

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

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Abstract. Hypothalamic kisspeptin neurons are master regulators of mammalian reproduction via direct stimulation of gonadotropin-releasing hormone and consequent gonadotropin release. Here, we generated novel *Kiss1* (kisspeptin gene)-*Cre* rats and investigated the developmental changes and sex differences in visualized *Kiss1* neurons of *Kiss1-Cre*-activated tdTomato reporter rats. First, we validated *Kiss1-Cre* rats by generating *Kiss1*-expressing cell-specific *Kiss1* knockout (*Kiss1-KpKO*) rats, which were obtained by crossing the current *Kiss1-Cre* rats with *Kiss1*-floxed rats. The resulting male *Kiss1-KpKO* rats lacked *Kiss1* expression in the brain and exhibited hypogonadotropic hypogonadism, similar to the hypogonadal phenotype of global *Kiss1* KO rats. Histological analysis of *Kiss1* neurons in *Kiss1-Cre*-activated tdTomato reporter rats revealed that tdTomato signals in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) were not affected by estrogen, and that tdTomato signals in the ARC, AVPV, and medial amygdala (MeA) were sexually dimorphic. Notably, neonatal AVPV tdTomato signals were detected only in males, but a larger number of tdTomato-expressing cells were detected in the AVPV and ARC, and a smaller number of cells in the MeA was detected in females than in males at postpuberty. These findings suggest that *Kiss1*-visualized rats can be used to examine the effect of estrogen feedback mechanisms on *Kiss1* expression in the AVPV and ARC. Moreover, the *Kiss1-Cre* and *Kiss1*-visualized rats could be valuable tools for further detailed analyses of sexual differentiation in the brain and the physiological role of kisspeptin neurons across the brain in rats.

Key words: Hypogonadotropic hypogonadism, Kisspeptin, Neuroendocrinology, Reproduction, Sex differences

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Kisspeptin is a neuropeptide encoded by the *Kiss1* gene that plays an indispensable role in reproductive functions such as puberty onset, gametogenesis, and ovulation, via the direct stimulation of gonadotropin-releasing hormone (GnRH) secretion in the mammalian hypothalamus [1–4]. Pioneering studies by Seminara *et al.* and de Roux *et al.* demonstrated that mutations in *GPR54* (kisspeptin receptor gene) caused hypogonadotropic hypogonadism in humans [5, 6]. Topaloglu *et al.* subsequently showed that a mutation of *KISS1* also replicated hypogonadotropic hypogonadism in humans [7]. *Kiss1* global knockout (KO) rats [8] and mice [9,10] showed pubertal failure and infertility in both sexes. Kisspeptin administration potently stimulates gonadotropin release in all mammalian species, such as rodents [11–13], sheep [14], goats [15], cows [16], and primates [17], including humans [18]. These results suggest that kisspeptin neurons are targets for therapeutic approaches in hypothalamic reproductive disorders, such as premature ovarian failure and anovulation disease. Indeed, it has been reported that at least 25% of women suffer from reproductive disorders due to hypothalamic dysfunction [19–21].

The cell bodies of kisspeptin neurons are primarily located in two distinct regions of the rodent hypothalamus: the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) [13, 22–25]. ARC kisspeptin neurons are involved in the generation of pulsatile GnRH/gonadotropin release, which controls gametogenesis and steroidogenesis in male and female rodents [26–32]. ARC kisspeptin neurons are a major site of gonadal steroid negative feedback that suppresses tonic (pulsatile) luteinizing hormone (LH) release because ARC kisspeptin neurons express estrogen receptor α (ER α) and estrogen downregulates ARC *Kiss1* expression in female rats and mice [24, 33]. Estrogen failed to suppress ARC *Kiss1* expression in kisspeptin neuron-specific ER α KO mice [34]. Notably, LH pulses and gonadectomy-induced increase in LH and follicle-stimulating hormone (FSH) completely disappeared in *Kiss1* global KO male and female rats [8]. On the other hand, kisspeptin neurons in the AVPV in rodents or the preoptic area (POA) in other species are responsible for estrogen positive feedback that generates a GnRH/LH surge to trigger ovulation in female mammals [2–4, 35]. Estrogen upregulates *Kiss1* expression in AVPV/POA kisspeptin neurons in rodents [13, 24, 33, 36], pigs [37], and Japanese monkeys [38]. Estrogen activates AVPV/POA kisspeptin neurons in rats, goats, and Japanese monkeys [24, 38, 39] and fails to upregulate AVPV *Kiss1* expression in kisspeptin neuron-specific ER α KO mice [34]. Indeed, *Kiss1* global KO rats fail to show spontaneous or estrogen-induced LH surge [8].

AVPV/POA kisspeptin neurons exhibit sexual dimorphism in

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rodents and other mammals. A larger number of AVPV/POA kisspeptin neurons are found in adult females than in males in mice [40, 41], rats [36, 42], and Japanese monkeys [38]. The sexual dimorphism of AVPV kisspeptin neurons is responsible for sex differences in their ability to exert a GnRH/LH surge induced by the preovulatory level of estrogen in rodents. Estrogen largely induces AVPV *Kiss1*/kisspeptin expression and triggers an LH surge in female rats, but not in male rats [42]. Notably, AVPV kisspeptin neurons play a physiological role in male rats because mating behavior increases the number of AVPV kisspeptin neurons in male rats [43]. AVPV *Kiss1* expression was examined in adult animals in most studies. On the other hand, some studies reported AVPV *Kiss1* expression on postnatal Day 11 in female and male rats [44] and postnatal Day 10 in female and male mice [45]. Notably, kisspeptin-immunoreactive (IR) cells were evident from embryonic Day 17 (E17) to postnatal Day 1 in the AVPV of male mice, but few kisspeptin-IR cells were found in the AVPV of female mice during the perinatal and prepubertal periods [46]. Pre- and postnatal AVPV *Kiss1* induction in male rodents may be due to the enhanced effect of estrogen derived from perinatal testosterone in rodents [47].

ARC kisspeptin neurons do not exhibit any apparent sexual dimorphism. ARC kisspeptin expression is evident during the late embryonic period in rodents of both sexes. Kisspeptin-IR cells were found in the ARC of female and male mice at E13 [48], and in female and male rats at E14.5 [49]. The physiological role of the embryonic or perinatal ARC *Kiss1*/kisspeptin has not been clarified in female rodents, but neonatal ARC *Kiss1*/kisspeptin expression is responsible for the neonatal testosterone surge in male rodents [50], which masculinizes the male rodent brain [47]. Notably, ARC *Kiss1*/kisspeptin expression is suppressed during the prepubertal period in female rats, and an increase in ARC *Kiss1*/kisspeptin expression triggers puberty onset [51, 52].

Kiss1 expression has also been reported in several brain regions of adult male and female rats and mice, including the medial amygdala (MeA) [53]. MeA kisspeptin neurons may modulate behavioral responses to olfactory cues, sexual partner preferences, and anxiety [54, 55]. Weak *Kiss1* expression was reported in the ventromedial nucleus of the hypothalamus (VMH) on postnatal Days 7 and 19 in male and female rats [44], and a small number of *Kiss1*/kisspeptin-IR cells were detected in the VMH of adult rats [56]. Our previous study revealed that *Kiss1*-tdTomato rats show little tdTomato fluorescence in the VMH of adult female rats [8]. However, the physiological role of *Kiss1* cells in the VMH is not yet fully understood.

Kiss1 neurons were reported in the lateral septum (LS) [57] in γ -aminobutyric acid (GABA) B1 receptor KO male and female mice, but few *Kiss1* neurons were detected in the LS of either sex of wild-type mice. Kisspeptin-IR cell bodies are also found in the bed nucleus of the stria terminalis (BNST) of male and female rats [56] and *Kiss1*-expressing cells are found in the BNST of mice of both sexes [57]. Kisspeptin-IR cell bodies were also found in the dorsal part of the dorsomedial nucleus (DMD) of male and female rats [58] and female mice [59], as well as the posterior hypothalamus (PH) of female mice [60]. Kisspeptin-IR fibers were detected in the paraventricular nucleus (PVN), reticular thalamic nucleus (Rt), medial POA (MPOA), retrochiasmatic area (RCh) [58], and medial tuberal nucleus (MTu) of female rats [56] and the MPOA [60] and PVN [41, 59, 60] of male and female mice. *Kiss1-Cre* reporter signals were detected in the cortex of female mice [61]. However, little is known about the physiological role of *Kiss1*/kisspeptin neurons in the brain nuclei, except for the AVPV and ARC, because weak *Kiss1* expression in most of these nuclei, such as the MeA, VMH,

LS, and BNST, makes it difficult to detect *Kiss1*-expressing cells. Therefore, genetically engineered *Kiss1*-visualized rats could be a useful model to investigate the physiological roles of potential *Kiss1*/kisspeptin neurons in these nuclei because *Kiss1*-visualized rats allow us to identify potential kisspeptin neurons by tdTomato fluorescence, regardless of *Kiss1*/kisspeptin expression.

The present study generated novel *Kiss1-Cre* rats and investigated developmental changes and sex differences in visualized *Kiss1* neurons in *Kiss1-Cre*-activated tdTomato reporter rats. First, we validated *Kiss1-Cre* rats by generating *Kiss1*-expressing cell-specific *Kiss1* knockout (*Kiss1-KpKO*) rats, which were obtained by crossing *Kiss1-Cre* rats with *Kiss1*-floxed rats [26]. The resulting male *Kiss1-KpKO* rats were subjected to the evaluation of reproductive indicators, such as testicular weights, ARC *Kiss1* expression, pulsatile LH release and pituitary *Lhb*, *Fshb* and *Gnrhr* mRNA expression. We examined sex differences in the distribution and number of kisspeptin neurons in the brain during several developmental periods using *Kiss1-Cre*-activated tdTomato reporter rats. We generated *Kiss1*-visualized rats by crossing *Kiss1-Cre* rats with *Cre*-dependent tdTomato reporter rats [LE-Tg(Gt(ROSA)26SorCAG-tdTomato)24Jfhy] [62]. The resulting *Kiss1*-visualized female rats underwent histological analysis by double staining for *Kiss1* expression and tdTomato immunoreactivity to examine whether tdTomato signals co-localized with *Kiss1*-expressing cells in the AVPV and ARC. *Kiss1-Cre*-activated tdTomato fluorescence signals were examined in adult gonadectomized male and female rats treated with or without estrogen to confirm whether tdTomato fluorescence was detectable in an estrogen-independent manner. The tdTomato signals were examined in the hypothalamic and extra-hypothalamic areas of male and female rats during several developmental periods, such as postnatal Days 1 and 7, 3 weeks (prepubertal), 7 weeks (postpubertal), and 10–13 weeks (adulthood), to determine developmental changes and sex differences in tdTomato-positive cells. We further analyzed sex differences in the distribution and number of tdTomato-positive cells in detail in the AVPV, where kisspeptin neurons exhibit sexual dimorphism.

Materials and Methods

A detailed description of the methodologies, including the generation of gene-modified rats, radioimmunoassay, qRT-PCR and histological analysis, is provided in the Supplementary Material.

Results

Kiss1-KpKO male rats obtained by crossing newly generated *Kiss1-Cre* rats with *Kiss1*-floxed rats showed hypogonadotropic hypogonadism

We generated *Kiss1-Cre* rats in which the *Cre* recombinase was inserted into the endogenous *Kiss1* locus by homologous recombination (Fig. 1A and Supplementary Fig. 1). To confirm that the *Cre-loxP* system functions in *Kiss1*-expressing cells in *Kiss1-Cre* rats, *Kiss1-Cre* rats were crossed with *Kiss1*-floxed rats (Fig. 1B) [26]. No *Kiss1*-expressing cells were detected in the ARC of castrated (Cast) *Kiss1-KpKO* male rats ($n = 4$), whereas many *Kiss1*-expressing cells were found in the ARC of Cast *Cre* (-)/*Kiss1*-floxed control male rats ($n = 4$) during adulthood (8 weeks of age) (Figs. 1C, D). Cast *Cre* (-)/*Kiss1*-floxed control adult male rats showed frequent LH pulses, whereas Cast *Kiss1-KpKO* adult male rats showed severe suppression of pulsatile LH release (Fig. 1E). The mean LH levels and LH pulse frequency in Cast *Kiss1-KpKO* male rats were significantly lower than those in *Cre* (-)/*Kiss1*-floxed control male rats ($n = 4$) ($P < 0.05$;

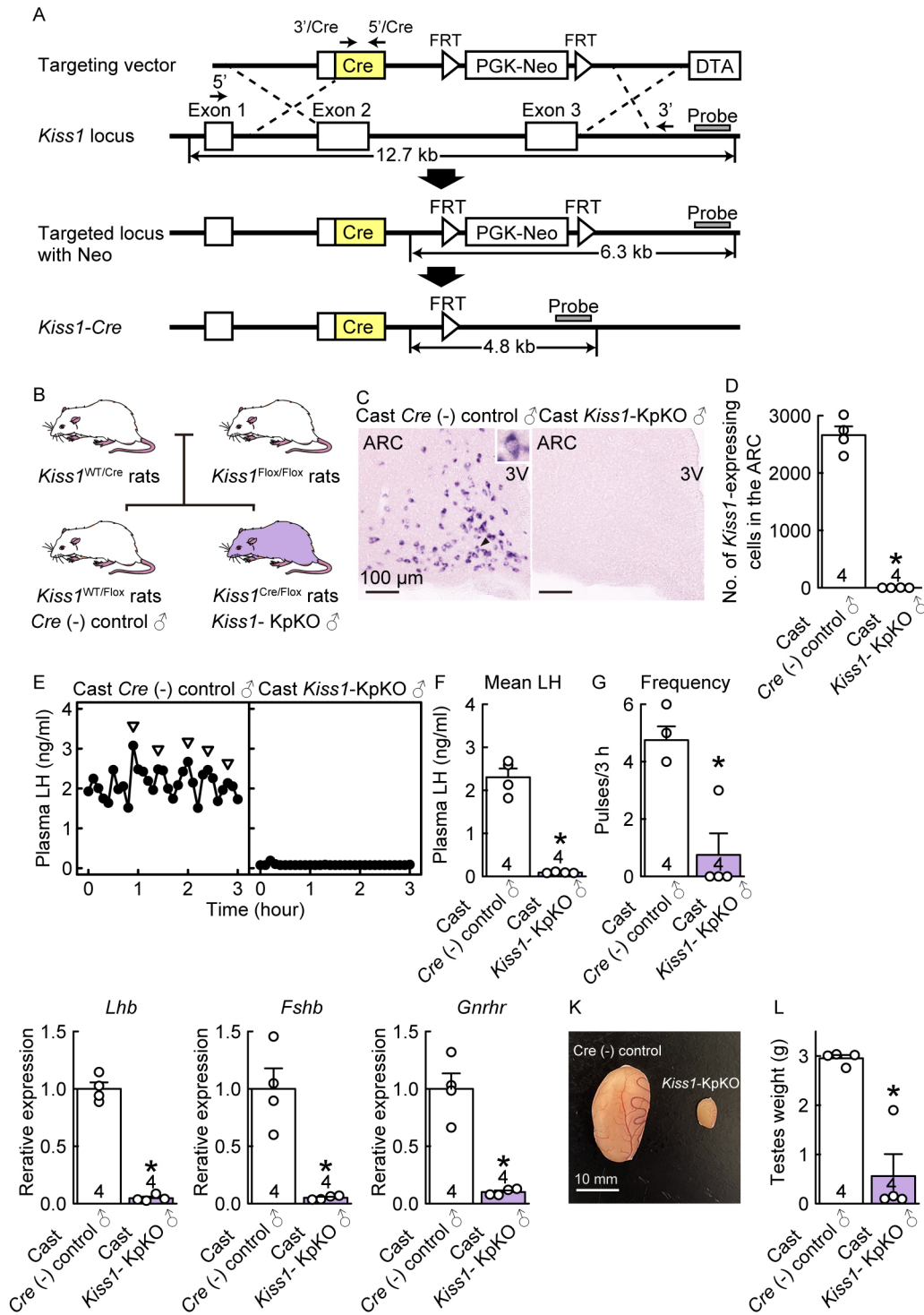


Fig. 1. *Kiss1*-expressing neuron-specific *Kiss1* knockout (*Kiss1*-KpKO) male rats obtained by crossing newly generated *Kiss1*-*Cre* rats with *Kiss1*-floxed rats showed hypogonadotropic hypogonadism. (A) Structures of the targeting vector for the generation of *Kiss1*-*Cre* rats, the wild-type *Kiss1* allele, and the targeted *Kiss1* allele with a neomycin resistance (Neo) selection cassette, resulting from replacement at dotted lines. Structure of the targeted *Kiss1* allele without Neo resulting from enhanced flippase (FLPe)-flippase recognition target (FRT) recombination in rat embryonic stem (ES) cells. A diphtheria toxin A (DTA) expression cassette in the targeting vector was used for negative selection. (B) Schematic illustration showing the generation of *Kiss1*-KpKO rats. To generate *Kiss1*-KpKO rats and Cre (-)/*Kiss1*-floxed control rats, *Kiss1*-*Cre* heterozygous rats were crossed with *Kiss1*-floxed homozygous rats. (C) *Kiss1* mRNA-expressing cells in the ARC of a representative Cast Cre (-)/*Kiss1*-floxed control rat (left) and *Kiss1*-KpKO rat (right) in adulthood. Scale bars, 100 μm. (D) The numbers of *Kiss1* mRNA-expressing cells in the ARC of Cast Cre (-)/*Kiss1*-floxed control rats and *Kiss1*-KpKO rats in adulthood. (E) Plasma LH profiles in a representative Cast Cre (-)/*Kiss1*-floxed control rat (left) and *Kiss1*-KpKO rat (right) in adulthood. Arrowheads indicate LH pulses identified by the PULSAR computer program. Mean LH concentration (F) and the frequency (G) of LH pulses in Cast Cre (-)/*Kiss1*-floxed control rat and *Kiss1*-KpKO rats in adulthood. Pituitary *Lhb* (H), *Fshb* (I), and *Gnhrh* (J) mRNA expression. (K) Photographs of testes obtained from a representative Cast Cre (-)/*Kiss1*-floxed control rat (left) and *Kiss1*-KpKO rat (right) in adulthood. Scale bars, 10 mm. (L) Testicular weights in *Kiss1*-KpKO rats and Cast Cre (-)/*Kiss1*-floxed control rats in adulthood. Sample size, n = 4. Values are the means ± SEMs. * Significant difference between groups (P < 0.05, Welch's *t* test). See also Supplementary Fig. 1.

Figs. 1F, G). Furthermore, pituitary *Lhb*, *Fshb*, and *Gnrhr* mRNA expression levels were significantly lower in Cast *Kiss1*-KpKO male rats than in Cast *Cre* (-)/*Kiss1*-floxed control male rats ($P < 0.05$; Figs. 1H, I, J). The testes of *Kiss1*-KpKO rats were smaller than those of *Cre* (-)/*Kiss1*-floxed control rats (Fig. 1K), and the testicular weights of *Kiss1*-KpKO rats were significantly lower than those of *Cre* (-)/*Kiss1*-floxed control rats ($n = 4$) ($P < 0.05$; Fig. 1L). These findings suggest that the Cre-loxP system functions in *Kiss1*-expressing cells in the current *Kiss1*-*Cre* rats.

Colocalization of Kiss1 and Kiss1-Cre-activated tdTomato-IR signals and the lack of the effect of estrogen on tdTomato fluorescence signals in the AVPV and ARC of Kiss1-visualized rats

Theoretically, cells that have expressed *Kiss1/Cre* at least once should persistently express tdTomato fluorescence following Cre recombination, regardless of *Kiss1* expression in *Kiss1-Cre*-activated tdTomato reporter rats. To confirm that tdTomato fluorescence co-localized with *Kiss1* in the AVPV and ARC, we used *in situ* hybridization for *Kiss1* expression and immunohistochemistry for tdTomato-IR. Immunohistochemistry for tdTomato was used only for double staining, because tdTomato fluorescence could not be detected after *Kiss1 in situ* hybridization. A number of *Kiss1*-expressing and tdTomato-IR cells were found in the AVPV of estradiol-17 β (E2)-treated ovariectomized (OVX) adult (8–16 weeks of age) female rats (Fig. 2, A and B). Most ($82.0 \pm 3.0\%$) *Kiss1*-expressing cells co-expressed tdTomato-IR signals in the AVPV, and approximately half ($49.8 \pm 4.3\%$) of tdTomato-IR cells co-expressed *Kiss1* signals in the AVPV of the E2-treated OVX female rats (Fig. 2C). Many *Kiss1*-expressing and tdTomato-IR cells were found in the ARC of adult OVX female rats (Fig. 2, D and E), and most ($86.4 \pm 1.3\%$) *Kiss1*-expressing cells co-expressed tdTomato-IR signals in the ARC of OVX female rats (Fig. 2F). Approximately two-thirds ($68.3 \pm 7.1\%$) of tdTomato-IR cells co-expressed *Kiss1* signals in the ARC of OVX female rats (Fig. 2F).

To confirm that circulating E2 did not affect tdTomato expression in the AVPV and ARC, we compared the number of AVPV and ARC tdTomato fluorescence-positive cells in OVX females and Cast males with and without E2 treatment in adulthood (8–16 weeks of age). Many tdTomato fluorescence-positive cells were found in the AVPV of OVX and Cast rats regardless of E2 treatment (Fig. 2, G and H), whereas *Kiss1* expression was found only in the AVPV of E2-treated OVX rats (Fig. 2I). E2 treatment did not significantly affect the number of AVPV tdTomato fluorescence-positive cells in the OVX females or Cast males (Fig. 2H). The number of tdTomato fluorescence-positive cells in the AVPV was significantly higher in females than in males, regardless of E2 treatment ($P < 0.05$; Fig. 2H). Detailed analyses of the distribution of AVPV tdTomato cells revealed that the number of tdTomato fluorescence-positive cells in the medial part of the AVPV (0–100 μ m lateral to the third ventricle [3V]) was significantly higher in females than in males, regardless of E2 treatment (Fig. 2J), whereas no sex difference was found in the number of tdTomato fluorescence-positive cells in the lateral part of the AVPV (100–300 μ m lateral to the 3V). Notably, colocalization of *Kiss1* and tdTomato-IR signals was primarily found in the medial part of the AVPV of E2-treated OVX females (0–100 μ m lateral to the 3V) (Supplementary Fig. 2).

Many tdTomato fluorescence-positive cells were found in the ARC of OVX and Cast rats, regardless of E2 treatment (Fig. 2, K and L), whereas *Kiss1*-expressing cells were found only in the ARC of OVX and Cast rats without E2 treatment (Fig. 2M). E2

treatment did not significantly affect the number of ARC tdTomato fluorescence-positive cells in OVX females or Cast males. The number of tdTomato fluorescence-positive cells in the ARC was significantly higher in female rats than in male rats, regardless of E2 treatment ($P < 0.05$, Fig. 2L). Many ARC *Kiss1*-expressing cells were found in OVX and Cast rats, and E2 treatment significantly reduced these numbers ($P < 0.05$; Fig. 2M).

Developmental increase and sex difference in Kiss1-Cre-activated tdTomato fluorescence signals in the AVPV and ARC with earlier AVPV tdTomato expression in males and higher AVPV and ARC tdTomato expression in females during the postpubertal period

To examine sex differences in the distribution and number of potential kisspeptin neurons during the developmental periods, we compared the number of tdTomato fluorescence-positive cells in the AVPV and ARC between females and males on postnatal Days 1 and 7, and 3 (prepubertal), 7 (postpubertal), and 10–13 weeks (adulthood) of age in gonad-intact rats. Few AVPV tdTomato-positive cells were found on postnatal Days 1 and 7 in female rats, and many AVPV tdTomato fluorescence-positive cells were found at 3, 7, and 10–13 weeks of age when the females were in the diestrus phase (Fig. 3A). Notably, AVPV tdTomato fluorescence-positive cells were found on postnatal Days 1 and 7 and thereafter (3, 7, and 10–13 weeks of age) in male rats. The number of AVPV tdTomato fluorescence-positive cells significantly increased in both sexes during the developmental period and was significantly higher in females than in males at 7 and 10–13 weeks of age ($P < 0.05$; Fig. 3B). In the ARC, tdTomato fluorescence-positive cells were found in gonad-intact female and male rats on postnatal Day 1 and thereafter (Day 7 and 3, 7, and 10–13 weeks of age) (Fig. 3C). The number of ARC tdTomato fluorescence-positive cells increased significantly in both sexes with increasing age and was significantly higher in females than in males at 7 and 10–13 weeks of age ($P < 0.05$; Fig. 3D).

Developmental increase and sex difference in Kiss1-Cre-activated tdTomato fluorescence signals in the MeA of rats with higher MeA tdTomato expression in males than in females in the postpubertal period

Because we found a large number of tdTomato fluorescence-positive cells in the MeA region of *Kiss1*-visualized rats, we examined *Kiss1* expression and tdTomato fluorescence signals in the MeA of gonadectomized adult male and female rats in the presence or absence of E2. No *Kiss1*-expressing cells were detected in the MeA of OVX females and Cast males regardless of E2 treatment (Figs. 4A and 4B); however, some *Kiss1-Cre*-activated tdTomato fluorescence-positive cells were detected in the MeA of both sexes regardless of E2 treatment in adulthood (Fig. 4C). E2 treatment did not significantly affect the number of MeA tdTomato fluorescence-positive cells in OVX females or Cast males (Fig. 4D). Notably, the number of MeA tdTomato fluorescence-positive cells was significantly higher in males than in females ($P < 0.05$; Fig. 4D).

We further examined the developmental changes and sex differences in the number of tdTomato fluorescence-positive cells in the MeA of gonad-intact male and female rats. No tdTomato fluorescence-positive cells were found in the MeA of either sex on postnatal Days 1 or 7, whereas tdTomato-positive cells were found in the MeA of both sexes at 3 weeks of age and thereafter (7 and 10–13 weeks of age) (Fig. 4E). The number of MeA tdTomato fluorescence-positive cells significantly increased in both sexes with increasing age and was significantly higher in males than in females at 7 and 10–13 weeks

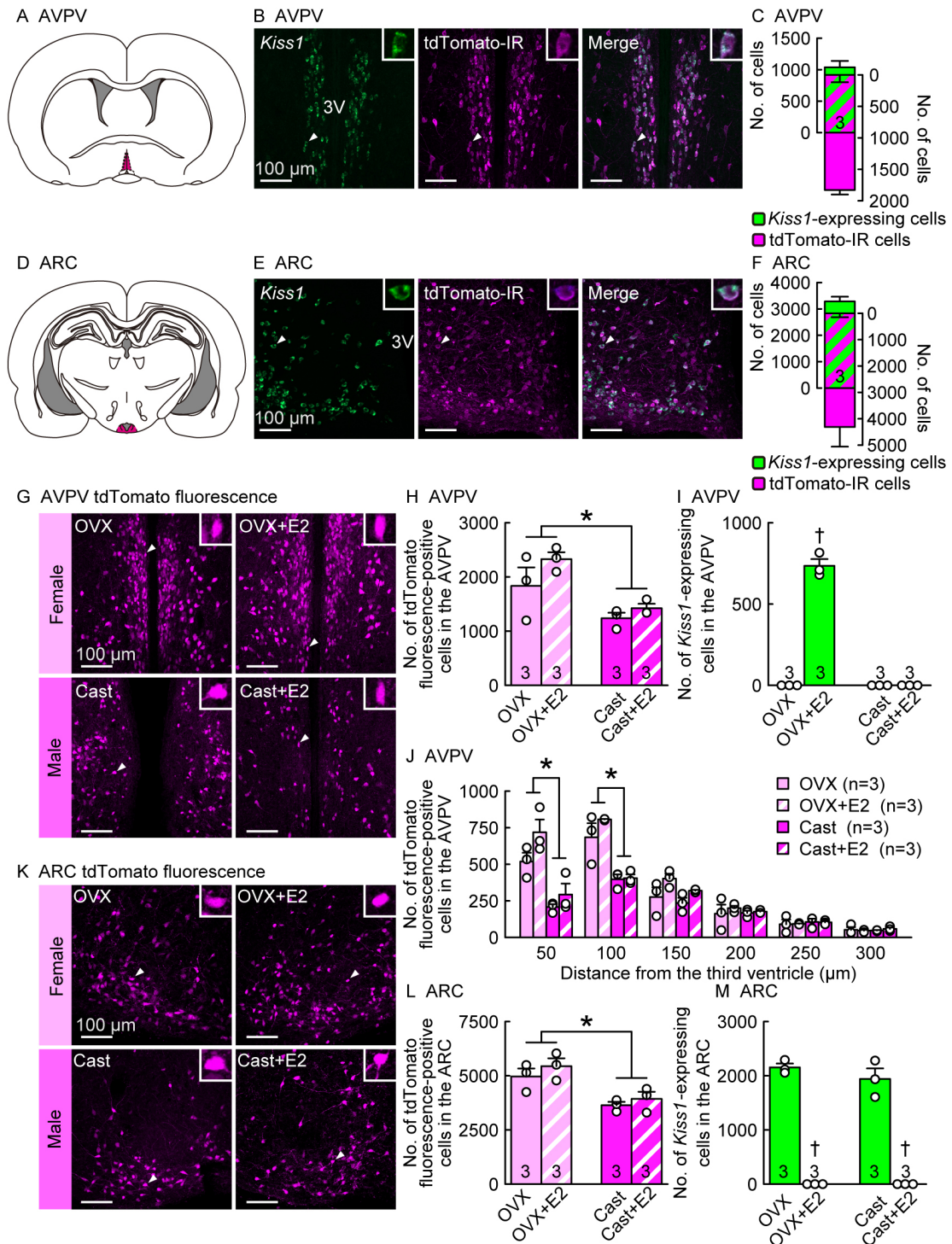


Fig. 2. Colocalization of *Kiss1* and *Kiss1*-Cre-activated tdTomato-immunoreactive (IR) signals and lack of estrogen effect on tdTomato fluorescence signals in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) of rats. (A) An illustrated brain section showing the AVPV highlighted in magenta. (B) *Kiss1* mRNA-expressing (green) and tdTomato-IR (magenta) cells in the AVPV of a representative ovariectomized (OVX) rat treated with a positive feedback level of estradiol-17 β (E2) in adulthood (8–16 weeks of age). Insets are magnified images showing a representative *Kiss1* and tdTomato-IR double-positive cell (indicated by arrowheads in main images). (C) The number of *Kiss1*-expressing (green), tdTomato-IR (magenta), or double-positive (striped) cells in the AVPV of OVX rats in adulthood. (D) An illustration of the brain section showing the ARC highlighted in magenta. (E) *Kiss1*-expressing (green) and tdTomato-IR (magenta) cells in the ARC of a representative OVX rat in adulthood. (F) The number of *Kiss1*-expressing (green), tdTomato-IR (magenta), or double-positive (striped) cells in the ARC of OVX rats in adulthood. (G) *Kiss1*-Cre-activated tdTomato fluorescence in the AVPV of representative OVX and castrated (Cast) rats with or without E2 in adulthood. (H and I) The numbers of (H) tdTomato fluorescence-positive cells and (I) *Kiss1*-expressing cells in the AVPV of OVX and Cast rats with or without E2 in adulthood. (J) The number of tdTomato fluorescence-positive cells in every 50- μ m layer (from the medial to lateral) of the AVPV of OVX and Cast rats with or without E2. (K) *Kiss1*-Cre-activated tdTomato fluorescence in the ARC of representative OVX and Cast rats with or without E2 in adulthood. (L and M) The number of (L) tdTomato fluorescence-positive cells and (M) *Kiss1*-expressing cells in the ARC of OVX and Cast rats with or without E2 in adulthood. Sample size, $n = 3$. Scale bars, 100 μ m. 3V, third ventricle. Values are the means \pm SEMs. * Significant difference between sexes ($P < 0.05$, two- or three-way ANOVA followed by Tukey–Kramer’s multiple comparisons test). † Significant difference by E2 treatment ($P < 0.05$, two-way ANOVA followed by Tukey–Kramer’s multiple comparisons test). See also Supplementary Fig. 2.

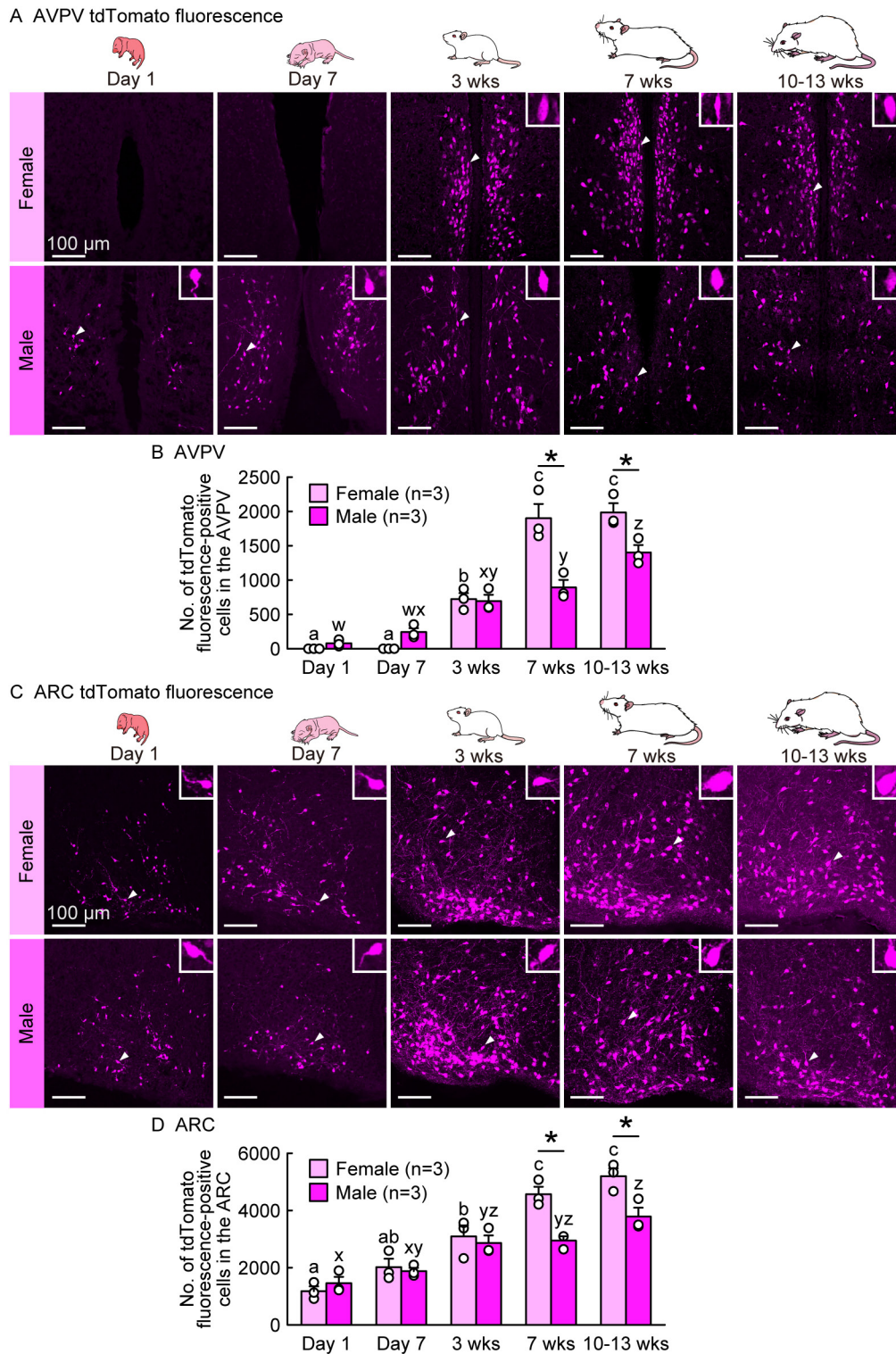


Fig. 3. Developmental increases and sex differences in *Kiss1-Cre*-activated tdTomato fluorescence signals in the AVPV and ARC with earlier AVPV tdTomato expression in males and higher AVPV and ARC tdTomato expression in females during the postpubertal period. (A) *Kiss1-Cre*-activated tdTomato fluorescence in the AVPV of representative gonad-intact female and male rats at postnatal Days 1 and 7, and 3 (prepubertal), 7 (postpubertal), and 10–13 weeks (adulthood) of age (in the diestrus phase for 7- and 10–13-week-old females). Insets are magnified images showing a representative *Kiss1-Cre*-activated tdTomato fluorescence-positive cell (indicated by arrowheads in main images). (B) Developmental changes in the number of tdTomato fluorescence-positive cells in the AVPV of gonad-intact female and male rats. (C) *Kiss1-Cre*-activated tdTomato fluorescence in the ARC of representative gonad-intact female and male rats. (D) Developmental changes in the number of tdTomato fluorescence-positive cells in the ARC of female and male rats. Sample size, $n = 3$. Scale bars, 100 μm . Values are the means \pm SEMs. Values with different letters (a–c for females and w–z for males) are significantly different between ages ($P < 0.05$, two-way ANOVA followed by Tukey–Kramer’s multiple comparisons test). * Significant difference between sexes ($P < 0.05$, two-way ANOVA followed by Tukey–Kramer’s multiple comparisons test).

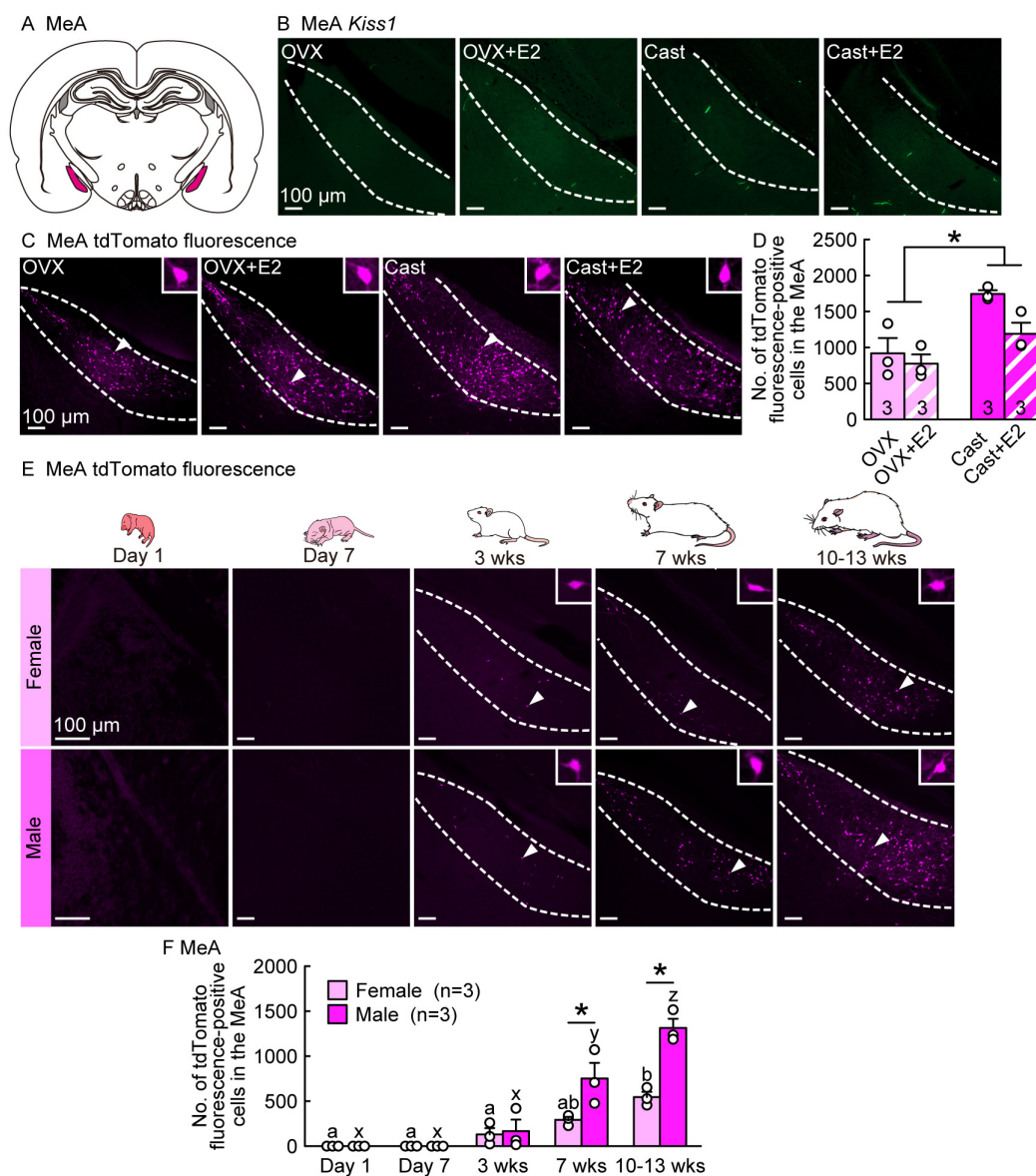


Fig. 4. Developmental increases and sex differences in *Kiss1-Cre*-activated tdTomato fluorescence signals in the medial amygdala (MeA) of rats with higher MeA tdTomato expression in males than in females in the postpubertal period. (A) An illustrated brain section showing the MeA highlighted in magenta. (B) Few *Kiss1*-expressing cells were detected in the MeA of OVX and Cast rats with or without E2 in adulthood (8–16 weeks of age). (C) *Kiss1-Cre*-activated tdTomato fluorescence in the MeA of representative OVX and Cast rats with or without E2 in adulthood. Insets are magnified images showing a tdTomato fluorescence-positive cell (indicated by arrowheads in main images). (D) The numbers of tdTomato fluorescence-positive cells in the MeA of OVX and Cast rats with or without E2 in adulthood. (E) *Kiss1-Cre*-activated tdTomato fluorescence-positive cells in the MeA of representative gonad-intact female and male rats at postnatal Days 1 and 7, and 3 (prepubertal), 7 (postpubertal), and 10–13 weeks (adulthood) of age (in the diestrus phase for 7- and 10–13-week-old females). (F) Developmental changes in the number of tdTomato-positive cells in the MeA of gonad-intact female and male rats. Sample size, $n = 3$. Scale bars, 100 μm . Values are the means \pm SEMs. * Significant difference between sexes ($P < 0.05$, two-way ANOVA followed by Tukey–Kramer’s multiple comparisons test). Values with different letters (a–b for females and x–z for males) are significantly different ($P < 0.05$, two-way ANOVA followed by Tukey–Kramer’s multiple comparisons test).

of age ($P < 0.05$; Fig. 4F).

Developmental increase but no sex difference in *Kiss1-Cre*-activated tdTomato fluorescence signals in the VMH of female and male rats

We also examined *Kiss1* expression and tdTomato fluorescence signals in the VMH of male and female rats, because we found a large number of tdTomato fluorescence-positive cells in the VMH of *Kiss1*-visualized rats. Few *Kiss1*-expressing cells were detected in the VMH of adult OVX females and Cast males, regardless of E2

treatment (Figs. 5A and 5B), whereas a large number of tdTomato fluorescence-positive cells were found in the VMH of adult rats of both sexes (Fig. 5C). No sex differences or estrogen effects were observed in the number of tdTomato fluorescence-positive cells in the VMH (Fig. 5D).

We also examined developmental changes and sex differences in the number of VMH tdTomato fluorescence-positive cells in gonad-intact male and female rats. Few VMH tdTomato fluorescence-positive cells were found in both sexes at postnatal Days 1 and 7, whereas many VMH tdTomato fluorescence-positive cells were found in

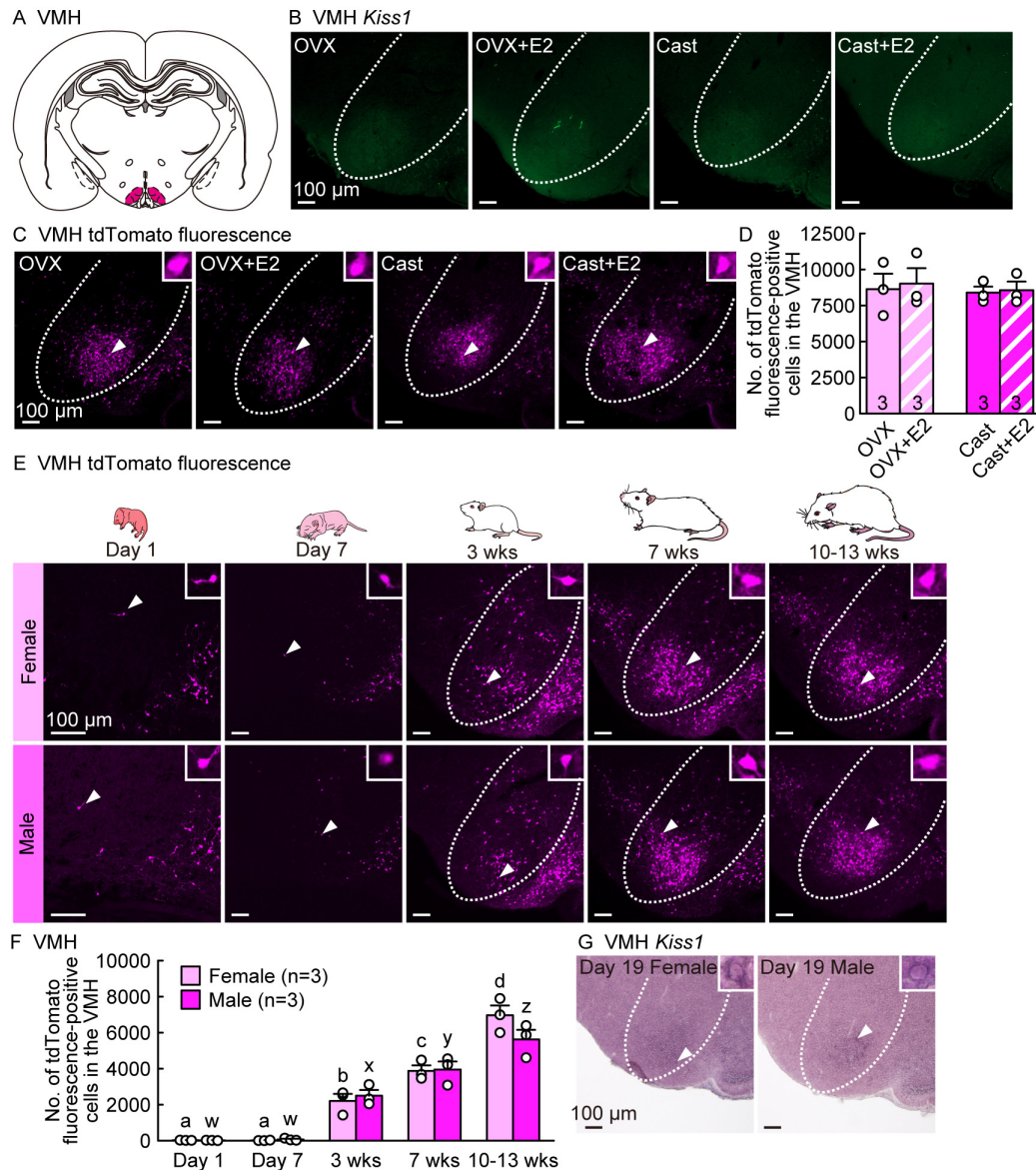


Fig. 5. Developmental increases but no sex differences in *Kiss1-Cre*-activated tdTomato fluorescence signals in the ventromedial nucleus of the hypothalamus (VMH) of female and male rats. (A) An illustrated brain section showing the VMH highlighted in magenta. (B) No *Kiss1*-expressing cells were detected in the VMH of OVX and Cast rats with or without E2 in adulthood (8–16 weeks of age). (C) *Kiss1-Cre*-activated tdTomato fluorescence-positive cells in the VMH of representative OVX and Cast rats with or without E2 in adulthood. Insets are magnified images showing tdTomato-positive cells (indicated by arrowheads in the main images). (D) The number of tdTomato-positive cells in the VMH of OVX and Cast rats with or without E2 in adulthood. (E) *Kiss1-Cre*-activated tdTomato fluorescence-positive cells in the VMH of representative gonad-intact female and male rats at postnatal Days 1 and 7, and 3 (prepubertal), 7 (postpubertal), and 10–13 weeks (adulthood) of age (in the diestrus phase for 7- and 10–13-week-old females). (F) Developmental changes in the number of tdTomato-positive cells in the VMH of gonad-intact female and male rats. (G) *Kiss1*-expressing cells in the VMH of representative female and male rats on postnatal Day 19. Insets are magnified images showing *Kiss1*-expressing cells. Sample size, $n = 3$. Scale bars, 100 μm . Values are the means \pm SEMs. Values with different letters (a–d for females and w–z for males) are significantly different ($P < 0.05$, two-way ANOVA followed by Tukey–Kramer’s multiple comparisons test).

both sexes at 3 weeks and thereafter (7 and 10–13 weeks of age) (Fig. 5E). The number of VMH tdTomato fluorescence-positive cells increased significantly in both sexes during the developmental period ($P < 0.05$; Fig. 5F), and no sex differences were observed in the number of VMH tdTomato fluorescence-positive cells throughout the developmental stages (Fig. 5F). Notably, we found weak *Kiss1* mRNA signals at 19 days of age in female and male gonad-intact rats (Fig. 5G), suggesting that transient *Kiss1* expression in the VMH during the prepubertal phase (19 days of age) triggers VMH tdTomato expression in both sexes.

Exploration of *Kiss1-Cre*-activated tdTomato-IR signals in other forebrain regions of the female rats

We also examined *Kiss1-Cre*-activated tdTomato and *Kiss1* mRNA signals in other brain regions of female rats using double-stained forebrain sections for *Kiss1* mRNA and tdTomato-IR. *Kiss1-Cre*-activated tdTomato-IR cells were found in the cortex (Fig. 6A), LS dorsal part (LSD, Fig. 6B), LS ventral part (LSV, Fig. 6C), BNST medial division anterior part (STMA, Fig. 6D), MPOA (Fig. 6E), Rt (Fig. 6F), lateroanterior hypothalamic nucleus (LA, Fig. 6G), RCh (Fig. 6H), MTu (Fig. 6I), DMD (Fig. 6J), and PH (Fig. 6K) in

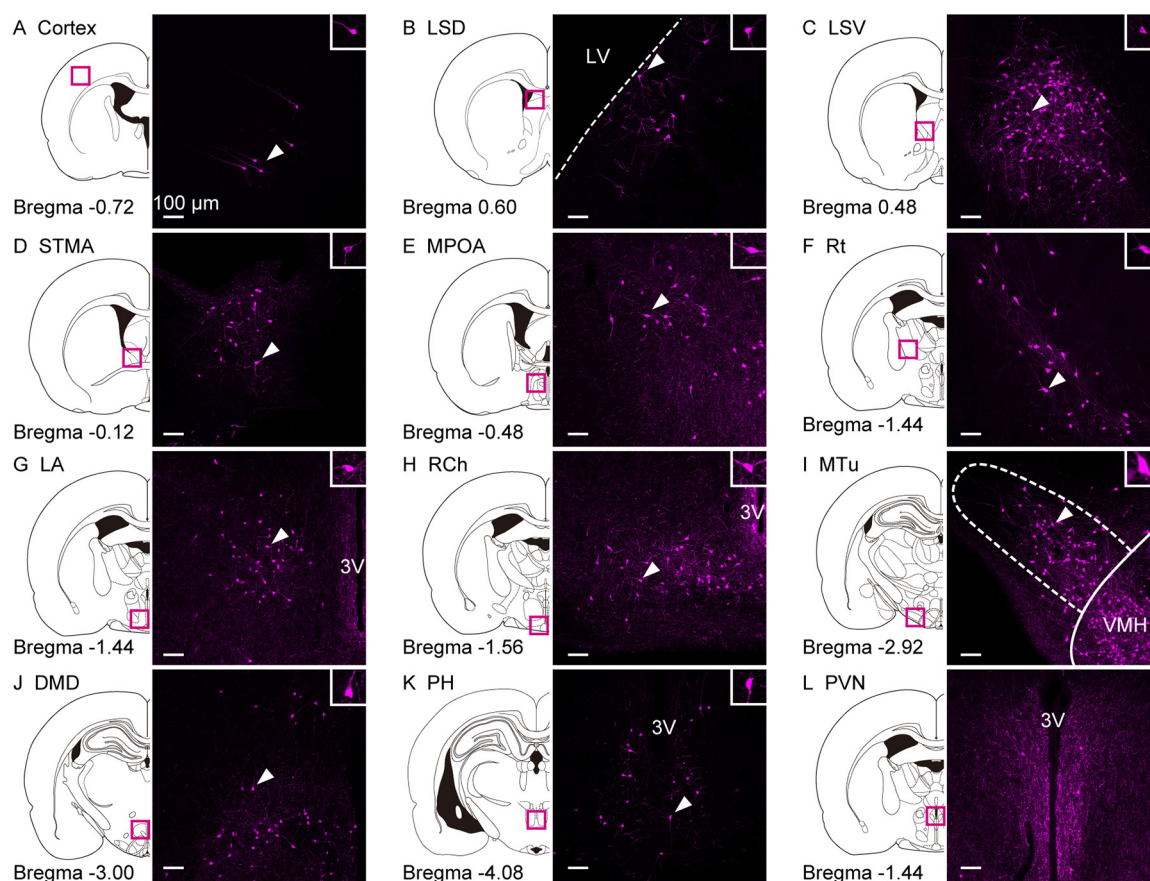


Fig. 6. Exploration of *Kiss1-Cre*-activated tdTomato-IR signals in other regions of the female rat forebrain. TdTomato-IR cells in the (A) cortex, (B) LSD, (C) LSV, (D) STMA, (E) MPOA, (F) Rt, (G) LA, (H) RCh, (I) MTu, (J) DMD, and (K) PH in representative OVX+E2 female rats in adulthood (8–16 weeks of age). Note that no *Kiss1* mRNA signals were detected in these nuclei by *in situ* hybridization. (L) The tdTomato-IR fibers in the PVN in a representative OVX+E2 female rat in adulthood. Insets are magnified images showing tdTomato-IR positive cells (indicated by arrowheads in the main images). LSD, lateral septal nucleus dorsal part; LSV, lateral septal nucleus ventral part; STMA, bed nucleus of the stria terminalis medial division anterior part; MPOA, medial preoptic area; Rt, reticular thalamic nucleus; LA, lateroanterior hypothalamic nucleus; RCh, retrochiasmatic area; MTu, medial tuberal nucleus; DMD, dorsal part of dorsomedial hypothalamic nucleus; PH, posterior hypothalamus; PVN, paraventricular hypothalamic nucleus. Scale bars, 100 μ m.

adult OVX + E2 female rats. However, the *Kiss1* mRNA was not detected in these regions. Dense tdTomato-IR fibers were observed in the medial part of the PVN in OVX + E2 female rats (Fig. 6L).

Discussion

The present study demonstrated the successful generation of *Kiss1-Cre* rats exhibiting genetically induced hypogonadotropic hypogonadism by crossing them with *Kiss1*-floxed rats. Specifically, adult *Kiss1-KpKO* male rats presented atrophic testes and lacked ARC *Kiss1* expression, with severe suppression of LH release even after castration and profound suppression of pituitary *Lhb*, *Fshb*, and *Gnrhr* mRNA expression. These findings suggest that Cre recombinase in the *Kiss1-Cre* rats successfully knocked out the *Kiss1* gene in *Kiss1*-floxed rats, resulting in a hypogonadotropic hypogonadism phenotype similar to that observed in global *Kiss1* KO rats [63]. Using *Kiss1-Cre* rats, we generated genetically modified rats with *Kiss1-Cre*-triggered cells visualized by tdTomato fluorescence in various brain regions and found that most of the *Kiss1*-expressing cells co-expressed tdTomato immunoreactivity in the AVPV of E2-treated OVX rats and the ARC of non-E2-treated OVX rats in adulthood. Notably, E2 treatment did not affect tdTomato fluorescence signals during adulthood. The

numbers of tdTomato fluorescence-positive cells in the AVPV and ARC were comparable between OVX and E2-treated OVX rats. In contrast, the number of AVPV *Kiss1*-expressing cells was decreased by ovariectomy, whereas E2 treatment decreased the number of ARC *Kiss1*-expressing cells in OVX *Kiss1-Cre*-activated tdTomato reporter rats. These results suggest that AVPV and ARC kisspeptin neurons can be identified by tdTomato fluorescence, regardless of E2-induced changes in *Kiss1* expression. The potential kisspeptin neurons visualized by tdTomato fluorescence provide a useful tool for investigating the mechanism mediating the downregulation of *Kiss1*/kisspeptin expression because *Kiss1*-visualized rats allowed us to identify potential kisspeptin neurons by tdTomato fluorescence regardless of *Kiss1* and kisspeptin expression. To the best of our knowledge, this is the first study to report the generation of *Kiss1-Cre* rats, which are useful for investigating the physiological and molecular mechanisms regulating kisspeptin neurons as key players in mammalian reproduction.

The current developmental analysis of *Kiss1-Cre*-activated tdTomato fluorescence-positive cells revealed that AVPV tdTomato expression during the early postnatal period, such as 1 or 7 days after birth, was found only in male rats, and that these tdTomato fluorescence-positive cells remained until adulthood (10–13 weeks of

age). This result suggests that *Kiss1* mRNA is transiently expressed in the AVPV of male rats and that kisspeptin neurons in the AVPV survive until the adult stage, when *Kiss1* expression is strongly suppressed by the organizing effect of perinatal testosterone in male rats. Perinatal testosterone likely transiently induces AVPV *Kiss1* expression and irreversibly inhibits AVPV *Kiss1* expression in male rats. In contrast, the earliest AVPV tdTomato fluorescence was observed at 3 weeks of age in females, and the number of tdTomato fluorescence-positive cells gradually increased by adulthood (10–13 weeks of age). The pubertal increase in the number of tdTomato fluorescence-positive cells was largely consistent with the increase in AVPV *Kiss1*-expressing cells in female rats, as reported in our previous study, which showed an increase in the number of AVPV *Kiss1*-expressing cells at puberty onset and thereafter in female rats [52]. Notably, compared to male rats, female rats showed a significantly greater number of tdTomato fluorescence-positive cells in the medial part of the AVPV region along the edge of 3V. This finding suggests that *Kiss1* neurons in the medial part of the AVPV are responsible for inducing a GnRH/LH surge in response to preovulatory estrogen in female rats. This hypothesis is consistent with our previous study showing that a preovulatory level of E2 induces *Kiss1* expression in the medial part of the AVPV, which is associated with an E2-induced LH surge in OVX rats and neonatally castrated male rats [42].

Kiss1-Cre-activated tdTomato fluorescence-positive cells in the ARC were found in the perinatal period (1 day of age) and thereafter in female and male rats, suggesting that ARC *Kiss1* expression is initiated in the perinatal period in both sexes. This result is consistent with a previous study showing that kisspeptin immunoreactivity is evident at E14.5 in female and male rats [49]. The number of ARC tdTomato fluorescence-positive cells continuously increased to the adult stage (10–13 weeks of age) in female rats, but was stable after puberty (7, 10–13 weeks of age) in male rats, which suggests that greater numbers of potential ARC kisspeptin neurons exist in female rats than in males in adulthood (10–13 weeks of age). This result is consistent with a previous study, which found a larger number of ARC kisspeptin neurons in gonad-intact female rats than in gonad-intact male rats during adulthood [64]. Notably, *Kiss1*/kisspeptin expression is largely suppressed by gonad-derived steroids during the prepubertal period in female rats [51]. Therefore, the current visualized potential ARC kisspeptin neurons would be a useful tool to investigate the inhibitory mechanism of the steroid-dependent prepubertal restraint of ARC *Kiss1*/kisspeptin expression in rodents.

Notably, in the present study, *Kiss1* mRNA signals were not detected in a large population of tdTomato fluorescence-positive cells, even in the AVPV of OVX females treated with a positive feedback level of E2, and in the ARC of OVX female rats without E2 treatment. This suggests that many potential kisspeptin neurons lacking *Kiss1* expression still exist in the nuclei of adult female rats. Alternatively, tdTomato expression driven by a strong CAG promoter allowed us to easily detect tdTomato signals, even if *Kiss1* mRNA expression was undetectable in some kisspeptin neurons. In this context, the coexistence of *Kiss1* and tdTomato signals may be detected through an increase in the sensitivity of *in situ* hybridization for *Kiss1*. Notably, a small population of *Kiss1*-expressing cells failed to show tdTomato immunoreactivity in the AVPV and ARC in female rats. Theoretically, cells that have expressed *Kiss1/Cre* at least once should exhibit persistent tdTomato expression after *Kiss1* expression in *Kiss1-Cre*-activated tdTomato reporter rats. We speculated that the tdTomato-IR signals might be below the threshold for tdTomato immunohistochemistry in some *Kiss1*-expressing

cells. Improvement of the methodology to detect *Kiss1* mRNA and tdTomato immunoreactivity may solve the inconsistency between *Kiss1*-expressing and tdTomato-IR cells in the future.

The current study found a large number of *Kiss1-Cre*-activated tdTomato fluorescence-positive cells in the MeA of adult male and female rats, while *Kiss1* mRNA signals were rarely detected in the nucleus. This suggests that potential MeA *Kiss1* neurons can be easily detected using the tdTomato signal as an indicator. The earliest tdTomato fluorescence signals in the MeA were observed at 3 weeks of age, and the number of MeA tdTomato-positive cells increased thereafter in the adult stage (10–13 weeks of age). The number of MeA tdTomato fluorescence-positive cells showed sex differences, with a larger number of tdTomato-positive cells in males than in females. This result is largely consistent with previous studies, which found that the number of MeA *Kiss1* neurons was higher in male rats than in female rats [53] and that the number of MeA *Kiss1*-expressing cells increased throughout development in male mice [65]. MeA kisspeptin neurons receive neuronal projections from the accessory olfactory bulb (AOB), where pheromonal information from the vomeronasal organ is relayed [54]. A previous study showed that selective activation of the posterior dorsal subnucleus of the MeA (MePD) kisspeptin neurons by the DREADD system enhanced sexual partner preference and social interaction and decreased anxiety in male mice [55]. Taken together, the potential kisspeptin neurons visualized by tdTomato fluorescence provide a useful tool for investigating the role of MeA kisspeptin neurons as modulators of behavioral responses to pheromones in male rodents.

The present study found a large number of *Kiss1-Cre*-activated tdTomato fluorescence-positive cells in the VMH of female and male rats, suggesting that potential kisspeptin neurons are abundantly located in the VMH. Previous studies showed *Kiss1* promoter-dependent tdTomato fluorescence in the VMH of adult female rats [8], and *Kiss1* expression in the VMH of adult OVX female rats treated with high-dose E2 [56]. The current study detected weak *Kiss1* expression on postnatal Day 19 in both sexes of wild-type rats as reported in a previous study [44]. These findings suggest that an increase in sex steroids released from the gonads during the prepubertal period (around postnatal Day 19) triggers *Kiss1-Cre*-activated tdTomato fluorescence in the VMH because plasma E2 levels increase in female and male rats at 2 weeks of age [66–68]. Interestingly, there was no obvious *Kiss1-Cre*-dependent reporter gene expression in the VMH of *Kiss1-Cre* mice [40], suggesting species-specific differences in VMH *Kiss1* expression. Taken together, *Kiss1* was temporally expressed in the VMH during the prepubertal period and *Kiss1* expression was turned off in most potential VMH kisspeptin neurons in rats. Further studies are required to clarify the physiological role of kisspeptin neurons in the VMH of both sexes.

The current study demonstrates that a large number of potential kisspeptin cells visualized by *Kiss1-Cre*-activated tdTomato exist in several other regions of the rat forebrain. Specifically, tdTomato-IR cell bodies were detected in the cortex, LS, BNST, MPOA, RCh, Mtu, DMD, and PH, where *Kiss1* and/or *Kiss1-Cre* reporter signals were previously reported in mice and rats [56, 57, 61]. TdTomato-IR fibers were detected in the medial part of the PVN and kisspeptin-IR fibers were reported in the mouse brain [41, 59, 60]. Therefore, the current *Kiss1-Cre*-activated tdTomato reporter rats may be useful models for investigating kisspeptin neuronal projections that cannot be examined using *Kiss1 in situ* hybridization. The current study also showed tdTomato-IR cell bodies in the Rt and LA, where *Kiss1*- and/or kisspeptin-positive cells have not been previously reported. Further studies are needed to clarify the physiological roles of potential

kisspeptin neurons originating from the cortex, LS, BNST, MPOA, RCh, Mtu, DMD, and PH, and neurons projecting to the PVN.

In conclusion, the present study demonstrated that the newly generated *Kiss1-Cre* rats and *Kiss1-Cre*-activated tdTomato reporter rats could be useful tools for further detailed analyses of potential *Kiss1*/kisspeptin neurons across the brain. Specifically, AVPV and ARC kisspeptin neurons identified by tdTomato fluorescence, regardless of E2 levels, could be used to investigate the mechanisms mediating the positive and negative effects of E2 on *Kiss1*/kisspeptin expression in the AVPV and ARC, respectively. Furthermore, *Kiss1-Cre* rats have an advantage over *Kiss1-Cre* mice because of their larger body size, which enables frequent blood sampling to detect LH pulses and E2-induced LH surges, and the monitoring of activities of ARC or AVPV kisspeptin neurons using *Kiss1-Cre*-dependent expression of calcium indicators, such as GCaMP. Moreover, the current *Kiss1-Cre* rats may also be used to investigate the physiological role of kisspeptin neurons located in brain regions other than the AVPV and ARC, because *Kiss1-Cre*-activated tdTomato fluorescence-positive cells are more easily detected than *Kiss1* mRNA signals. In addition, *Kiss1-Cre*-dependent infection of tracer genes, such as sensor probe genes, and/or the deletion of certain genes using the Cre/loxP system in potential *Kiss1* neurons in *Kiss1-Cre* rats would provide powerful tools to further improve our understanding of the role of kisspeptin neurons as master regulators of mammalian reproduction.

Conflicts of interests: The authors declare no conflict of interest.

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References

- Uenoyama Y, Nagae M, Tsuchida H, Inoue N, Tsukamura H. Role of KNDy Neurons Expressing Kisspeptin, Neurokinin B, and Dynorphin A as a GnRH Pulse Generator Controlling Mammalian Reproduction. *Front Endocrinol (Lausanne)* 2021; 12: 724632. [Medline] [CrossRef]
- Uenoyama Y, Inoue N, Nakamura S, Tsukamura H. Kisspeptin neurons and estrogen-estrogen receptor α signaling: Unraveling the mystery of steroid feedback system regulating mammalian reproduction. *Int J Mol Sci* 2021; 22: 9229. [Medline] [CrossRef]
- Tsukamura H. Kobayashi Award 2019: The neuroendocrine regulation of the mammalian reproduction. *Gen Comp Endocrinol* 2022; 315: 113755. [Medline] [CrossRef]
- Goodman RL, Herbison AE, Lehman MN, Navarro VM. Neuroendocrine control of gonadotropin-releasing hormone: Pulsatile and surge modes of secretion. *J Neuroendocrinol* 2022; 34: e13094. [Medline]
- Seminara SB, Messenger S, Chatzidaki EE, Thresher RR, Acierno JS Jr, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinf KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O’Rahilly S, Carlton MB, Crowley WF Jr, Aparicio SA, Colledge WH. The GPR54 gene as a regulator of puberty. *N Engl J Med* 2003; 349: 1614–1627. [Medline] [CrossRef]
- de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci USA* 2003; 100: 10972–10976. [Medline] [CrossRef]
- Topaloglu AK, Tello JA, Kotan LD, Ozbek MN, Yilmaz MB, Erdogan S, Gurbuz F, Temiz F, Millar RP, Yuksel B. Inactivating KiSS1 mutation and hypogonadotropic hypogonadism. *N Engl J Med* 2012; 366: 629–635. [Medline] [CrossRef]
- Uenoyama Y, Nakamura S, Hayakawa Y, Ikegami K, Watanabe Y, Deura C, Minabe S, Tomikawa J, Goto T, Ieda N, Inoue N, Sanbo M, Tamura C, Hirabayashi M, Maeda K-I, Tsukamura H. Lack of pulse and surge modes and glutamatergic stimulation of luteinising hormone release in Kiss1 knockout rats. *J Neuroendocrinol* 2015; 27: 187–197. [Medline] [CrossRef]
- Lapatto R, Pallais JC, Zhang D, Chan YM, Mahan A, Cerrato F, Le WW, Hoffman GE, Seminara SB. Kiss1^{-/-} mice exhibit more variable hypogonadism than Gpr54^{-/-} mice. *Endocrinology* 2007; 148: 4927–4936. [Medline] [CrossRef]
- d’Anglemont de Tassigny X, Fagg LA, Dixon JPC, Day K, Leitch HG, Hendrick AG, Zahn D, Franceschini I, Caraty A, Carlton MBL, Aparicio SAJR, Colledge WH. Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. *Proc Natl Acad Sci USA* 2007; 104: 10714–10719. [Medline] [CrossRef]
- Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* 2004; 145: 4073–4077. [Medline] [CrossRef]
- Navarro VM, Castellano JM, Fernández-Fernández R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. *Endocrinology* 2004; 145: 4565–4574. [Medline] [CrossRef]
- Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K. Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. *Endocrinology* 2005; 146: 4431–4436. [Medline] [CrossRef]
- Messenger S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MBL, Colledge WH, Caraty A, Aparicio SAJR. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci USA* 2005; 102: 1761–1766. [Medline] [CrossRef]
- Ohkura S, Takase K, Matsuyama S, Mogi K, Ichimaru T, Wakabayashi Y, Uenoyama Y, Mori Y, Steiner RA, Tsukamura H, Maeda KI, Okamura H. Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. *J Neuroendocrinol* 2009; 21: 813–821. [Medline] [CrossRef]
- Naniwa Y, Nakatsukasa K, Setsuda S, Oishi S, Fujii N, Matsuda F, Uenoyama Y, Tsukamura H, Maeda K, Ohkura S. Effects of full-length kisspeptin administration on follicular development in Japanese Black beef cows. *J Reprod Dev* 2013; 59: 588–594. [Medline] [CrossRef]
- Shahab M, Mastrorandi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. *Proc Natl Acad Sci USA* 2005; 102: 2129–2134. [Medline] [CrossRef]
- Dhillon WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatel MA, Bloom SR. Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. *J Clin Endocrinol Metab* 2005; 90: 6609–6615. [Medline] [CrossRef]
- Robert L. Barbieri. Female infertility. In: Jerome F. Strauss, Barbieri RL (eds.), Yen and Jaffe’s Reproductive Endocrinology, Elsevier; 2019: 556–581.
- Barbieri RL. Clinical applications of GnRH and its analogues. *Trends Endocrinol Metab* 1992; 3: 30–34. [Medline] [CrossRef]
- Smith S, Pfeifer SM, Collins JA. Diagnosis and management of female infertility. *JAMA* 2003; 290: 1767–1770. [Medline] [CrossRef]
- Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology* 2005; 146: 2976–2984. [Medline] [CrossRef]
- Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA. Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. *J Neurosci* 2006; 26: 6687–6694. [Medline] [CrossRef]
- Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K. Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. *J Reprod Dev* 2007; 53: 367–378. [Medline] [CrossRef]
- Clarkson J, d’Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci* 2008; 28: 8691–8697. [Medline] [CrossRef]
- Nagae M, Uenoyama Y, Okamoto S, Tsuchida H, Ikegami K, Goto T, Majorune S, Nakamura S, Sanbo M, Hirabayashi M, Kobayashi K, Inoue N, Tsukamura H. Direct

- evidence that KNDy neurons maintain gonadotropin pulses and folliculogenesis as the GnRH pulse generator. *Proc Natl Acad Sci USA* 2021; **118**: 1–11. [Medline] [CrossRef]
27. Clarkson J, Han SY, Piet R, McLennan T, Kane GM, Ng J, Porteous RW, Kim JS, Colledge WH, Iremonger KJ, Herbison AE. Definition of the hypothalamic GnRH pulse generator in mice. *Proc Natl Acad Sci USA* 2017; **114**: E10216–E10223. [Medline] [CrossRef]
 28. Ikegami K, Goto T, Nakamura S, Watanabe Y, Sugimoto A, Majarune S, Horihata K, Nagae M, Tomikawa J, Imamura T, Sanbo M, Hirabayashi M, Inoue N, Maeda K-I, Tsukamura H, Uenoyama Y. Conditional kisspeptin neuron-specific Kiss1 knockout with newly generated Kiss1-floxed and Kiss1-Cre mice replicates a hypogonadal phenotype of global Kiss1 knockout mice. *J Reprod Dev* 2020; **66**: 359–367. [Medline] [CrossRef]
 29. Minabe S, Nakamura S, Fukushima E, Sato M, Ikegami K, Goto T, Sanbo M, Hirabayashi M, Tomikawa J, Imamura T, Inoue N, Uenoyama Y, Tsukamura H, Maeda KI, Matsuda F. Inducible Kiss1 knockdown in the hypothalamic arcuate nucleus suppressed pulsatile secretion of luteinizing hormone in male mice. *J Reprod Dev* 2020; **66**: 369–375. [Medline] [CrossRef]
 30. Moore AM, Coolen LM, Lehman MN. In vivo imaging of the GnRH pulse generator reveals a temporal order of neuronal activation and synchronization during each pulse. *Proceedings of the National Academy of Sciences* 2022; **119** [CrossRef].
 31. Han SY, Kane G, Cheong I, Herbison AE. Characterization of GnRH pulse generator activity in male mice using GCaMP fiber photometry. *Endocrinology* 2019; **160**: 557–567. [Medline] [CrossRef]
 32. McQuillan HJ, Han SY, Cheong I, Herbison AE. GnRH pulse generator activity across the estrous cycle of female mice. *Endocrinology* 2019; **160**: 1480–1491. [Medline] [CrossRef]
 33. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology* 2005; **146**: 3686–3692. [Medline] [CrossRef]
 34. Dubois SL, Acosta-Martínez M, DeJoseph MR, Wolfe A, Radovick S, Boehm U, Urban JH, Levine JE. Positive, but not negative feedback actions of estradiol in adult female mice require estrogen receptor α in kisspeptin neurons. *Endocrinology* 2015; **156**: 1111–1120. [Medline] [CrossRef]
 35. Kauffman AS. Neuroendocrine mechanisms underlying estrogen positive feedback and the LH surge. *Front Neurosci* 2022; **16**: 953252. [Medline] [CrossRef]
 36. Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M. Sexual differentiation of Kiss1 gene expression in the brain of the rat. *Endocrinology* 2007; **148**: 1774–1783. [Medline] [CrossRef]
 37. Tomikawa J, Homma T, Tajima S, Shibata T, Inamoto Y, Takase K, Inoue N, Ohkura S, Uenoyama Y, Maeda K, Tsukamura H. Molecular characterization and estrogen regulation of hypothalamic KISS1 gene in the pig. *Biol Reprod* 2010; **82**: 313–319. [Medline] [CrossRef]
 38. Watanabe Y, Uenoyama Y, Suzuki J, Takase K, Suetomi Y, Ohkura S, Inoue N, Maeda KI, Tsukamura H. Oestrogen-induced activation of preoptic kisspeptin neurones may be involved in the luteinising hormone surge in male and female Japanese monkeys. *J Neuroendocrinol* 2014; **26**: 909–917. [Medline] [CrossRef]
 39. Matsuda F, Nakatsukasa K, Suetomi Y, Naniwa Y, Ito D, Inoue N, Wakabayashi Y, Okamura H, Maeda KI, Uenoyama Y, Tsukamura H, Ohkura S. The luteinising hormone surge-generating system is functional in male goats as in females: involvement of kisspeptin neurones in the medial preoptic area. *J Neuroendocrinol* 2015; **27**: 57–65. [Medline] [CrossRef]
 40. Yeo SH, Kyle V, Morris PG, Jackman S, Sinnett-Smith LC, Schacker M, Chen C, Colledge WH. Visualisation of Kiss1 neurone distribution using a Kiss1-CRE transgenic mouse. *J Neuroendocrinol* 2016; **28**: 1–12. [Medline] [CrossRef]
 41. Marraudino M, Miceli D, Fariñetti A, Ponti G, Panzica G, Gotti S. Kisspeptin innervation of the hypothalamic paraventricular nucleus: sexual dimorphism and effect of estrous cycle in female mice. *J Anat* 2017; **230**: 775–786. [Medline] [CrossRef]
 42. Homma T, Sakakibara M, Yamada S, Kinoshita M, Iwata K, Tomikawa J, Kanazawa T, Matsui H, Takatsu Y, Ohtaki T, Matsumoto H, Uenoyama Y, Maeda K, Tsukamura H. Significance of neonatal testicular sex steroids to defeminize anteroventral periventricular kisspeptin neurons and the GnRH/LH surge system in male rats. *Biol Reprod* 2009; **81**: 1216–1225. [Medline] [CrossRef]
 43. Watanabe Y, Ikegami K, Nakamura S, Uenoyama Y, Ozawa H, Maeda K-I, Tsukamura H, Inoue N. Mating-induced increase in Kiss1 mRNA expression in the anteroventral periventricular nucleus prior to an increase in LH and testosterone release in male rats. *J Reprod Dev* 2020; **66**: 579–586. [Medline] [CrossRef]
 44. Cao J, Patisaul HB. Sexually dimorphic expression of hypothalamic estrogen receptors α and β and Kiss1 in neonatal male and female rats. *J Comp Neurol* 2011; **519**: 2954–2977. [Medline] [CrossRef]
 45. Semaan SJ, Murray EK, Poling MC, Dhamija S, Forger NG, Kauffman AS. BAX-dependent and BAX-independent regulation of Kiss1 neuron development in mice. *Endocrinology* 2010; **151**: 5807–5817. [Medline] [CrossRef]
 46. Clarkson J, Busby ER, Kirilov M, Schütz G, Sherwood NM, Herbison AE. Sexual differentiation of the brain requires perinatal kisspeptin-GnRH neuron signaling. *J Neurosci* 2014; **34**: 15297–15305. [Medline] [CrossRef]
 47. Nakamura S, Watanabe Y, Goto T, Ikegami K, Inoue N, Uenoyama Y, Tsukamura H. Kisspeptin neurons as a key player bridging the endocrine system and sexual behavior in mammals. *Front Neuroendocrinol* 2022; **64**: 100952. [Medline] [CrossRef]
 48. Knoll JG, Clay CM, Bouma GJ, Henion TR, Schwarting GA, Millar RP, Tobet SA. Developmental profile and sexually dimorphic expression of kiss1 and kiss1r in the fetal mouse brain. *Front Endocrinol (Lausanne)* 2013; **4**: 140. [Medline] [CrossRef]
 49. Desrozières E, Droguerre M, Bentsen AH, Robert V, Mikkelsen JD, Caraty A, Tillet Y, Duittoz A, Franceschini I. Embryonic development of kisspeptin neurones in rat. *J Neuroendocrinol* 2012; **24**: 1284–1295. [Medline] [CrossRef]
 50. Chen J, Minabe S, Munetomo A, Magata F, Sato M, Nakamura S, Hirabayashi M, Ishihara Y, Yamazaki T, Uenoyama Y, Tsukamura H, Matsuda F. Kiss1-dependent and independent release of luteinizing hormone and testosterone in perinatal male rats. *Endocr J* 2022; **69**: 797–807. [Medline] [CrossRef]
 51. Takase K, Uenoyama Y, Inoue N, Matsui H, Yamada S, Shimizu M, Homma T, Tomikawa J, Kanda S, Matsumoto H, Oka Y, Tsukamura H, Maeda KI. Possible role of oestrogen in pubertal increase of Kiss1/kisspeptin expression in discrete hypothalamic areas of female rats. *J Neuroendocrinol* 2009; **21**: 527–537. [Medline] [CrossRef]
 52. Majarune S, Nima P, Sugimoto A, Nagae M, Inoue N, Tsukamura H, Uenoyama Y. Ad libitum feeding triggers puberty onset associated with increases in arcuate Kiss1 and Pdyn expression in growth-retarded rats. *J Reprod Dev* 2019; **65**: 397–406. [Medline] [CrossRef]
 53. Kim J, Semaan SJ, Clifton DK, Steiner RA, Dhamija S, Kauffman AS. Regulation of Kiss1 expression by sex steroids in the amygdala of the rat and mouse. *Endocrinology* 2011; **152**: 2020–2030. [Medline] [CrossRef]
 54. Pineda R, Plaisier F, Millar RP, Ludwig M. Amygdala kisspeptin neurons: putative mediators of olfactory control of the gonadotropic axis. *Neuroendocrinology* 2017; **104**: 223–238. [Medline] [CrossRef]
 55. Adekunbi DA, Li XF, Lass G, Shetty K, Adegoke OA, Yeo SH, Colledge WH, Lightman SL, O'Byrne KT. Kisspeptin neurones in the posterodorsal medial amygdala modulate sexual partner preference and anxiety in male mice. *J Neuroendocrinol* 2018; **30**: e12572. [Medline] [CrossRef]
 56. Xu Z, Kaga S, Mochiduki A, Tsubomizu J, Adachi S, Sakai T, Inoue K, Adachi AA. Immunocytochemical localization of kisspeptin neurons in the rat forebrain with special reference to sexual dimorphism and interaction with GnRH neurons. *Endocr J* 2012; **59**: 161–171. [Medline] [CrossRef]
 57. Di Giorgio NP, Semaan SJ, Kim J, López PV, Bettler B, Libertun C, Lux-Lantos VA, Kauffman AS. Impaired GABAB receptor signaling dramatically up-regulates Kiss1 expression selectively in nonhypothalamic brain regions of adult but not prepubertal mice. *Endocrinology* 2014; **155**: 1033–1044. [Medline] [CrossRef]
 58. Brailoiu GC, Dun SL, Ohsawa M, Yin D, Yang J, Chang JK, Brailoiu E, Dun NJ. Kiss-1 expression and metastin-like immunoreactivity in the rat brain. *J Comp Neurol* 2005; **481**: 314–329. [Medline] [CrossRef]
 59. Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus: sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology* 2006; **147**: 5817–5825. [Medline] [CrossRef]
 60. Clarkson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. *J Neuroendocrinol* 2009; **21**: 673–682. [Medline] [CrossRef]
 61. Cravo RM, Margatho LO, Osborne-Lawrence S, Donato J Jr, Atkin S, Bookout AL, Rovinsky S, Frazão R, Lee CE, Gautron L, Zigman JM, Elias CF. Characterization of Kiss1 neurons using transgenic mouse models. *Neuroscience* 2011; **173**: 37–56. [Medline] [CrossRef]
 62. Igarashi H, Koizumi K, Kaneko R, Ikeda K, Egawa R, Yanagawa Y, Muramatsu S, Onimaru H, Ishizuka T, Yawo H. A novel reporter rat strain that conditionally expresses the bright red fluorescent protein tdTomato. *PLoS One* 2016; **11**: e0155687. [Medline] [CrossRef]
 63. Uenoyama Y, Nakamura S, Hayakawa Y, Ikegami K, Watanabe Y, Deura C, Minabe S, Tomikawa J, Goto T, Ieda N, Inoue N, Sanbo M, Tamura C, Hirabayashi M, Maeda KI, Tsukamura H. Lack of pulse and surge modes and glutamatergic stimulation of luteinising hormone release in Kiss1 knockout rats. *J Neuroendocrinol* 2015; **27**: 187–197. [Medline] [CrossRef]
 64. Takumi K, Iijima N, Ozawa H. Developmental changes in the expression of kisspeptin mRNA in rat hypothalamus. *J Mol Neurosci* 2011; **43**: 138–145. [Medline] [CrossRef]
 65. Stephens SBZ, Chahal N, Munaganuru N, Parra RA, Kauffman AS. Estrogen stimulation of Kiss1 expression in the medial amygdala involves estrogen receptor- α but not estrogen receptor- β . *Endocrinology* 2016; **157**: 4021–4031. [Medline] [CrossRef]
 66. Bell MR. Comparing postnatal development of gonadal hormones and associated social behaviors in rats, mice, and humans. *Endocrinology* 2018; **159**: 2596–2613. [Medline] [CrossRef]
 67. Döhler KD, Wuttke W. Changes with age in levels of serum gonadotropins, prolactin and gonadal steroids in prepubertal male and female rats. *Endocrinology* 1975; **97**: 898–907. [Medline] [CrossRef]
 68. Walker DM, Kirson D, Perez LF, Gore AC. Molecular profiling of postnatal development of the hypothalamus in female and male rats. *Biol Reprod* 2012; **87**: 129. [Medline] [CrossRef]