—Original Article—

Inducible *Kiss1* knockdown in the hypothalamic arcuate nucleus suppressed pulsatile secretion of luteinizing hormone in male mice

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Abstract. Accumulating evidence suggests that kisspeptin-GPR54 signaling is indispensable for gonadotropin-releasing hormone (GnRH)/gonadotropin secretion and consequent reproductive functions in mammals. Conventional *Kiss1* knockout (KO) mice and rats are reported to be infertile. To date, however, no study has investigated the effect of inducible central *Kiss1* KO/knockdown on pulsatile gonadotropin release in male mammals. Here we report an *in vivo* analysis of inducible conditional *Kiss1* knockdown male mice. The mice were generated by a bilateral injections of either adeno-associated virus (AAV) vectors driving Cre recombinase (AAV-Cre) or AAV vectors driving GFP (AAV-GFP, control) into the hypothalamic arcuate nucleus (ARC) of *Kiss1*-floxed male mice, in which exon 3 of the *Kiss1* gene were floxed with loxP sites. Four weeks after the AAV-Cre injection, the mice showed a profound decrease in the both number of ARC *Kiss1*-expressing cells and the luteinizing hormone (LH) pulse frequency. Interestingly, pulsatile LH secretion was apparent 8 weeks after the AAV-Cre injection despite the suppression of ARC *Kiss1* expression. The control *Kiss1*-floxed mice infected with AAV-GFP showed apparent LH pulses and *Kiss1* expression in the ARC at both 4 and 8 weeks after the AAV-GFP injection. These results with an inducible conditional *Kiss1* knockdown in the ARC of male mice suggest that ARC kisspeptin neurons are responsible for pulsatile LH secretion in male mice, and indicate the possibility of a compensatory mechanism that restores GnRH/LH pulse generation.
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Pulsatile gonadotropin-releasing hormone (GnRH)/gonadotropin secretion is considered to be indispensable for controlling gonadal functions, such as gametogenesis and steroidogenesis, and has been found in various mammalian species, such as rats [1], guinea pigs [2], pigs [3], sheep [4], goats [5], cows [6], horses [7], and rhesus monkeys [8]. In order to stimulate the production and release of gonadotropin such as luteinizing hormone (LH), a pulsatile pattern of GnRH release is required at the median eminence (ME) of the mediobasal hypothalamus (MBH) into the hypophyseal portal circulation [4]. A historical experiment by Halasz and Pupp demonstrated that complete deafferentation of the MBH failed to affect testis weight and structure in male rats, indicating the presence of a hypophysiotropic area within the MBH [9], encompassing a center for tonic LH secretion called, "the GnRH/LH pulse generator". This notion was supported by later studies showing that pulsatile LH secretion was kept normally even after complete hypothalamic deafferentation in rats [1, 10] and monkeys [11]. In rats and goats, rhythmic electrical bursts of multiple unit activity are accompanied by LH pulses at regular intervals in the hypothalamic arcuate nucleus (ARC)/ME—an area that contains few, if any, GnRH neuronal cell bodies [12, 13]. These facts strongly suggest that pulsatile GnRH/LH secretion is regulated by a pacemaker source (other than GnRH neurons) located in the ARC.

Kisspeptin-GPR54 signaling is accepted to play a critical role in regulation of reproduction via direct stimulation of GnRH and subsequent gonadotropin release in mammals such as humans [14, 15], primates [16], ruminants [13, 17] and rodents [18]. Male mice and rats with conventional knockout (KO) of the kisspeptin-encoding *Kiss1* gene exhibit atrophied testes, low levels of testosterone, and a suppression of LH pulses [18, 19]. In rodents of both sexes, *Kiss1*/kisspeptin expression is negatively regulated by estradiol and/or testosterone [20–22]. ARC kisspeptin neurons may therefore be a site for the negative feedback action of gonadal steroids that exert an inhibitory effect on LH pulses in both males and females, and are the

most likely candidate for the GnRH/LH pulse generator. However, conditional *Kiss1* KO or knockdown is still necessary to prove this hypothesis, as another population of kisspeptin neurons exists in the anteroventral periventricular nucleus (AVPV) in female rodents. In male rodents, kisspeptin neurons are mainly located only in the ARC since the AVPV *Kiss1* expression is known to be masculinized and/or defeminized by perinatal testosterone surge to cause failure of AVPV *Kiss1* expression as well as occurrence of LH surge [23]. In this context, inducible *Kiss1* KO/knockdown models with male rodents can be used to investigate the definite role of ARC kisspeptin neurons in pulsatile LH secretion.

In this study, we generated inducible ARC-specific *Kiss1* knockdown mice by infecting adeno-associated virus (AAV) vectors driving Cre recombinase (AAV-Cre-GFP) into the brains of *Kiss1*-floxed adult male mice. We analyzed pulsatile LH secretion and determined the long-term effects of ARC *Kiss1* knockdown on male reproductive functions by examining the phenotypes 4 and 8 weeks after the AAV-Cre-GFP injection.

## Materials and Methods

Animals

Kiss1-floxed mice were bred in-house from homozygous breeding pairs of *Kiss1*-floxed mice, in which exon 3 of the Kiss1 gene were floxed with LoxP sites, as described in detail elsewhere [24]. Animals were housed in a controlled environment (14L:10D, lights on at 0500 h, at $22 \pm 2°C$) with free access to food and water. Surgical procedures for the animals were performed under isoflurane anesthesia. The study was approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University; and the institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Stereotaxic injection of AAV into the ARC of male mice

We used a Cre/loxP system to induce region-specific *Kiss1* knockdown. For ARC-specific deletion of *Kiss1*, we stereotaxically injected *cre*-expressing AAV8 (AAV-Cre-GFP, Vector BioLabs, PA, USA) or control AAV8 (AAV-GFP, Vector BioLabs) into the ARC of *Kiss1*-floxed male mice (15–25 weeks old). Each mouse was bilaterally injected with a 26G stainless cannula, with the tip placed 2.3 and 2.7 mm posterior, ± 0.35 mm lateral, and –5.6 mm ventral to the bregma according to the Paxinos mouse brain atlas [25] (Fig. 1A). We slowly infused 3 µL of phosphate-buffered saline (PBS) with AAV (2×10^{12} GC /ml) into the ARC at a rate of 200 nl/min. After the infusion, the injector was kept in place for an additional 5 min before withdrawal to allow the virus solution to diffuse and to prevent backflow through the needle track. Post-surgery mice were housed individually for up to 8 weeks to allow recovery and sufficient *Kiss1* gene deletion before further experiments.

In vivo analysis of reproductive functions in the inducible ARC Kiss1 knockdown male mice

Pulsatile LH secretions were analyzed by frequent blood sampling in free-moving male mice as described previously [26]. Mice were bilaterally castrated one week before frequent blood sampling to detect LH pulses without the negative feedback effects (if any) of
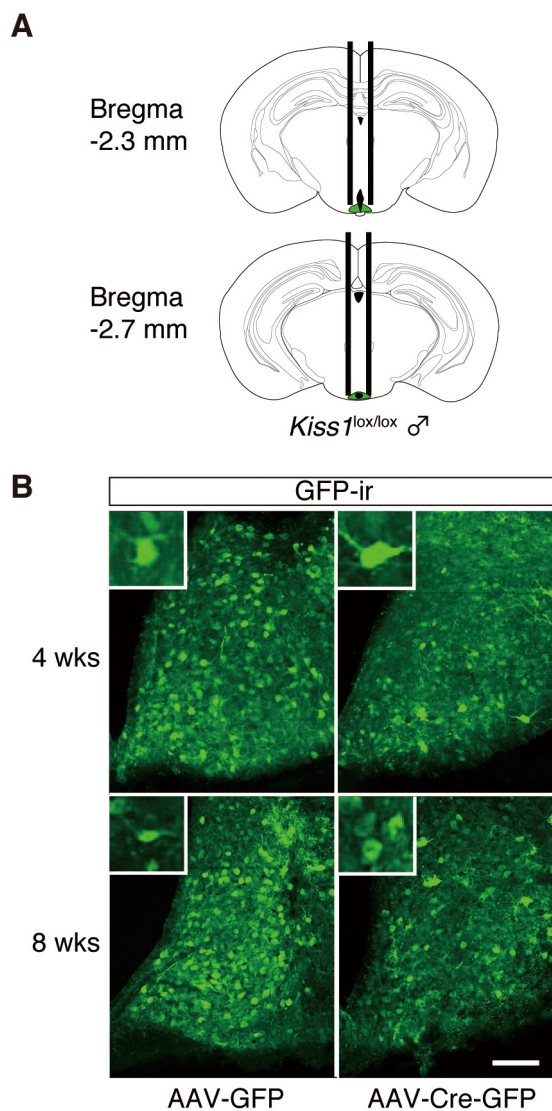
Fig. 1. Transgene expression in the arcuate nucleus (ARC) of *Kiss1*-floxed (*Kiss1*$^{lox/lox}$) male mouse brain 4 or 8 weeks after the injection of adeno-associated virus (AAV) vector into the ARC. (A) Schematic representation of the site of injection (*green*, the ARC) adapted from the Paxinos and Franklin [25] mouse brain atlas. (B) Representative photomicrographs showing the distribution of GFP immunoreactivity (GFP-ir) in the ARC of a *Kiss1*-floxed male mouse injected with AAV-GFP or AAV-Cre-GFP. Insets: GFP-ir cells at a higher magnification. Scale bar, 100 µm. AAV-GFP, *Kiss1*-floxed male mice transduced with AAV-GFP in the ARC; AAV-Cre-GFP, *Kiss1*-floxed male mice transduced with AAV-Cre-GFP in the ARC; 4 wks, *Kiss1*-floxed male mice analyzed 4 weeks after AAV injection; 8 wks, floxed male mice analyzed in 8 weeks after AAV injection.

steroid hormones. To analyze plasma testosterone concentrations, blood samples (50 µl) were obtained from the caudal vein under anesthesia immediately before castration. The testes were weighed, fixed with 4% paraformaldehyde PBS solution, and their paraffin sections (4 µm) were histologically examined under a microscope

after general staining with hematoxylin-eosin (HE). To quantify testes morphology, two cross-sections were made from the middle of the testis. Tubular diameters were imaged at 4 × with a light microscope (BX50; Olympus, Tokyo, Japan) and analyzed across their shortest axis from cross-sectional profiles using ImageJ software (National Institutes of Health, Bethesda, MD, USA). To analyze the percentage of mature spermatids in total tubules, seminiferous tubules containing step 16 spermatids (elongated spermatids that line the lumen [27]) were counted under a 10 × ocular and 20 × objective of a light microscope (BX50; Olympus).

Each animal then received an indwelling atrial cannula made of silicon tubing (internal diameter, 0.5 mm; outer diameter, 1.0 mm; length, 22 cm; Shin-Etsu Polymer, Tokyo, Japan) through the right jugular vein, 2 days before blood sampling. In order to determine LH pulses, blood samples (20 µl) were collected through the intra-atrial cannula at 3-min intervals for 1 h starting at 1300 h. To keep the hematocrit values constant, each blood sample was replaced with an equivalent volume of washed red blood cells obtained from other ICR strain wild-type mice. Plasma was separated by centrifugation at 3,000 rpm for 10 min at 4ºC, and 10 µl aliquots were stored at –30ºC until the LH assay. To confirm no changes in their estimated total body fluid volume, the body weights of each mouse was measured before and after the blood sampling.

Visualization of Kiss1 and GFP

Castrated *Kiss1*-floxed mice treated with AAV-Cre-GFP or AAV-GFP in the ARC were deeply anesthetized with sodium pentobarbital and perfused with 0.05 M PBS, followed by 4% paraformaldehyde-PB through the right atrium, clamping the thoracic vena cava to prevent fixative from perfusing the lower body. Brains were removed immediately, postfixed in the same fixative overnight at 4°C, and then immersed in 30% sucrose in 0.05 M PB at 4°C. Serial coronal sections (50 µm in thickness) of the hypothalamus containing the ARC were obtained using a cryostat. Every fourth section of the whole ARC (1.23–2.79 mm posterior to the bregma)— taken from each mouse according to the brain atlas [25]— was subjected to histological analysis for *Kiss1* and/or GFP expression.

Mouse *Kiss1* mRNA expression in the ARC was detected by free-floating *in situ* hybridization using specific digoxigenin (DIG)-labeled probes, as described elsewhere [28]. DIG-labeled anti-sense cRNA probes for mouse *Kiss1* (position 60–372; GenBank accession no. NM_178260) were synthesized by *in vitro* transcription from the mouse hypothalamic cDNA using a DIG-RNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany). To detect the DIG-labeled *Kiss1* probe after hybridization, alkaline phosphatase-conjugated anti-DIG antibody (1:1000; Roche Diagnostics, Indianapolis, IN, USA) and a chromagen solution (337 µg/ml 4-Nitroblue tetrazolium chloride, 175 µg/ml 5-Bromo-4-chloro-3-indoyl-phosphate) were used. The sections were mounted and examined by an optical microscope (BX51; Olympus). Specificity of *Kiss1* probes was confirmed by the absence of signal in ARC sections hybridized with the sense *Kiss1* cRNA probe (data not shown), while *Kiss1* expression was detected exclusively with anti-sense probe.

To visualize GFP, the sections were incubated with anti-GFP chicken polyclonal antibody (1:2000, ab13970, Abcam, Cambridge, UK) and then incubated with secondary antibodies conjugated with Alexa Fluor 488 (1:800, A21202, Invitrogen, Carlsbad, CA, USA). Fluorescence images were obtained with laser scanning confocal microscopy (LSM5 Pascal; Carl Zeiss, Oberkochen, Germany).

LH and testosterone assays

Plasma LH concentrations were determined with a mouse LH radioimmunoassay kit provided by the National Hormone and Peptide Program (Bethesda, MD, USA). LH concentrations were expressed in terms of NIDDK mouse LH-RP AFP-5306A. The least detectable level was 0.78 ng/ml for 10 µl plasma, and the intra- and inter-assay coefficients of variation were 5.3% at 4.9 ng/ml and 10.7% at 2.03 ng/ml, respectively.

Plasma testosterone concentrations were determined by an enzyme immunoassay as described previously [29]. Testosterone was measured after diethyl-ether extraction, followed by an overnight incubation with sheep anti-testosterone antibody (1: 60,000, GDN #250, kindly donated by Dr GD Niswander, Colorado University, CO, USA) at 4°C in 96-well plates pre-coated with rabbit anti-sheep IgG antibody (0.5 µg/100 µl/well, #613-4128, Rockland Immunochemicals, PA. USA). After incubation with horseradish peroxidase (HRP)-labeled testosterone for 4 h at 4°C, HRP activity was visualized using a 3, 3', 5, 5'-tetrame-thylbenzidine substrate. The least detectable level was 0.125 ng/ml for 10 µl plasma, and the intra- and inter-assay coefficients of variation were 2.6% and 13.3% at 5.08 ng/ml, respectively.

Data analysis and statistics

To quantify the number of *Kiss1*-expressing cells, every fourth section of the whole ARC region was used for counting. The numbers of *Kiss1*-expressing cells in the ARC were statistically analyzed. LH pulses were identified by the PULSAR computer program [30] as previously described [31]. Statistical differences between AAV-GFP and AAV-Cre-GFP groups in terms of testicular weight, tubular diameters, percentage of mature spermatids in total tubules, each LH pulse parameter, and the number of cells expressing *Kiss1* mRNA were determined by Student's *t*-tests. Statistical difference in plasma testosterone levels between the AAV-GFP and AAV-Cre-GFP groups was determined by the Mann-Whitney *U* test.

Results

Confirmation of region-specific Kiss1 knockdown in the ARC of adult male mice

Kiss1-floxed male mice were bilaterally injected with AAV8 into the middle (Fig. 1A, upper panel) and posterior (Fig. 1A, lower panel) part of the ARC. Fig. 1B shows the immunohistochemical localization of GFP immunoreactivity (ir) in the ARC of representative *Kiss1*-floxed male mice injected with AAV-GFP or AAV-Cre-GFP. GFP-ir cells were observed in the ARC in both AAV-GFP and AAV-Cre-GFP groups at 4 or 8 weeks after the injection.

Fig. 2A shows *Kiss1*-expressing cells in the ARC of representative *Kiss1*-floxed male mice injected AAV-GFP or AAV-Cre-GFP. Few *Kiss1*-expressing cells were found in the ARC of AAV-Cre-GFP-injected mice, while such cells were abundant in control mice (Fig. 2A). The numbers of *Kiss1*-expressing cells in AAV-Cre-GFP mice (468 ± 57 cells at 4 weeks and 331 ± 34 cells at 8 weeks) were significantly lower ($P < 0.05$ at 4 weeks and $P < 0.01$ at 8 weeks,

A

B
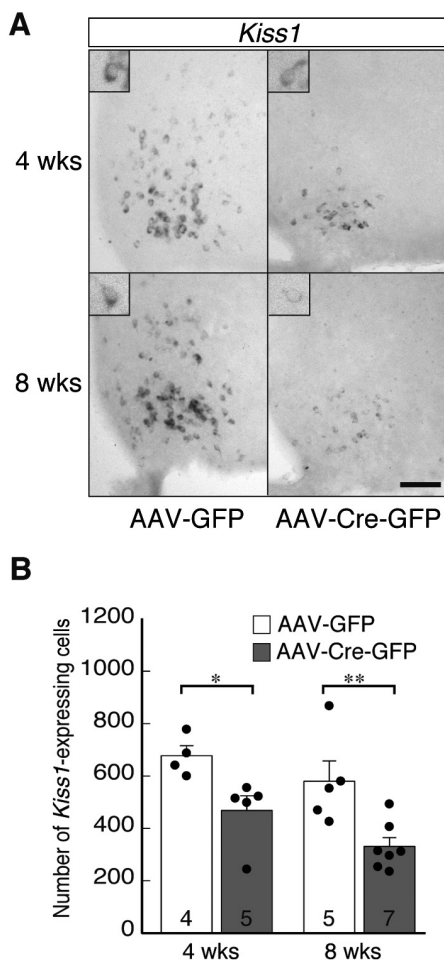
Fig. 2. Inducible knockdown of *Kiss1* expression in the arcuate nucleus (ARC) of castrated male *Kiss1*-floxed mice. (A) Representative photomicrographs showing the distribution of *Kiss1* mRNA in the ARC of each group. Scale bar, 100 μm. (B) Number of *Kiss1*-expressing cells in the ARC, with the mean ± SEM and individual data points overlaid on the bar charts. The numbers in or on each column indicate the sample size of the group. * P < 0.05; ** P < 0.01 (*vs.* AAV-GFP-injected controls, Student's *t*-test). Abbreviations are the same as in Fig. 1.

Student's *t*-test) compared to those in the corresponding groups of control mice (678 ± 38 cells at 4 weeks and 580 ± 77 cells at 8 weeks) (Fig. 2B).

Suppression of LH pulses by the inducible ARC Kiss1 knockdown in male mice

Fig. 3A shows the profiles of LH release in representative *Kiss1*-floxed male mice in each group. Apparent LH pulses were found in control *Kiss1*-floxed mice at both 4 and 8 weeks after AAV-GFP injection, but pulsatile LH release was profoundly suppressed in *Kiss1*-floxed mice 4 weeks after AAV-Cre-GFP injection (Fig. 3A). In contrast, LH pulses were normal in *Kiss1*-floxed mice 8 weeks after the injection (Fig. 3A). The frequency of LH pulses was significantly different between AAV-Cre-GFP-injected mice and their control

counterparts at 4 weeks after injection (P < 0.05), but not at 8 weeks (Fig. 3B). The mean and baseline LH levels, as well as the amplitude of LH pulses in AAV-Cre-GFP-injected mice were comparable to those in control mice (Fig. 3B).

The effect of inducible ARC Kiss1 knockdown on testis morphology and plasma testosterone levels in male mice

Fig. 4A shows representative photomicrographs of HE-stained testes obtained from AAV-GFP- or AAV-Cre-GFP-injected *Kiss1*-floxed male mice 4 or 8 weeks after the injection. No obvious changes in testis morphology were observed between AAV-Cre-GFP-injected mice and control mice (Fig. 4A). Plasma testosterone levels of AAV-Cre-GFP-injected *Kiss1*-floxed mice tended to be lower (P = 0.1) at 4 weeks after the AAV injection, but no significant difference was found between groups (Fig. 4B, left). Plasma testosterone levels at 8 weeks after injection showed no significant difference between groups. Testicular weights were comparable between the control and AAV-Cre-GFP groups at both 4 and 8 weeks after injection (Fig. 4B, right). Additionally, both seminiferous tubule diameters and percentage of mature spermatids in total tubules were comparable between the control and AAV-Cre-GFP groups at both 4 and 8 weeks after injection (Fig. 4C). Body weight also remained unaffected in AAV-Cre-GFP-injected mice 4 weeks (AAV-GFP: 47.48 ± 2.01 g *vs.* AAV-Cre-GFP: 46.68 ± 1.76 g) or 8 weeks (AAV-GFP: 46.33 ± 3.00 g *vs.* AAV-Cre-GFP: 49.97 ± 2.77 g) after injection.

Discussion

This study demonstrates that the inducible ARC *Kiss1* knockdown male mice exhibited suppression of pulsatile LH release, suggesting that the ARC kisspeptin neurons might serve as a GnRH/LH pulse generator in mice. These results are consistent with our previous study that reported complete suppression of pulsatile LH secretion in rats after a conventional *Kiss1* KO [18]. An advantage of the current inducible *Kiss1* knockdown male mice model is that it minimizes the potential confounding effect of developmental compensation. Although technical limitations resulted in the acute deletion of only 30% of total ARC *Kiss1* expression, the results indicate that this partial deletion was sufficient for significant suppression of GnRH/LH pulse generation. Accumulating evidence suggests that neurokinin B (NKB) and dynorphin A —expressed in the ARC kisspeptin neurons— facilitate and inhibit neuronal activity, respectively, and hence generate the rhythmic activity of the neurons [13]. Our results —demonstrating that targeting only *Kiss1* knockdown lowered LH pulse frequency— suggest that in addition to these neuropeptides, kisspeptin-GPR54 signaling may also be involved in the GnRH/LH pulse generation. This notion is also supported by previous studies showing that administration of kisspeptin increases LH pulse frequency in ewes and humans, in which NKB inactivation significantly lowers the LH pulse frequency [32, 33]. GPR54 expression was abundantly detected in close proximity to the ARC kisspeptin neurons, but not in the ARC kisspeptin-ir cells in female rats [34], suggesting that ARC kisspeptin neurons might construct neuronal circuits with other GPR54-expressing cells in order to affect GnRH/LH pulse generation.

In this study, testicular morphology and plasma testosterone
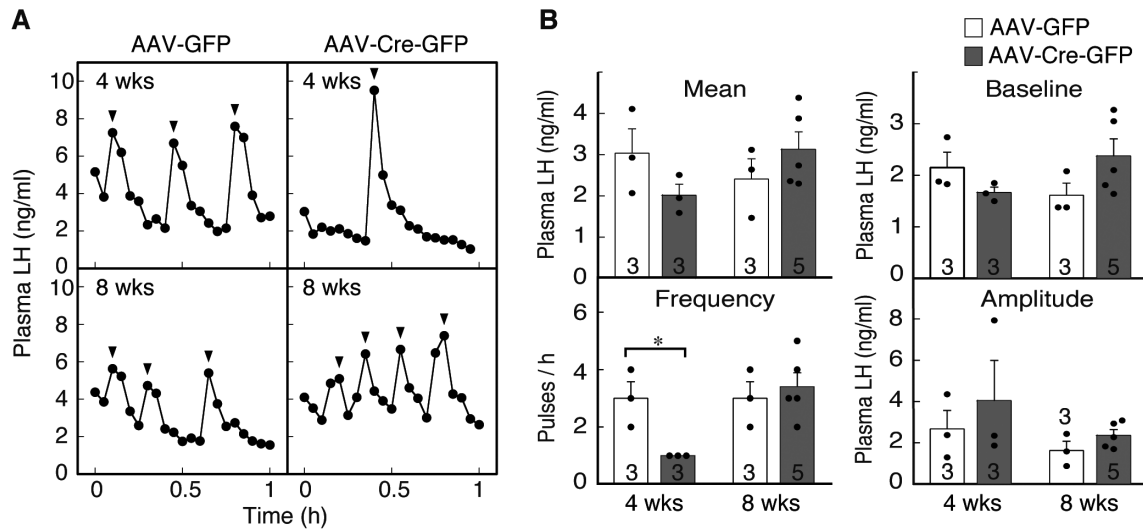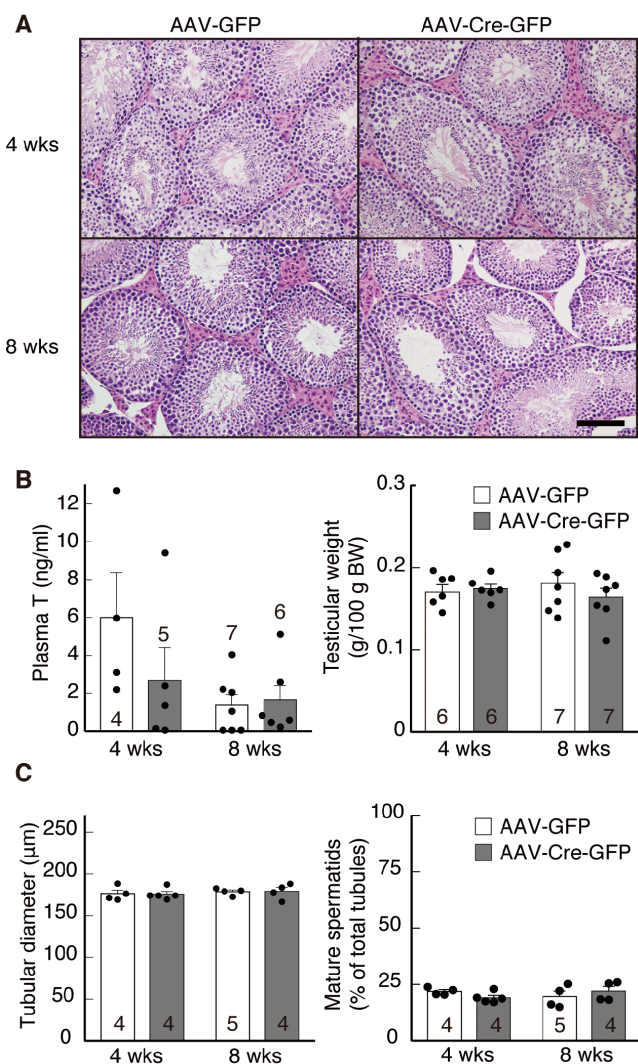
Fig. 3. Effect of AAV-GFP or AAV-Cre-GFP injection on pulsatile luteinizing hormone (LH) secretion in castrated male *Kiss1*-floxed mice. (A) Plasma LH profiles in representative animals from each group. Blood samples were collected every 3 min for 1 h a week after the castration. Arrowheads indicate the peaks of LH pulses as identified by the PULSAR computer program. (B) Mean and baseline LH concentrations, and the frequency and amplitude of LH pulses were calculated for a 1-h sampling period. Bar charts portray the mean ± SEM with individual data points overlaid. The numbers in or on each column indicate the sample size of the group. * $P < 0.05$ (*vs.* AAV-GFP-injected controls, Student's *t*-test). Abbreviations are the same as in Fig. 1.

levels were comparable between control and AAV-Cre-GFP groups despite a reduction of pulsatile LH secretion in *Kiss1*-floxed mice 4 weeks after the AAV injection. The discrepancy between LH pulses and testicular functions in the inducible *Kiss1* knockdown mice could be because their mean and baseline LH levels and amplitude of LH pulses were comparable to the control groups. GnRH/LH pulsatility is essential for pituitary responsiveness to GnRH because gonadotropin synthesis and GnRH receptor gene expression in the pituitary gonadotroph depend on the pulsatile pattern of GnRH signals [35, 36], whereas testicular function could be maintained by a certain plasma level of LH. This interpretation is supported by the fact that hypophysectomized male rats injected with LH twice daily show an increase in plasma testosterone levels [37]. Since the inducible *Kiss1* knockdown mice showed significant suppression of ARC *Kiss1* expression even though 70% of ARC kisspeptin neurons remained, the remaining kisspeptin neurons might be sufficient to maintain testicular functions.

Interestingly, pulsatile LH secretion was detected 8 weeks after the AAV-Cre injection despite the suppression of ARC *Kiss1* expression, indicating the possibility of a compensatory mechanism against the reduction of kisspeptin neurons. Complete *Kiss1* KO showed hypogonadotropic hypogonadism in male mice [19], and several mice models with conventional *Kiss1* KO have also shown that a compensatory mechanism during the developmental period may rescue reproductive functions in global *Kiss1* KO mice [38, 39]. While it is still unclear how LH pulses in the inducible *Kiss1* knockdown mouse model are rescued; we suggest two possible explanations. First, the residual kisspeptin neurons in this study may have acquired full functionality as a GnRH pulse generator. This

interpretation is supported by a previous study which determined that male homozygous *Kiss1*[Cre/Cre] mice with only 5% of the normal *Kiss1* transcript can sire normal-sized litters [40]. Suppression of LH pulses 4 weeks after the AAV-Cre injection suggests that residual kisspeptin neurons reconstruct their neuronal network for GnRH pulse generator. Such a neuronal network reconstruction in adulthood has been reported in GnRH neurons; transplants of GnRH neurons into GnRH-deficient mice increased gonadotropin levels, gonadal development, and spermatogenesis [41]. Second, the reduction of *Kiss1* may be compensated by systems other than kisspeptin neurons, which modulate GnRH secretions. Seminara *et al.* suggested the existence of a kisspeptin-independent GnRH-stimulatory pathway by showing that treatment with a GnRH antagonist significantly decreased serum gonadotropin levels and spermatogenesis in *Kiss1* KO mice with hypogonadotropic hypogonadism compared to vehicle treatment [38]. Thus, these compensatory changes in the hypothalamic GnRH/LH pulse generation system might cause LH pulse recovery, and subsequently induce spermatogenesis.

Our results demonstrate that the ARC kisspeptin neurons are responsible for pulsatile LH secretion in male mice, despite our technical limitations that were only able to achieve partial knockdown of ARC *Kiss1* expression. Our results also show possibility of potential compensatory mechanism to restore GnRH/LH pulse generation in adulthood. This study improves our understanding of how a highly redundant system of GnRH/LH pulse generation with central kisspeptin ensures reproductive success during adulthood in male mice.

Fig. 4. Effect of AAV-GFP or AAV-Cre-GFP injection on testicular functions in male *Kiss1*-floxed mice. (A) Photomicrographs of testicular sections stained by hematoxylin and eosin in a representative animal from each group. Insets: *Kiss1*-expressing cells at a higher magnification. Scale bar, 100 μm. (B) Plasma testosterone (T) levels (*left*) and testicular weights (*right*) in each group. (C) Tubular diameters (*left*) and percentage of mature spermatids in total tubules (*right*) in each group. Bar charts portray the mean ± SEM with individual data points overlaid. Numbers in or on each column indicate the sample size of the group. Abbreviations are the same as in Fig. 1.

Conflict of Interests: The authors have nothing to disclose.

Acknowledgments

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