Journal of Reproduction and Development, Vol. 64, No 6, 2018

—SRD Outstanding Research Award 2017—

The roles of kisspeptin in the mechanism underlying reproductive functions in mammals

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Abstract. Kisspeptin, identified as a natural ligand of GPR54 in 2001, is now considered as a master regulator of puberty and subsequent reproductive functions in mammals. Our previous studies using *Kiss1* knockout (KO) rats clearly demonstrated the indispensable role of kisspeptin in gonadotropin-releasing hormone (GnRH)/gonadotropin secretion. In addition, behavioral analyses of *Kiss1* KO rats revealed an organizational effect of kisspeptin on neural circuits controlling sexual behaviors. Our studies using transgenic mice carrying a region-specific *Kiss1* enhancer-driven reporter gene provided a clue as to the mechanism by which estrogen regulates *Kiss1* expression in hypothalamic kisspeptin neurons. Analyses of *Kiss1* expression and gonadotropin secretion during the pubertal transition shed light on the mechanism triggering GnRH/gonadotropin secretion at the onset of puberty in rats. Here, we summarize data obtained from the aforementioned studies and revisit the physiological roles of kisspeptin in the mechanism underlying reproductive functions in mammals.
Key words: Estrogen, Gonadotropin-releasing hormone (GnRH), *Kiss1*, Luteinizing hormone (LH), Puberty
(**J. Reprod. Dev. 64:** 469–476, 2018)

Introduction

It is widely accepted that the hypothalamus plays a pinnacle role in the hierarchical mechanism regulating the gonadal axis through controlling the anterior pituitary gland in mammals. After the discovery of the gonadotropin-releasing hormone (GnRH) in the hypothalamus of domestic animals by two independent groups led by Schally and Guillemin in 1971 [1, 2], neuroendocrinologists have been keen to find out how GnRH release is controlled in mammals. The majority of GnRH neurons are scattered in the septum, preoptic and anterior hypothalamic regions of the brain in mammals [3-5], and most of them send axonal projections to the median eminence [6]. GnRH is secreted into the pituitary portal circulation at the median eminence, and activates the gonadotropin-secreting cells of the anterior pituitary gland through GnRH receptors [7, 8].

There are two modes of GnRH secretion in female mammals: one is pulsatile GnRH secretion [9, 10] and the other is the surge-mode of GnRH secretion [11, 12]. It is well known that pulsatile GnRH secretion controls tonic gonadotropin secretion [9, 10], which is found at most stages of the estrous cycle and is responsible for follicular development and steroidogenesis in the follicles and corpora lutea. Since the classical study performed by Moore and Price [13], it has been well accepted that pulsatile GnRH secretion is controlled by the negative feedback action of steroid hormones secreted from the follicles and corpora lutea [14]. On the other hand, the surge-mode of GnRH secretion induces a luteinizing hormone (LH) surge [11, 12], which is found in the preovulatory stage of the estrous cycle and evokes ovulation and corpus luteum formation. It is apparent that the surge-mode of GnRH secretion is induced by the positive feedback action of high levels of estrogen secreted by mature follicles [15]. During the last three decades of the 20th century, intensive studies were performed to address the involvement of various neurotransmitters and neuropeptides in the regulation of two modes of GnRH/gonadotropin secretion; however the mechanisms controlling GnRH/gonadotropin secretion were not fully elucidated until the discovery of kisspeptin at the beginning of the 21st century.

Kisspeptin, first named metastin, was originally found in human placenta as an endogenous ligand for GPR54, an orphan G-protein coupled receptor, in 2001 [16, 17]. The peptide was identical to the partial amino acid sequence of the *KISS1* metastasis suppressor gene product reported elsewhere [18] and inhibited the chemotaxis, invasion, and metastasis of GPR54-expressing melanomas [16]. Kisspeptin was identified as a 52-amino-acid peptide in rats, and the amino acid sequences are highly conserved in mammals studied to date [16, 17, 19–24]. In particular, the C-terminal amidated 10-amino-acid sequences of kisspeptin, which are considered to be essential and sufficient for receptor interaction [16], are identical among mammals, except for a C-terminal tyrosine changed to phenylalanine in primates.

Two years after the discovery of kisspeptin [16, 17], a role for kisspeptin–GPR54 signaling as a key regulator for mammalian reproduction emerged. Two clinical studies independently revealed that loss-of-function mutations of the *GPR54* gene caused hypogonadotropic hypogonadism, characterized by absence of sexual maturation, and low circulating gonadotropins and gonadal steroids

Received: September 10, 2018
Accepted: September 23, 2018
Published online in J-STAGE: October 8, 2018

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[25, 26], suggesting that kisspeptin–GPR54 signaling plays a key role as a gatekeeper of reproduction in humans. To date, patients carrying loss-of-function mutations of the *KISS1* gene recapitulated the phenotypes seen in patients carrying *GPR54* mutations [27]. The incipient line of *Gpr54* knockout (KO) mice also recapitulated human hypogonadal phenotypes [26]. Subsequently, several groups made efforts to generate *Gpr54* or *Kiss1* KO mice [26, 28–31] and further substantiated the essential role of kisspeptin–GPR54 signaling in puberty and fertility in mammals. However, detailed analyses of gonadotropin secretion in *Gpr54* or *Kiss1* KO mice were limited [32], because mice are simply too small to collect frequent blood samples for detailed analyses of the plasma profiles of gonadotropins. To overcome this disadvantage, a skillful frequent blood collection and a highly sensitive radioimmunoassay or enzyme-immunoassay are required for the successful detection of pulsatile and surge modes of gonadotropin secretion, as previously reported by ourselves and in other studies [32, 33].

The present article focuses on the roles of hypothalamic kisspeptin in the central mechanism regulating reproductive functions in mammals. First, we review the phenotypes of our *Kiss1* KO rat model that have clearly demonstrated the indispensable role of kisspeptin in mammalian reproduction via controlling pulsatile and surge modes of GnRH/gonadotropin secretion. Second, we propose the molecular mechanisms by which estrogen regulates *Kiss1* expression and consequently controls GnRH/gonadotropin secretion. Finally, we discuss a possible mechanism regulating pubertal changes in hypothalamic *Kiss1* expression to trigger GnRH/gonadotropin secretion in rodents. A better understanding of the mechanism regulating reproductive functions in mammals would provide us with the basis for therapeutic approaches to solve infertility in livestock species.

Kiss1 KO Rats: An Animal Model to Demonstrate the Indispensable Role of Kisspeptin in Governing the Pulsatile and Surge Modes of GnRH/Gonadotropin Secretion

We generated *Kiss1* KO rats by gene targeting in rat embryonic stem (ES) cells to demonstrate the indispensable role of kisspeptin in GnRH/gonadotropin secretion [34]. The gene-modified rat model has an advantage over the mouse model, as its large body size allows a detailed analysis of hormonal profiles in an individual. Briefly, a targeting vector carrying a tandem dimer Tomato (tdTomato) —a variant red fluorescent protein— reporter gene in the rat *Kiss1* locus, was generated in order to disrupt the *Kiss1* gene of rat ES cells by homologous recombination. Chimera rats were generated by microinjection of the *Kiss1*-targeted ES cells into the cavity of recipient rat blastocysts. Germline pups carrying the disrupted *Kiss1* locus heterozygously were fertile and successfully intercrossed to produce *Kiss1* KO rats.

Kiss1 KO rats clearly reproduced the hypogonadal phenotypes of human and mouse models carrying *KISS1*/*Kiss1* mutations [34]. Namely, *Kiss1* KO rats showed pubertal failure and atrophic gonads in both sexes without growth retardation. Female *Kiss1* KO rats showed a complete inhibition of both LH and follicle-stimulating hormone (FSH) secretion, whereas wild-type female littermates showed cyclic changes in plasma LH and FSH levels during their regular 4-day estrous cycle (Fig. 1A). Our more detailed analysis of plasma LH profiles with frequent blood collection, clearly showed that *Kiss1* KO rats exhibited a complete suppression of pulsatile LH secretion even after ovariectomy (Fig. 1B). In addition, *Kiss1* KO female rats exhibited no LH surge when animals received preovulatory levels of estradiol-17β (E2) (Fig. 1C). These findings clearly demonstrated that kisspeptin plays an indispensable role in controlling both the pulsatile and surge mode of secretion of GnRH/gonadotropin that regulate pubertal onset and subsequent normal reproductive functions in mammals. In addition, the administration of major stimulatory neurotransmitters, such as monosodium glutamate, N-methyl-D-aspartate (an agonist of a class of ionotropic glutamate receptor), or norepinephrine, failed to stimulate LH secretion in *Kiss1* KO rats, though each stimulatory neurotransmitter stimulated LH secretion in wild-type littermates [34]. These findings suggest that kisspeptin neurons function as a hub, integrating major stimulatory neural inputs to GnRH neurons.

In addition to the indispensable role of kisspeptin in GnRH/gonadotropin secretion, our behavioral analyses of *Kiss1* KO rats revealed the involvement of kisspeptin in the defeminization/masculinization of neural circuits controlling sexual behaviors in male rats [35]. Namely, *Kiss1* KO male rats exhibited no male-type sexual behaviors, such as mounting, intromission, and ejaculation, even though they received testosterone replacement in adulthood. The mounting and intromission in *Kiss1* KO male rats were recovered by long-term testosterone replacement from the peripubertal period to adulthood, suggesting that kisspeptin is required for testosterone secretion from pubertal onset to adulthood to consequently induce masculinization of the neural circuits controlling male-type sexual behaviors. Notably, *Kiss1* KO male rats exhibited the lordosis reflex, a female-type sexual behavior, as shown in wild-type females, when they received preovulatory levels of E2 in adulthood. The lordosis reflex in *Kiss1* KO male rats was reduced to levels seen in wild-type males by kisspeptin replacement at the neonatal period, suggesting that kisspeptin is required for defeminization of the neural circuits controlling female-type sexual behaviors at the so-called "critical period" for sexual differentiation of the brain. Interestingly, *Kiss1* KO female rats exhibited normal female-type sexual behaviors when they received preovulatory levels of E2 in adulthood. These findings clearly demonstrate the organizational effects of kisspeptin on the neural circuits controlling sexual behaviors in male rats.

Molecular Mechanism by which Estrogen Regulates *Kiss1* Expression in Two Populations of Hypothalamic Kisspeptin Neurons

Distributions of kisspeptin neurons are largely similar in all mammals studied to date [20–22, 24, 36–46]. Our studies revealed the distribution of hypothalamic kisspeptin neurons in several mammals, such as rats [37, 41], Japanese monkeys [24], goats [20, 46], pigs [21, 45], and musk shrews (*Suncus murinus*) [22]. In rodents, kisspeptin neurons are mainly localized in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) [36, 37, 40, 41]. The distribution of kisspeptin neurons in rodents is largely based on studies in females, because males have few kisspeptin neurons in the AVPV [40, 41, 47, 48]. The sexual dimorphism of AVPV kisspeptin

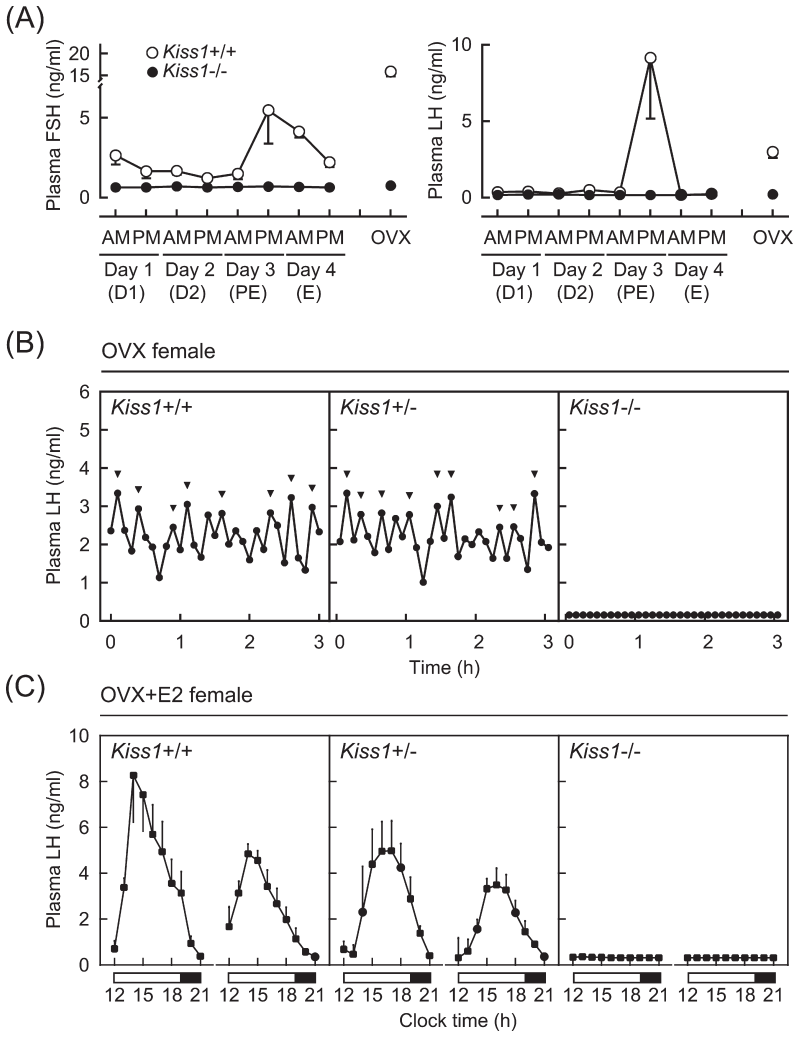

Fig. 1. Lack of pulsatile and surge modes of gonadotropin secretion in *Kiss1* knockout (*Kiss1*$^{-/-}$) rats. (A) Plasma follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels in wild-type (*Kiss1*$^{+/+}$) and *Kiss1*$^{-/-}$ female rats. Plasma samples were collected from gonad-intact animals at 1000 h (AM) and 1700 h (PM) (1410 h light/dark cycle, light on 0500 h) for four consecutive days, and ovariectomized (OVX) rats at 1000 h. Stages of the estrous cycle were determined by vaginal smears in *Kiss1*$^{+/+}$ rats. D1, diestrus 1; D2, diestrus 2; PE, proestrus; E, estrus. (B) Individual 3-h plasma LH profiles of representative OVX *Kiss1*$^{+/+}$, heterozygous (*Kiss1*$^{+/-}$), and *Kiss1*$^{-/-}$ rats. Arrowheads indicate LH pulses identified with the PULSAR computer program [70]. (C) Nine-hour plasma LH profiles of *Kiss1*$^{+/+}$, *Kiss1*$^{+/-}$, and *Kiss1*$^{-/-}$ female rats, which were OVX and received preovulatory levels of estradiol-17β (E2). Plasma samples were collected for two consecutive days. Open and closed horizontal bars indicate light and dark periods. Originally published in Uenoyama *et al*. (2015) [34].

neurons provide a clue to their axonal projections; at least a portion of the AVPV kisspeptin neurons probably send axonal projections to GnRH cell bodies in the preoptic area (POA) [37, 40], because kisspeptin fibers adjusted to GnRH neuronal cell bodies were more apparent in female mice than in males [40]. On the other hand, ARC kisspeptin neurons probably send axonal projections to the median eminence, because our previous studies showed a close proximity of kisspeptin and GnRH axons in the median eminences of goats and rats [49, 50]. A schematic illustration of axonal projections of kisspeptin neurons is shown in Fig. 2.

Estrogen robustly and differentially regulates *Kiss1* expression in a region-specific manner: *Kiss1* expression in the AVPV is up-regulated by estrogen treatment [36, 41], while ARC *Kiss1* expression is down-regulated by this treatment [36, 37, 41]. The two-way regulation of *Kiss1* expression by estrogen is suggested to be mediated by estrogen receptor-α (ERα), because estrogen had no effect on the *Kiss1* expression in both the AVPV and ARC of ovariectomized (OVX) ERα KO mice [36, 51], in which it failed to exert its positive and negative feedback effects on gonadotropin secretion [52]. These studies suggest that AVPV kisspeptin neurons are responsible for the positive feedback action of estrogen to induce the surge mode of GnRH/LH secretion and that ARC kisspeptin neurons are involved in the negative feedback action of estrogen to modulate pulsatile GnRH/gonadotropin secretion in mammals. Given the potent stimulatory

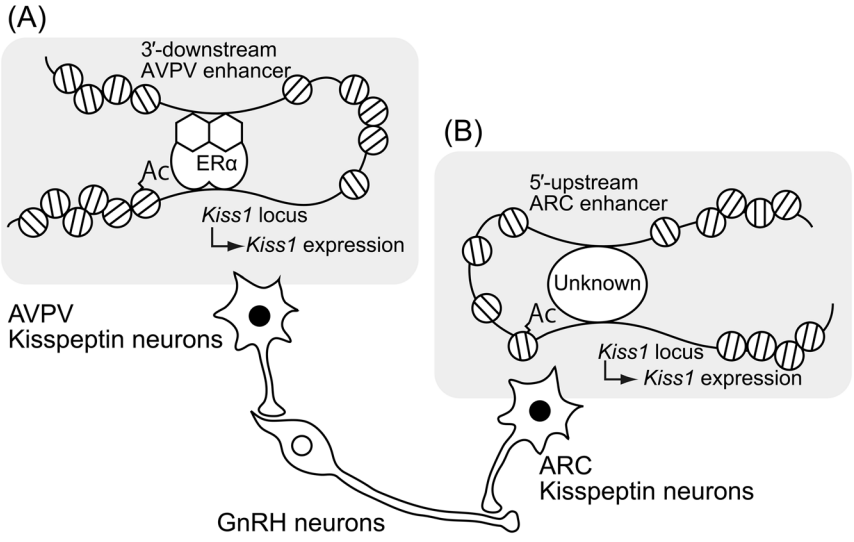

Fig. 2. Schematic illustrations of axonal projections of two populations of hypothalamic kisspeptin neurons and the molecular and epigenetic mechanism regulating *Kiss1* expression in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC). (A) Estrogen-ERα complex bound on the *Kiss1* promoter region induces histone acetylation in the *Kiss1* promoter region and forms chromatin loops between the 3′-downstream region and promoter region of the *Kiss1* locus to activate AVPV *Kiss1* expression. (B) Histone acetylation of the *Kiss1* promoter region and chromatin loops formed between the 5′-upstream region and promoter region of the *Kiss1* locus via unknown transcriptional factor(s) appeared to be involved in ARC *Kiss1* expression in the absence of estrogen. Ac, histone acetylation.

effect of kisspeptin on LH secretion and the successful blockade of spontaneous and E2-induced LH surges by the POA infusion of anti-kisspeptin antibody [37, 41, 53], AVPV kisspeptin neurons probably serve as a so-called "GnRH surge generator". We recently reviewed the role of sexual dimorphic AVPV kisspeptin neurons in GnRH/LH surge generation [54]. Additionally, our previous studies in goats suggest that ARC kisspeptin neurons provably serve as a part of the GnRH pulse generating mechanism, because periodic bursts of multiple unit activity, corresponding to LH pulses, were successfully recorded from electrodes placed near the cluster of ARC kisspeptin neurons in goats [20, 55]. The current understanding of the molecular mechanism of GnRH pulse generation has been recently reviewed elsewhere [56, 57].

Our *in vivo* reporter assay using *Kiss1*-green fluorescent protein (GFP) reporter transgenic mice suggests the presence of region-specific enhancers of the *Kiss1* locus and provides a clue as to the epigenetic and molecular mechanisms regulating *Kiss1* expression in the AVPV and ARC. Briefly, transgenic mice carrying a so-called "full-length" *Kiss1*-GFP reporter construct (approximately 33 kb) successfully exhibited GFP signals in both populations of kisspeptin neurons localized in the AVPV and ARC [58]. On the other hand, transgenic mice carrying a 3′- downstream-truncated *Kiss1*-GFP reporter construct (approximately 28 kb) and transgenic mice carrying the 5′-upstream-truncated *Kiss1*-GFP reporter construct (approximately 13 kb) exhibited GFP signals only in the kisspeptin neurons localized in the ARC and AVPV, respectively [58, 59]. The results of this *in vivo* reporter assay suggest that the 3′-downstream region of the *Kiss1* locus serves as an AVPV-specific enhancer for estrogen-induced increase in AVPV *Kiss1* expression, whereas the 5′-upstream region of the *Kiss1* locus serves as an ARC-specific enhancer for increase in ARC *Kiss1* expression in the absence of estrogen. This scenario is supported by our previous gene conformation analyses, which indicated that the formation of chromatin loops between the *Kiss1* promoter region and each putative region-specific enhancer is associated with the induction of *Kiss1* expression in the AVPV and ARC [58, 59].

We have also demonstrated estrogen-dependent ERα recruitment and histone acetylation, an active histone modification, in the *Kiss1* promoter region in the AVPV [58]. Interestingly, histone acetylation in the *Kiss1* promoter region was also associated with an increase in ARC *Kiss1* expression, because histones of the *Kiss1* promoter region were highly acetylated in the absence of estrogen [59]. Further, the *in vitro* expression of *Kiss1* was induced by a histone deacetylase inhibitor in the non-*Kiss1*-expressing immortalized neural cell line [58]. Thus, we estimate that AVPV *Kiss1* expression is likely up-regulated by histone acetylation of the *Kiss1* promoter region, and forms chromatin loops between the 3′-downstream region and promoter region of the *Kiss1* locus under the control of an estrogen-ERα complex bound in the *Kiss1* promoter region (Fig. 2A). Moreover, ARC *Kiss1* expression is likely up-regulated by histone acetylation of the *Kiss1* promoter region, and chromatin loops formed between the 5′-upstream region and promoter region of the *Kiss1* locus in the absence of estrogen (Fig. 2B). The intracellular mechanism involved in the estrogen-dependent region-specific histone modification and consequent *Kiss1* expression in AVPV and ARC kisspeptin neurons remains to be determined. Further studies are needed to address these issues.

Mechanism Regulating Pubertal Augmentation of ARC *Kiss1* Expression to Trigger Pulsatile GnRH/Gonadotropin Secretion

As described above, kisspeptin–GPR54 signaling plays a key role

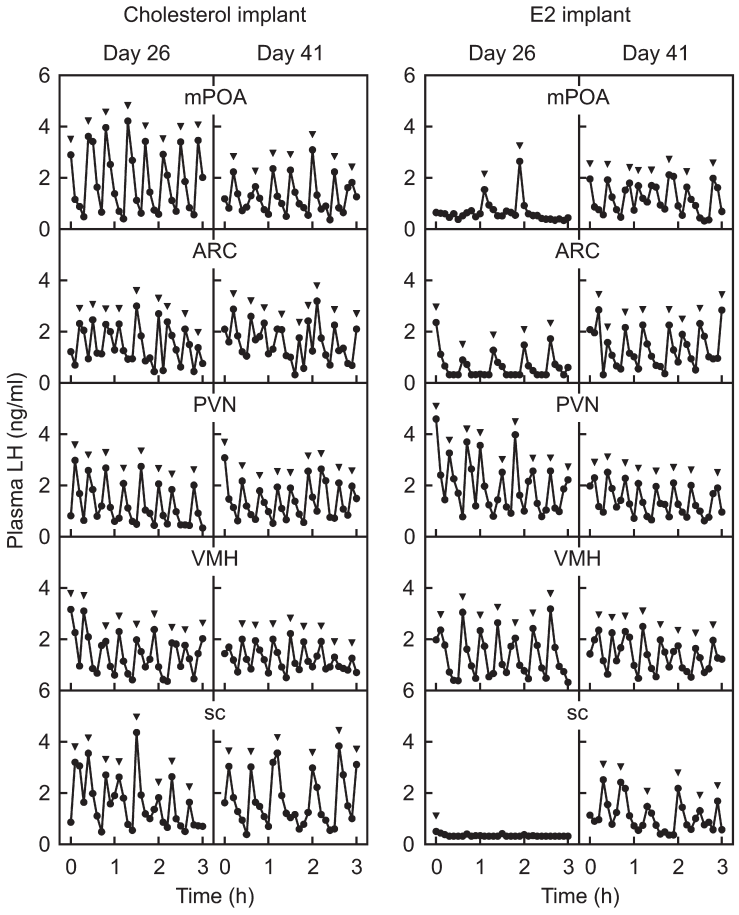

Fig. 3. Estrogen-dependent prepubertal suppression of pulsatile LH secretion in female rats. Plasma LH profiles in cholesterol- (left panel) or E2-implanted (right panel) OVX rats at 26 (prepubertal period) and 41 (postpubertal period) days of age. Note that E2 implanted into the medial preoptic area (mPOA), ARC or subcutaneous (sc) space, but not into the paraventricular nucleus (PVN) or ventromedial nucleus (VMH), suppressed LH pulses in OVX rats in the prepubertal period. Arrowheads indicate LH pulses identified with the PULSAR computer program [70]. Originally published in Uenoyama *et al*. (2015) [69].

as a gatekeeper of pubertal onset in mammals. Sexual maturation in the pubertal period seems to be timed by an increase in pulsatile GnRH/gonadotropin secretion in mammals [60–64], indicating a promising role of ARC kisspeptin neurons in the pubertal augmentation of GnRH/gonadotropin secretion in mammals. Classical studies highlighted the negative feedback action of estrogen in the mechanism controlling peripubertal GnRH/gonadotropin secretion in female rodents. Namely, Ramirez and McCann [65] and Eldridge *et al*. [66] proposed the 'gonadostat hypothesis', which states that a decrease in the sensitivity to the negative feedback action of estrogen would be associated with the pubertal augmentation of GnRH/gonadotropin secretion in rodents, because a lower amount of estrogen was required for the suppression of gonadotropin secretion in prepubertal animals compared to that in matured animals [65, 66]. After the discovery of kisspeptin, therefore, we hypothesized that pubertal changes in *Kiss1* expression are robustly controlled by the negative feedback action of estrogen in female rats in a prepubertal period-specific manner [67]. Not surprisingly, ARC *Kiss1* expression and pulsatile LH secretion increased immediately after ovariectomy in prepubertal rats. Estrogen replacement reverted female rats to the immature state. The strong suppression of ARC *Kiss1* expression and pulsatile LH secretion was found only in the prepubertal period. Taken together, these findings suggest that the pulsatile GnRH/gonadotropin-secreting system seems to be already equipped before the onset of puberty, and the decrease in sensitivity to the negative feedback action of estrogen on ARC *Kiss1* expression may trigger the pubertal augmentation of pulsatile GnRH/gonadotropin secretion in female rats.

The negative feedback action of estrogen seems to directly inhibit *Kiss1* expression via ERα expressed in ARC kisspeptin neurons, because kisspeptin neuron-specific ERα KO mice showed precocious ARC *Kiss1* expression at birth, resulting in the precious pubertal onset [68]. Interestingly, our previous study [69] suggested another action site of estrogen negative feedback to suppress GnRH/LH secretion in the prepubertal period, because micro-implants of E2 in the POA, as well as the ARC, partly suppressed pulsatile LH secretion in prepubertal OVX rats (Fig. 3). It is unlikely that E2 implanted in the POA leaked into the ARC, because E2 implants in the nuclei close to the ARC, such as the paraventricular nucleus

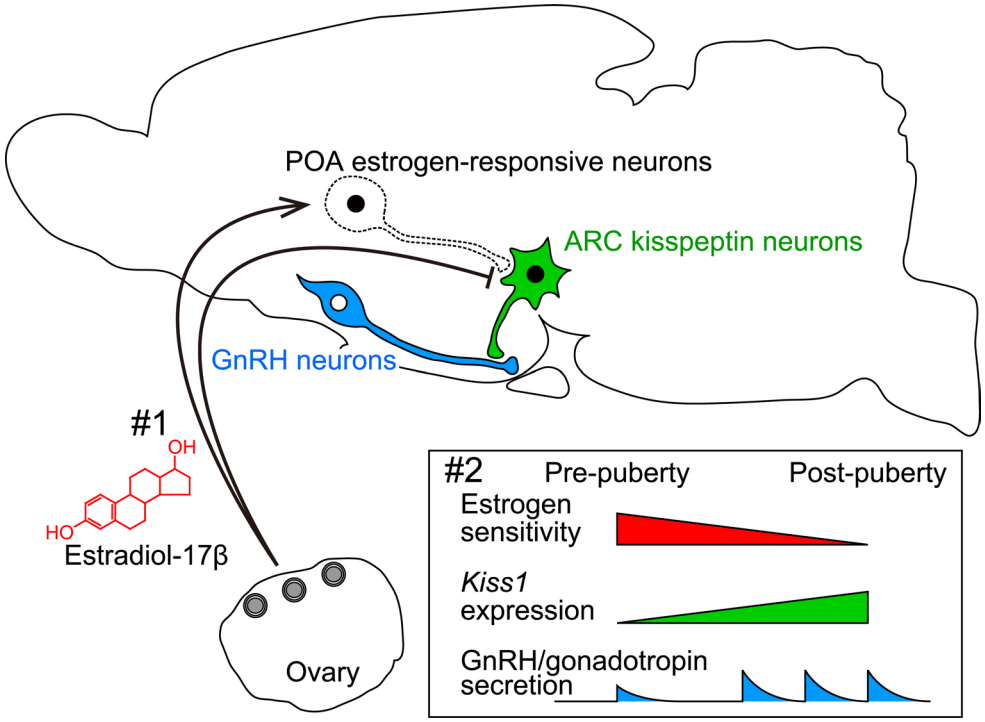

Fig. 4. Schematic illustration showing a possible mechanism regulating the pubertal augmentation of ARC *Kiss1* expression to trigger pulsatile GnRH/gonadotropin secretion. #1, Estrogen strongly suppresses ARC *Kiss1* expression via direct and indirect pathways: estrogen-responsive neurons in the POA mediate estrogen negative feedback action to ensure the prepubertal suppression of ARC *Kiss1* expression. #2, During the pubertal transition, the sensitivity to the estrogen negative feedback action on ARC *Kiss1* expression somehow decreases, which results in an increase in *Kiss1* expression, and the subsequent secretion of kisspeptin triggers GnRH/gonadotropin secretion at pubertal onset.

or ventromedial nucleus, showed no suppression of pulsatile LH secretion in prepubertal female rats (Fig. 3). Thus, we envision that POA estrogen-responsive neurons may mediate the negative feedback action of estrogen to insure the prepubertal suppression of ARC *Kiss1* expression and subsequent pulsatile GnRH/gonadotropin secretion in female rats.

Taken together, the decrease in sensitivity to estrogen negative feedback action on ARC *Kiss1* expression plays a critical role in triggering GnRH/gonadotropin secretion at the onset of puberty in female rats (Fig. 4). We speculate that a decrease in sensitivity to estrogen negative feedback action would occur in both POA estrogen-responsive neurons and ARC kisspeptin neurons during pubertal transition in rodents, because micro-implants of E2 in the POA and ARC failed to suppress pulsatile LH secretion in postpubertal OVX rats (Fig. 3). It is unlikely that the pubertal decrease in the sensitivity to estrogen is simply caused by a decrease in ERα expression, because ERα mRNA expression and the number of ERα-expressing cells in the POA and ARC of female rats were comparable between prepubertal and postpubertal periods [69]. Further studies are warranted to clarify the molecular mechanism decreasing the sensitivity of estrogen negative feedback action on ARC *Kiss1* expression at the onset of puberty.

Conclusions and Perspective

Over the past 15 years, after the discovery of the key role of kisspeptin–GPR54 signaling in pubertal onset in humans, intensive studies on hypothalamic kisspeptin–GPR54 signaling elucidated the mechanism underlying reproductive functions in several mammalian species. As described in this article, kisspeptin plays indispensable roles in puberty and subsequent normal reproductive functions in mammals via controlling the pulse and surge modes of GnRH/gonadotropin secretion. Further analyses of *Kiss1* expression and kisspeptin secretion at the cellular and molecular levels would provide a better understanding of the mechanism underlying reproductive functions in mammals. We envisage that these findings on the central mechanism of reproduction in mammals can be used as the basis for future therapeutic approaches to solve infertility of livestock species.

Acknowledgments

We would like to express our gratitude to the Society for Reproduction and Development (SRD) for awarding YU the SRD Outstanding Research Award in 2017. We would like to thank all the collaborators who contributed to this study, and Dr Nicola Skoulding for the editorial assistance. The present study was supported in part by a grant from the Program for Promotion of Basic Research Activities for Innovative Biosciences and the Cooperative Study

Program of the National Institute for Physiological Sciences (to KM), and by JSPS KAKENHI Grant Numbers 24380157 (to KM), 16K07987 (to NI), and 18H03973 (to HT).

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