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

Kana IKEGAMI1)*, Teppei GOTO1, 2)*, Sho NAKAMURA3), Youki WATANABE1), Arisa SUGIMOTO1), Sutisa MAJARUNE1), Kei HORIHATA1), Mayuko NAGAE1), Junko TOMIKAWA1), Takuya IMAMURA4), Makoto SANBO2), Masumi HIRABAYASHI2), Naoko INOUE1), Kei-ichiro MAEDA3), Hiroko TSUKAMURA1) and Yoshihisa UENOYAMA1)

1)Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan
2)Center for Genetic Analysis of Behavior, National Institute for Physiological Sciences, Okazaki 444-8585, Japan
3)Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan
4)Graduate School of Integrated Sciences for Life, Hiroshima University, Hiroshima 739-8526, Japan

Abstract. The present study aimed to evaluate whether novel conditional kisspeptin neuron-specific \textit{Kiss1} knockout (KO) mice utilizing the Cre-loxP system could recapitulate the infertility of global \textit{Kiss1} KO models, thereby providing further evidence for the fundamental role of hypothalamic kisspeptin neurons in regulating mammalian reproduction. We generated \textit{Kiss1}-floxed mice and hypothalamic kisspeptin neuron-specific Cre-expressing transgenic mice and then crossed these two lines. The conditional \textit{Kiss1} KO mice showed pubertal failure along with a suppression of gonadotropin secretion and ovarian atrophy. These results indicate that newly-created hypothalamic \textit{Kiss1} KO mice obtained by the Cre-loxP system recapitulated the infertility of global \textit{Kiss1} KO models, suggesting that hypothalamic kisspeptin, but not peripheral kisspeptin, is critical for reproduction. Importantly, these \textit{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

Expression recovered reproductive function in \textit{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 \textit{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 [24, 25]; optogenetic stimulation or inhibition of ARC kisspeptin neurons could stimulate or inhibit pulsatile LH secretion in \textit{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

Received: February 26, 2020
Accepted: April 2, 2020
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Correspondence: Y Uenoyama (e-mail: uenoyama@nagoya-u.jp)
* K Ikegami and T Goto contributed equally to this paper.

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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 SLIC, 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 ± 3°C). 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'-cacagagatgaagaagagca-3' | Reverse, 5'-aattagctgaccagacagtca-3' |
| Kiss1-Cre | Forward, 5'-agagaacttgtagttcgcgat-3' | Reverse, 5'-aattagctgaccagacagtca-3' |
| ES selection | |
| 5'-region | Forward, 5'-gtgatttgatggaatgatgagc-3' | Reverse, 5'-gggaaaactgttcaagagcg-3' |
| 3'-region | Forward, 5'-gaggactgtttctgtaaatg-3' | Reverse, 5'-aacaacatctttcgagac-3' |
| loxP site | Forward, 5'-gacgctttgtgctggatagtt-3' | Reverse, 5'-gagctttatgagttgac-3' |
| Probe preparation | Forward, 5'-agagactttctttgtgatucagttg-3' | Reverse, 5'-aattagctgaccagacagtca-3' |
| for southern blotting | Reverse, 5'-aattagctgaccagacagtca-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$^{fl/+}$) 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

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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<sup>fl/fl</sup> mice and Kiss1-Cre mice

The Kiss1-Cre mice were crossed onto Kiss1<sup>fl/fl</sup> 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<sup>fl/fl</sup> 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<sup>fl/fl</sup> 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 (E₂; Sigma-Aldrich, St. Louis, MO, USA) dissolved in peanut oil at 10 μg/ml. The E₂ 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 E₂ 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 in situ hybridization using corresponding sense probes, and no signals were detected with the sense probes.

Kiss1 KO and Cre-negative KO mice showed no vaginal opening as an external sign of pubertal onset by 40 days of age, whereas the Cre-negative Kiss1 fl/fl mice 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 mice by crossing Kiss1 KO female mice by crossing Kiss1 fl/fl with Cre-conditional mice expressing Cre under the control of the MMTV promoter. The conditional Kiss1 KO 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).

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) and specific primers for mouse Kiss1 (5'-gtcttattttgcattggttga-3' and 5'-gtcttattttgcattggttga-3'), Cre (5'-cagcaacatttgggccagcta-3' and 5'-cagcaacatttgggccagcta-3') and mouse Actb (5'-gagcgctgagcctgagt-3' and 5'-gagcgctgagcctgagt-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 95ºC) 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, and 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
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+E2 conditional Kiss1 KO mice and Cre-negative Kiss1fl/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 Kiss1fl/fl controls (Fig. 5A). The number of ARC Kiss1-expressing cells were significantly lower in the conditional Kiss1 KO mice compared with Cre-negative Kiss1fl/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 Kiss1fl/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 Kiss1fl/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 Kiss1fl/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 Kiss1fl/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 Kiss1fl/fl controls (Welch’s-t test).
Kiss1-positive cells were also significantly lower in the conditional Kiss1 KO mice than the Cre-negative Kiss\textsuperscript{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 Kiss\textsuperscript{fl/fl} OVX + E\textsubscript{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 Kiss\textsuperscript{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 Kiss\textsuperscript{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 Kiss\textsuperscript{fl/fl} controls (Welch’s-t test). Scale bars, 100 µm.
Kiss1 KO MICE WITH THE Cre-loxP SYSTEM

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.

Discussion

The present study demonstrates that the newly-created conditional kisspeptin neuron-specific Kiss1 KO mice generated by the Cre-loxP system recapitulated 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.

Acknowledgements

The authors are grateful to the National Hormone and Peptide Program (HNPP), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), and Dr AF Parlow for providing the LH and FSH assay kit. The radioimmunoassays were performed at the Nagoya University Radioisotope Center. We wish to thank Dr Nicola Skoulding for editorial assistance and Takashi Hirashima, Tatsuya Fukunuma, Moe Yanagihara, Hitomi Abe, Yuhei Takayama, Ren Ishigaki, Saki Okamoto and Koki Yamada for their technical support. This work was supported in part by a Grant-in-Aid for the JSPS Fellows (No. 26-4247 to KI); the Research Program...
on Innovative Technologies for Animal Breeding, Reproduction, and Vaccine Development Grant (RE02002 to HT); the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries, and Food Industry (to HT); a Grants-in-Aid from the Japan Society for the Promotion of Science (18H03973 and 18K19267 to HT); and the Cooperative Study Program of National Institute for Physiological Sciences.

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