) neurons. This is because a GnRH neuron-targeted deletion of *Gpr54* recapitulated the infertility of *Kiss1* or *Gpr54* KO animal models [8, 9]. Further, GnRH neuron-specific rescue of *Gpr54* expression recovered reproductive function in *Gpr54* KO mice [8].

Circumstantial evidence suggests that the hypothalamic kisspeptin neurons, located in two nuclei, such as the anteroventral periventricular nucleus-periventricular nucleus (AVPV-PeN) continuum (also known as the rostral periventricular region of the third ventricle, or RP3V) and the hypothalamic arcuate nucleus (ARC), are functionally distinct: AVPV-PeN kisspeptin neurons are indicated to be responsible for GnRH/luteinizing hormone (LH) surge generation in rodents [10–15], whereas the ARC ones are suggested to be involved in GnRH/LH pulse generation in rodents and ruminants [16–22]. Indeed, AVPV-PeN *Kiss1* ablation by neonatal sex steroid exposure resulted in a deficiency of the LH surge in female rats [14, 15]. As for ARC kisspeptin neurons, rhythmic increases in the multiple unit activity recorded by the electrodes placed in close proximity to the ARC kisspeptin neurons corresponded to LH pulses in goats [16, 17]; the pulsatile kisspeptin secretion detected at the median eminence largely corresponded to GnRH pulses in monkeys [23]; chronic estrogen exposure in the neonatal period caused an irreversible suppression of ARC *Kiss1* expression and LH pulses in male and female rats [24, 25]; optogenetic stimulation or inhibition of ARC kisspeptin neurons could stimulate or inhibit pulsatile LH secretion in *Kiss1*-Cre mice receiving adeno-associated virus (AAV) vectors carrying channelrhodopsin-2 or archaerhodopsin, respectively [26, 27]; ARC kisspeptin neurons exhibited a rhythmic increase in *in*

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vivo levels of intracellular Ca^{2+} that correspond to LH pulses in *Kiss1*-Cre mice receiving AAV vectors carrying GCaMP6, a Ca^{2+} biosensor [27]. In addition, previous studies showed that kisspeptin neurons are also located in the medial amygdala (MeA) of mice and rats and that kisspeptin administration into the MeA stimulated LH secretion, indicating that MeA kisspeptin neurons may integrate the limbic system and GnRH/LH secretion [28–31].

In addition to such an indispensable role of central kisspeptin in controlling pulsatile and surge-mode of GnRH/gonadotropin secretion, kisspeptin is now considered as a multi-functional molecule in the peripheral tissues [32–34]. Previous studies demonstrated that *Kiss1* and *Gpr54* expression were evident in the ovary and uterine of rodents and suggested local roles of kisspeptin signaling in follicular development, ovulation/corpus luteum formation, and implantation [35–37]. *KISS1*/*Kiss1* and *GPR54*/*Gpr54* expression were also found in the pancreas and adipose tissue of humans and rodents, wherein peripheral kisspeptin was suggested to be involved in metabolic function: Previous *in vitro* studies showed that kisspeptin increased glucose-induced insulin secretion from the pancreas and decreased glucose uptake and lipid accumulation via decreasing lipogenesis and increasing lipolysis in the adipose tissue [32, 34]. The Cre-loxP system for generating tissue- or cell type-specific *Kiss1* KO is increasingly important to further elucidate local roles of kisspeptin in those peripheral organs as well as the central nervous system.

The present study aimed to evaluate whether our newly-created conditional kisspeptin neuron-specific *Kiss1* KO mice obtained by the Cre-loxP system could recapitulate the infertility of global *Kiss1* KO animal models, thereby providing further evidence for the fundamental role of central kisspeptin signaling in regulating reproduction in mammals. For this purpose, we here have generated *Kiss1*-floxed mice (*Kiss1*$^{fl/fl}$ mice), which could be useful for a better understanding of the brain region, tissue- or cell type-specific roles of kisspeptin. We also generated hypothalamic kisspeptin neuron-specific *Cre*-expressing transgenic mice (*Kiss1*-Cre mice) based on our previous findings on the brain region-specific *Kiss1* enhancer [38, 39]. Further, we generated conditional *Kiss1* KO mice by crossing the aforementioned two mouse lines and analyzed the reproductive function of the conditional *Kiss1* KO mice to investigate if the mice replicate the phenotype, such as pubertal failure, suppression of gonadotropin secretion in global *Kiss1* KO mice.

Materials and Methods

Animals

Gene-modified mice and wild-type (ICR, Charles River Laboratories Japan, Kanagawa, Japan; and BDF1, Japan SLC, Shizuoka, Japan) mice were housed under a controlled environment (14 h of light and 10 h of darkness; lights on at 0500 h; temperature, 22 ± 3ºC). Animals were weaned at postnatal day 21 and allowed free access to standard laboratory mouse chow (CE-2; CLEA Japan, Tokyo, Japan) and water. Genotypes of animals were determined by polymerase chain reaction (PCR) analyses of genomic DNA extracted from the ear tissue. The primer sequences for genotyping are listed in Table 1. The present study was approved by the Committees on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University and the National Institute for Physiological Sciences.

Table 1. Primer sequences for genotyping of animals and embryonic stem (ES) cell selection

Purpose	Primers
Genotyping of animals	
Kiss1-floxed	Forward, 5'-cacaggatggaagcagagca-3'
	Reverse, 5'-actgcccttcccctaaatgc-3'
Kiss1-Cre	Forward, 5'-gcagaacctgaagatgttcgcgat-3'
	Reverse, 5'- aggtatctctgaccagagtcatcc-3'
ES selection	
5'-region	Forward, 5'-gttgtttggggtggaatgagtc-3'
	Reverse, 5'-gcgataccgtaaagcacgag-3'
3'-region	Forward, 5'- caggacgtgacaaatggaag-3'
	Reverse, 5'-accaaacattcctccagcag-3'
loxP site	Forward, 5'-gagccttgttgtctgtgaagtg-3'
	Reverse, 5'-ggagttccagttgtaggtggac-3'
Probe preparation for southern blotting	Forward, 5'-agaggctattcggctatgactg-3'
	Reverse, 5'-actcgtcaagaaggcgatagaa-3'

Generation of Kiss1$^{fl/fl}$ *mice*

The targeting vector harbored a floxed exon 3 of the *Kiss1* gene coding for the 52-amino-acid mouse kisspeptin and a floxed neomycin resistance cassette as shown in Fig. 1A. The targeting vector was electroporated into the TT2 (CBA × C57BL/6) line of mouse embryonic stem (ES) cells [40]. Successfully targeted ES cell clones were selected via a neomycin-supplemented medium. Genomic DNA was isolated to screen ES cell clones for homologous recombination of the *Kiss1* locus. The presence of the loxP site in the *Kiss1* locus was confirmed by PCR (Fig. 1B) and then confirmed by Southern blot analysis (Fig. 1C). The primer sequences for the probe preparation and PCR analyses for the ES selection are listed in Table 1. The targeted ES clones were injected into ICR 8-cell-stage embryos. The embryos containing the targeted ES clones were transplanted into the uterus of pseudopregnant foster mice. The resultant chimeric males were coupled with ICR females in order to test the germline transmission. *Kiss1*-floxed heterozygous mice (*Kiss1*$^{fl/+}$ mice) without a floxed neomycin resistance cassette were produced by an injection of Cre recombinase-expressing plasmid (pCre-Pac; kindly provided by Dr Yagi, Osaka University) [41] into the fertilized oocytes obtained from the germline offspring. The resultant *Kiss1*$^{fl/+}$ males and females were mated in order to generate *Kiss1*-floxed homozygous mice (*Kiss1*$^{fl/fl}$ mice). *Kiss1*$^{fl/fl}$ males and females were also fertile.

Generation of Kiss1-Cre mice

Kiss1-Cre mice, in which Cre recombinase is expected to be driven by the *Kiss1* promoter and the ARC-specific *Kiss1* enhancer identified in our previous study [39], were generated as follows: Cre recombinase gene was inserted into a pIRES-AcGFP vector (Takara Bio, Kusatsu, Japan) and the resultant Cre-IRES-AcGFP transgene was substituted for the site between the translational start point and 3' end of exon 2 of the *Kiss1* gene (accession no. AB666166) in a bacterial artificial chromosome (BAC) clone RP24-299J2 (BACPAC Resources, Oakland, CA, USA) by using a counterselection BAC modification kit (Gene Bridges, Heidelberg, Germany). The 3'-downstream-truncated

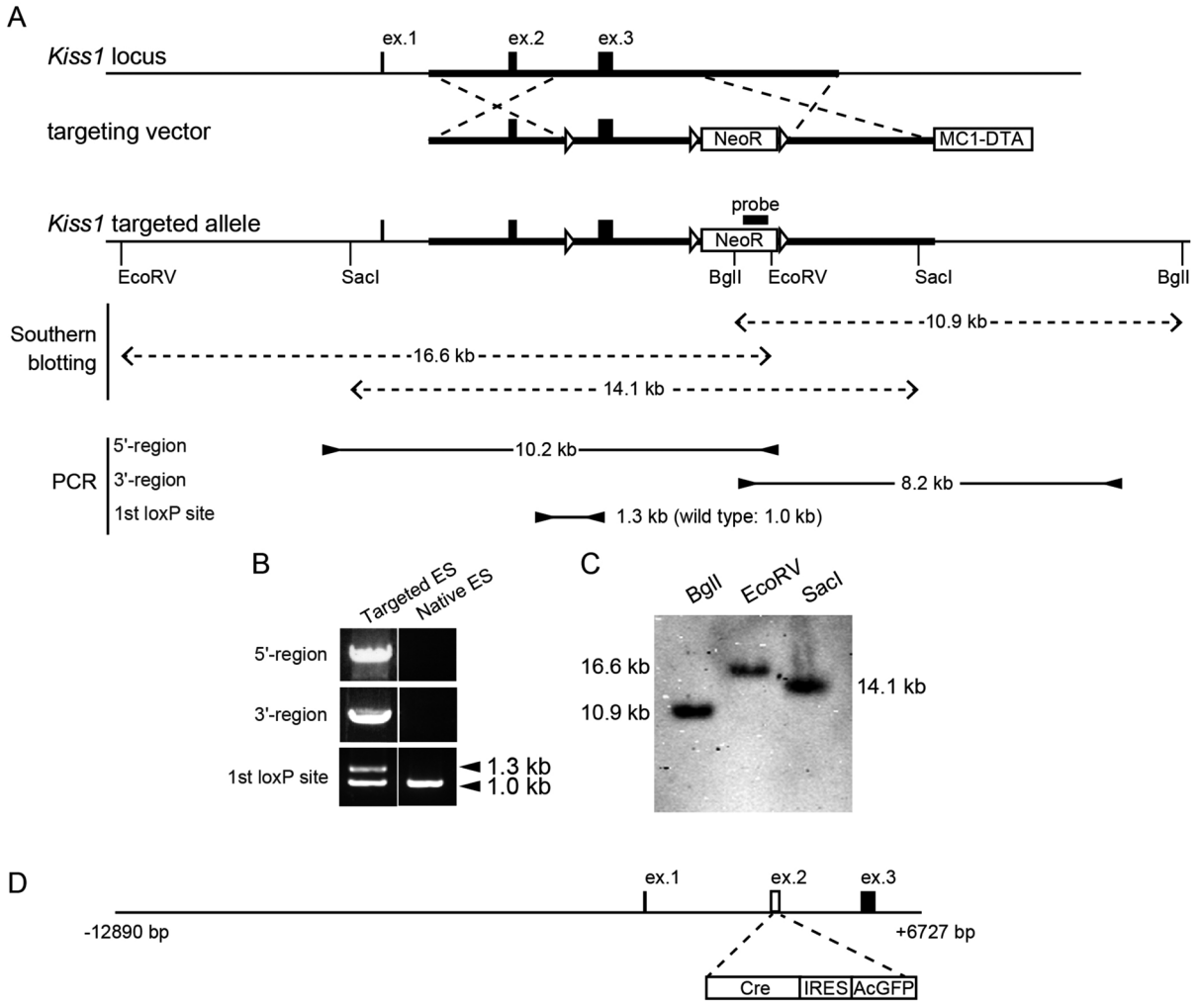


Fig. 1. Generation of *Kiss1*-floxed mice and *Kiss1*-Cre mice. (A) Structure of the wild-type *Kiss1* allele (top), targeting vector for the generation of *Kiss1*-floxed mice (middle), and *Kiss1* targeted allele (bottom), resulting from replacement at dotted lines. The *Kiss1* targeted allele was designed by insertion of three loxP sites (open triangles) and a neomycin resistance (NeoR) selection cassette. A diphtheria toxin A (DTA) expression cassette was used for negative selection in embryonic stem (ES) cells. Note that the NeoR selection cassette was removed by an injection of Cre recombinase-expressing plasmid into the fertilized oocytes obtained from the germline offspring. (B) Screening of ES cell clones by polymerase chain reaction (PCR) using three sets of primers (5'-region, 3'-region, and 1st loxP site). The locations of primers are shown by the arrowheads in panel A. The product sizes are also provided in the panel A. (C) Southern blot analysis of BglI-, EcoRV-, or SacI-digested DNA using the probe on the NeoR cassette detected 10.9-, 16.6-, and 14.1-kb fragments in the targeted allele. Predicted sizes of the DNA fragments are shown by dotted double arrows in panel A. (D) Structure of construct for *Kiss1*-Cre mice. The construct was designed by substitution of Cre, internal ribosome entry site (IRES), and *Aequorea coerulescens* green fluorescent protein (AcGFP) cassette (white boxes) for exon 2 of *Kiss1* gene in which transcriptional start site is located.

DNA construct (Fig. 1D) was linearized according to our previous study [39]. The transgenic mice were generated by microinjection of the linearized construct to pronuclear-stage oocytes of BDF1 mice as previously described elsewhere [39].

Generation of conditional Kiss1 KO mice by crossing the Kiss1$^{fl/fl}$ mice and Kiss1-Cre mice

The *Kiss1*-Cre mice were crossed onto *Kiss1*$^{fl/fl}$ mice two times to generate offspring, in which Cre recombinase theoretically deletes the floxed *Kiss1* exon 3, encoding a functional region of kisspeptin, in both alleles.

The vaginal opening was checked daily in the resultant conditional *Kiss1* KO mice and their littermate Cre-negative *Kiss1*$^{fl/fl}$ controls until 40 days of age. Animals were then subjected to the collection of the ovary, blood, and brain samples.

Ovary collection and estradiol treatment

The conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ control mice were bilaterally ovariectomized (OVX) under aseptic conditions with isoflurane anesthesia (1–3% in air). Animals then immediately

received subcutaneous Silastic implants (internal diameter: 1.57 mm; outer diameter: 3.18 mm; 3 mm in length; Dow Corning, Midland, MI, USA) that were filled with estradiol-17β (E_2; Sigma-Aldrich, St. Louis, MO, USA) dissolved in peanut oil at 10 μg/ml. The E_2 implant was chosen based on our previous studies [42–44] to visualize *Kiss1* gene expression in both the AVPV and ARC: the size and dose were adjusted according to the animal body weight. Ovaries were weighed and stored at –80ºC until analysis for *Kiss1* and *Cre* mRNA expression.

Brain sampling and in situ hybridization for Kiss1 and Cre mRNA expression

One week after the OVX and E_2 treatment, the animals were deeply anesthetized with pentobarbital (70 mg/kg, Kyoritsu Seiyaku, Tokyo, Japan), and then intracardially perfused with RNase-free 0.05 M phosphate-buffered saline (PBS; pH 7.5) followed by 4% paraformaldehyde (Sigma-Aldrich) in 0.05 M phosphate buffer (PB; pH 7.5). The brains were immediately removed, post-fixed with the same fixative for overnight at 4ºC, and then kept in 30% sucrose in 0.05 M PB until they sank at 4ºC under the RNase-free conditions. Frozen frontal sections (50-μm thickness) of the brain containing the AVPV, ARC and medial amygdala (MeA), in which the previous study showed *Kiss1* expression in mice [28, 29], were prepared using a cryostat (CM1800; Leica, Wetzlar, Germany) on the day or a day before the *in situ* hybridization and then stored in PBS at 4ºC. Every two AVPV section and every four ARC and MeA section were used for *in situ* hybridization to visualize *Kiss1* and *Cre* mRNA expression.

Digoxigenin (DIG)-labeled *Kiss1* cRNA probe (position 38-372, GenBank accession no. NM_178260) and DIG-labeled *Cre* cRNA probe (position 485-1516, GenBank accession no. X03453) were synthesized by using a DIG-labeling kit (Boehringer Mannheim, Mannheim, Germany). *Kiss1* and *Cre* expression was detected by free-floating *in situ* hybridization as described previously with slight modification [10]. Briefly, the sections were hybridized overnight at 60ºC with 1 μg/ml *Kiss1* or *Cre* cRNA probes. The DIG-labeled probes were detected by an alkaline phosphatase-conjugated anti-DIG antibody (1:1000; Roche Diagnostics, Indianapolis, IN, USA) and a chromogen (338 μg/ml 4-nitroblue tetrazolium chloride and 175 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate, Roche Diagnostics). The sections were mounted on gelatin-coated slides and cover-slipped with 90% glycerol in 0.05 M PB. The signals of *Kiss1* or *Cre* mRNA expression were observed under a light microscope (BX53; Olympus, Tokyo, Japan) and the numbers of *Kiss1*- or *Cre*-positive cells were counted throughout the AVPV and ARC. The specificity of signals was confirmed by *in situ* hybridization using corresponding sense probes, and no signals were detected with the sense probes.

Blood sampling and radioimmunoassay for LH and follicle-stimulating hormone (FSH)

Fifty-μl blood samples were collected from the descending aorta of both the conditional *Kiss1* KO and Cre-negative *Kiss1*$^{fl/fl}$ control mice under the anesthetized condition just before the brain perfusion.

Plasma LH concentrations in 25-μl plasma samples were determined with a mouse LH-RIA kit provided by the National Hormone and Peptide Program (Bethesda, MD, USA) as previously described [45]. LH concentrations were expressed in terms of NIDDK mouse LH-RP. The least detectable concentration of LH in 25-μl plasma samples was 0.156 ng/ml. The intra- and inter-assay coefficients of variation were 4.7 and 14.5% at 1.6 ng/ml, respectively.

Plasma FSH concentrations in 25-μl plasma samples were determined with a mouse FSH RIA kit provided by the National Hormone and Peptide Program. FSH concentrations were expressed in terms of NIDDK mouse FSH-RP. The least detectable concentration of FSH in 25-μl plasma samples was 1.25 ng/ml. The intra- and inter-assay coefficient of variation was 0.32 and 13.8% at 9.6 ng/ml, respectively.

Ovarian Kiss1 and Cre expression

DNA-free total RNA was purified from the ovary by using ISOGEN (Nippon Gene, Tokyo, Japan) and the cDNA was synthesized with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The quantitative PCR analysis was performed by using an ABI 7500 real-time system (Thermo Fisher Scientific) with Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan) with specific primers for mouse *Kiss1* (5'-gctgctgcttctcctct-gtgt-3' and 5'-gcataccgcgattccttttc-3'), *Cre* (5'-cagcaacatttgggccagcta-3' and 5'-ccgccgcataaccagtgaaac-3') and mouse *Actb* (5'-ggtgggaatgggt-cagaagg-3' and 5'-gtacatggctggggtgttga-3'). The cycling protocol was as follows: pre-denature for 1 min at 95ºC, 40 cycles amplification of 15 sec at 95ºC and 1 min at 60ºC. The specificity of the amplicons was confirmed by a dissociation curve analysis (60 to 95°C) after 40-cycle amplification. A distinct single peak was considered that only a single DNA sequence was amplified. The expression levels of *Kiss1* and *Cre* were normalized to that of *Actb*.

Statistical analysis

Statistical differences in ovarian weights, plasma gonadotropin concentrations, the number of hypothalamic *Kiss1*-expressing cells, as well as ovarian *Kiss1* and *Cre* expression levels between the conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ controls were determined by Welch's-*t* test (R version 3.6.0, http://www.R-project.org/).

Results

Pubertal failure and atrophy of ovaries in conditional Kiss1 KO mice

The conditional *Kiss1* KO female mice by crossing *Kiss1*-Cre mice and *Kiss1*$^{fl/fl}$ mice showed no vaginal opening as an external sign of pubertal onset by 40 days of age, whereas the Cre-negative *Kiss1*$^{fl/fl}$ controls showed vaginal opening at 28–34 postnatal days (Fig. 2A). The ovarian weight was significantly lower in the conditional *Kiss1* KO mice than the Cre-negative *Kiss1*$^{fl/fl}$ control mice ($P < 0.05$, Fig. 2B).

Reduction of plasma gonadotropin levels in the conditional Kiss1 KO mice

Plasma LH levels were undetectable in all conditional *Kiss1* KO female mice and the levels were significantly lower in the conditional *Kiss1* KO mice compared with those in the Cre-negative *Kiss1*$^{fl/fl}$ control mice ($P < 0.05$, Fig. 3A). Plasma FSH levels were undetectable in three out of five conditional *Kiss1* KO mice, resulting in significant lower levels of FSH in the conditional *Kiss1* KO mice compared

with those in the Cre-negative *Kiss1*$^{fl/fl}$ controls ($P < 0.05$, Fig. 3B).

Kiss1 expression in the brain of the conditional Kiss1 KO female mice and Cre expression in the brain of the Kiss1-Cre female mice

Figure 4 shows representative photomicrographs of *Cre*-expressing cells in the ARC (Fig. 4A) and AVPV (Fig. 4B) of *Kiss1*-Cre mice and Cre-negative controls. A number of *Cre*-expressing cells (432.9 ± 50.5, n = 4) were found in the ARC of *Kiss1*-Cre mice, but not in the Cre-negative control mice (Fig. 4A). Very few *Cre*-expressing cells (58.1 ± 8.3 cells, n = 4) with weak signals were found in the AVPV of *Kiss1*-Cre mice, but not in Cre-negative control mice (Fig. 4B).

Figure 5 shows representative photomicrographs of *Kiss1*-expressing cells in the ARC (Fig. 5A) and AVPV (Fig. 5C) of the OVX+E_2 conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ controls. No *Kiss1*-positive cells were found in the ARC of conditional *Kiss1* KO female mice, whereas a number of *Kiss1*-positive cells were found in the ARC of Cre-negative *Kiss1*$^{fl/fl}$ controls (Fig. 5A). The number of ARC *Kiss1*-expressing cells were significantly lower in the conditional *Kiss1* KO mice compared with Cre-negative *Kiss1*$^{fl/fl}$ controls ($P < 0.05$, Fig. 5B). Unexpectedly, no *Kiss1*-expressing cells were found in the AVPV of conditional *Kiss1* KO female mice, whereas a number of *Kiss1*-positive cells were found in the AVPV of Cre-negative *Kiss1*$^{fl/fl}$ controls (Fig. 5C). The number of AVPV

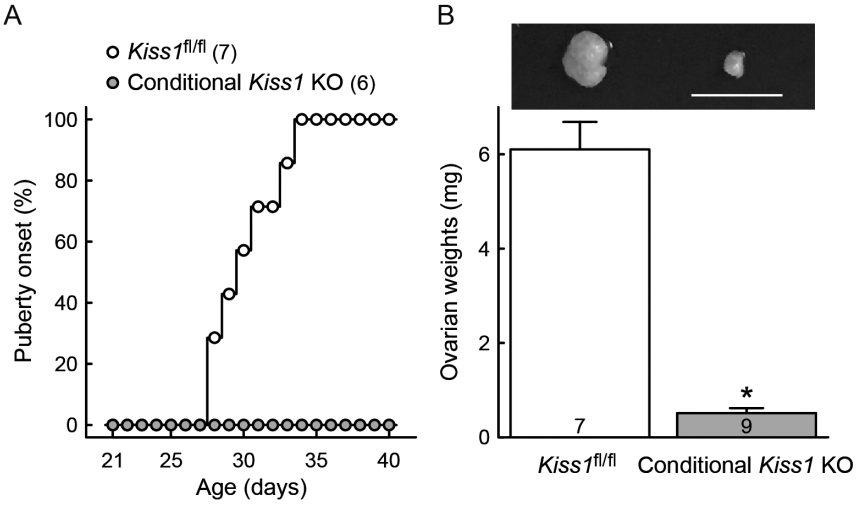


Fig. 2. The conditional *Kiss1* knockout (KO) mice failed to show puberty onset and ovarian atrophy. (A) Timing of vaginal opening as an external sign of pubertal onset is expressed as a percentage of the total number of animals for each genotype. Numbers in the parentheses indicate the number of animals used. (B) Representative photograph of ovary and ovarian weights in the conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ controls. Values are indicated as mean ± SEM. Numbers in each column indicate the number of animals used. * $P < 0.05$ between the conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ controls (Welch's-*t* test). Scale bar, 5 mm.

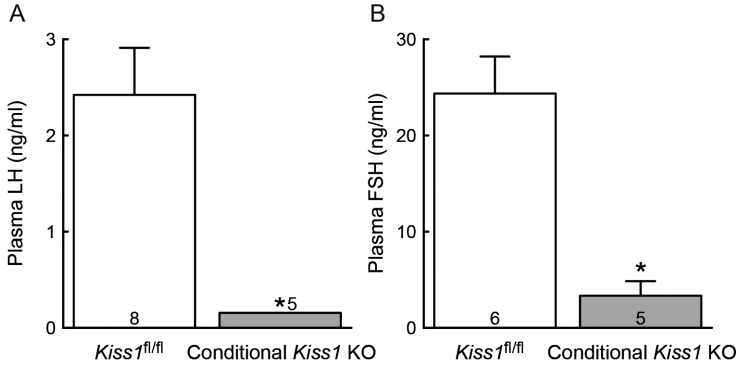
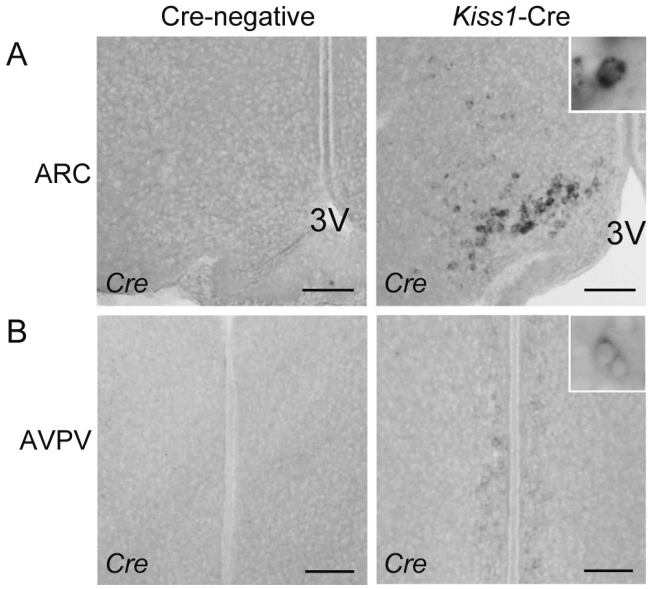


Fig. 3. The conditional *Kiss1* knockout (KO) mice showed suppression of gonadotropin secretion. Plasma luteinizing hormone (LH, A) and follicle-stimulating hormone (FSH, B) levels of the conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ controls. Values are indicated as mean ± SEM. Note that plasma LH levels were undetectable in all conditional *Kiss1* KO mice and expressed as the least detectable concentration of LH (0.156 ng/ml). Numbers in or on each column indicate the number of animals used. * $P < 0.05$ between the conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ controls (Welch's-*t* test).



Kiss1-positive cells were also significantly lower in the conditional *Kiss1* KO mice than the Cre-negative *Kiss1*$^{fl/fl}$ controls ($P < 0.05$, Fig. 5D).

No *Kiss1*-expressing cells were found in the MeA of both the conditional *Kiss1* KO and Cre-negative *Kiss1*$^{fl/fl}$ OVX + E_2 mice (Fig. 6A). In addition, no *Cre*-expressing cells were found in the MeA of *Kiss1*-Cre mice as well as Cre-negative controls (Fig. 6B).

Fig. 4. Determination of *Cre* expression in the hypothalamus of *Kiss1*-Cre mice. (A) *Cre*-expressing cells in the arcuate nucleus (ARC) of a representative *Kiss1*-Cre mouse (right panel). No *Cre*-expressing cells were found in the ARC of Cre-negative controls (left panel). 3V, third cerebroventricle. (B) Few *Cre*-expressing cells with weak signals in the anteroventral periventricular nucleus (AVPV) of a representative *Kiss1*-Cre mouse (right panel). No *Cre*-expressing cells were found in the AVPV of Cre-negative controls (left panel). Scale bars, 100 µm.

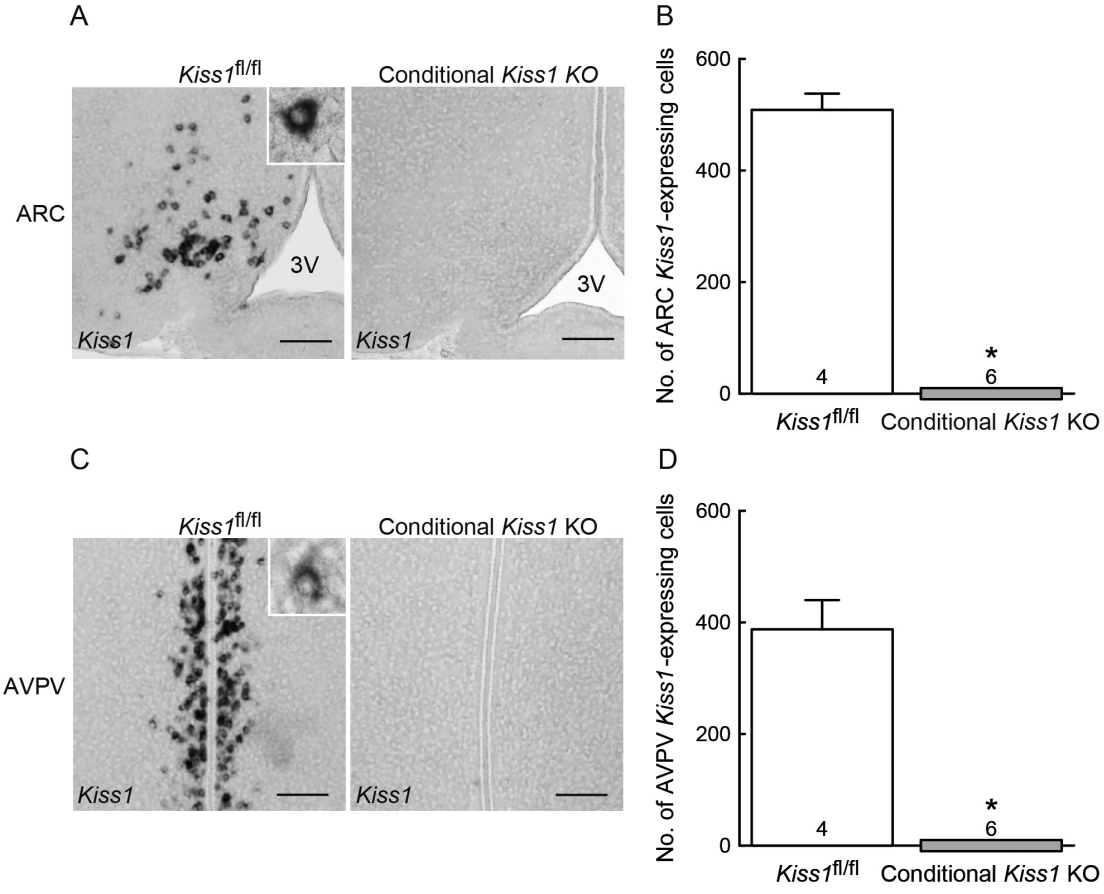


Fig. 5. The conditional *Kiss1* knockout (KO) mice showed completely suppression of *Kiss1* expression in the hypothalamus. (A) *Kiss1*-expressing cells in the arcuate nucleus (ARC) of representative conditional *Kiss1* KO mouse and Cre-negative *Kiss1*$^{fl/fl}$ control. 3V, third cerebroventricle. (B) The number of *Kiss1*-expressing cells throughout the ARC. Note that no *Kiss1*-expressing cells were found in the ARC of conditional *Kiss1* KO mice. (C) *Kiss1*-expressing cells in the anteroventral periventricular nucleus (AVPV) of representative conditional *Kiss1* KO mouse and Cre-negative *Kiss1*$^{fl/fl}$ control. (D) The number of *Kiss1*-expressing cells throughout the AVPV. Note that no *Kiss1*-expressing cells were found in the AVPV of conditional *Kiss1* KO mice. Values are indicated as mean ± SEM. Numbers in or on each column indicate the number of animals used. * $P < 0.05$ between the conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ controls (Welch's-*t* test). Scale bars, 100 µm.

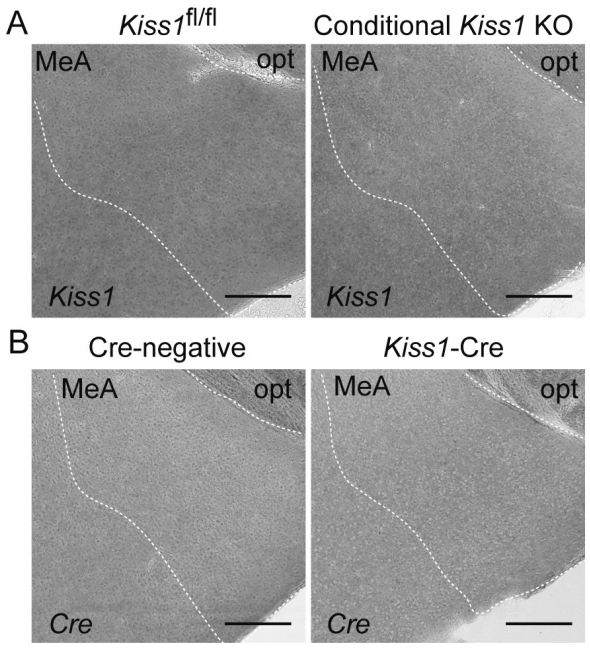


Fig. 6. Neither *Kiss1* nor *Cre* expression in the medial amygdala (MeA). (A) Representative photomicrographs showing no *Kiss1*-expressing cells in the MeA of both the conditional *Kiss1* knockout (KO) mice and Cre-negative *Kiss1*$^{fl/fl}$ controls. (B) Representative photomicrographs showing no *Cre*-expressing cells in the MeA of *Kiss1*-Cre mice and Cre-negative controls. opt, optic tract. Scale bars, 200 μm.

Kiss1 and Cre expression in the ovary of the conditional Kiss1 KO female mice

Kiss1 mRNA was slightly detected in the ovary of both conditional *Kiss1* KO mice (0.0031 ± 0.0012% of *Actb*) and Cre-negative *Kiss1*$^{fl/fl}$ control mice (0.0060 ± 0.0016% of *Actb*), and the levels were comparable between the conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ control mice. Note that *Kiss1* expression was highly detected in the wild-type mouse hypothalamus (1.02% of *Actb*, n = 2). *Cre* mRNA was slightly detected in the ovary of conditional *Kiss1* KO mice (0.050 ± 0.022% of *Actb*), whereas it was undetectable in Cre-negative *Kiss1*$^{fl/fl}$ control mice.

Discussion

The present study demonstrates that the newly-created conditional kisspeptin neuron-specific *Kiss1* KO mice generated by the Cre-loxP system replicated a hypogonadal phenotype of global *Kiss1* KO mice [5, 6], because the animals generated by crossing *Kiss1*$^{fl/fl}$ mice and *Kiss1*-Cre mice showed no puberty onset along with an undetectable level of plasma gonadotropin and ovarian atrophy. It should be noted that high *Cre* expression was found in the ARC, but little in the AVPV, MeA, and ovary of the current *Kiss1*-Cre mice. Indeed, ovarian *Kiss1* expression levels were comparable between the conditional *Kiss1* KO mice and Cre-negative *Kiss1*$^{fl/fl}$ control mice. Collectively, the present results provide further evidence that hypothalamic kisspeptin neurons are fundamental to puberty onset and subsequent reproductive function in mammals and suggest that the *Kiss1* expression outside of the hypothalamus may have a less important role for reproductive function in female mice.

The current result that *Kiss1*-Cre mice, which were generated by a microinjection of 3'-truncated *Kiss1* locus replaced with *Cre* gene, mainly expressed *Cre* mRNA in the ARC at adulthood, was consistent with our previous finding showing that 5'-upstream sequence of *Kiss1* locus serves as an ARC-specific *Kiss1* enhancer in mice [39]. As expected, the conditional *Kiss1* KO mice successfully lacked *Kiss1* mRNA expression in the ARC. A previous study showed that *Kiss1* is first expressed in the ARC during prenatal development: specifically, from embryonic day 12.5 in rats [46]. Taken together with this previous finding, the *Kiss1* KO by Cre-loxP recombination is likely to occur in the ARC during the prenatal period in the current conditional *Kiss1* KO female mice.

Interestingly, AVPV *Kiss1* expression was also deprived in the current conditional *Kiss1* KO female mice, even though only a few *Cre*-expressing cells were detected in the AVPV of the *Kiss1*-Cre mice. It is likely that such little *Cre* mRNA expression was enough to knock out *Kiss1* in AVPV kisspeptin neurons. On the other hand, it is tempting to speculate that the AVPV *Kiss1* mRNA expression would be somehow introduced depending on the ARC kisspeptin neurons. There are three possibilities to explain this result as follows: 1) AVPV kisspeptin neurons could be derived from ARC kisspeptin neurons. If this is the case, we envision that *Kiss1* expression had been already suppressed before the migration of *Kiss1*-expressing cells from the ARC to AVPV because of the *Kiss1* knocked out in the ARC by the Cre-loxP recombination; 2) Cre recombinase could be expressed in both the ARC and AVPV kisspeptin neurons during the fetal developmental period, although *Cre* mRNA expression was exclusively found only in the ARC at adulthood; 3) *Kiss1* expression in the ARC kisspeptin neurons may be required for *Kiss1* expression in the AVPV kisspeptin neurons at the adulthood, since a previous anterograde tracing study indicated the projection of ARC kisspeptin neurons toward AVPV kisspeptin neurons [47]. Further studies are needed to address this issue.

In summary, the current conditional kisspeptin neuron-specific *Kiss1* KO mice newly utilizing the Cre-loxP system recapitulated the infertility of global *Kiss1* KO animal models. The current *Kiss1*-floxed mice can be used as a valuable model for more elaborate analyses of the roles of distinct populations of kisspeptin neurons and kisspeptin-producing cells in the brain as well as the peripheral organs by the spatiotemporal manipulation of Cre expression.

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