

総合論文

Uncovering the role of arcuate kisspeptin neurons as a source of the gonadotropin-releasing hormone pulse generator using gene-modified rats

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Strategies for increasing reproductive performance are needed for domestic animals because for example the conception (pregnancy) rate has decreased in dairy cows around the world. Reproductive function is controlled by hormones released by the hypothalamus-pituitary-gonadal axis in mammals, including domestic animals. Of those hormones, tonic (pulsatile) gonadotropin-releasing hormone (GnRH) release is fundamentally important for gonadotropin release and gonadal activity. Therefore, uncovering the mechanism controlling GnRH pulses, that is GnRH pulse generator, is essential to improve reproductive technologies for domestic animals. The present review is focused on the indispensable role of arcuate nucleus (ARC) kisspeptin neurons (also known as KNDy neurons) as the GnRH pulse generator in mammals. First, we give a brief overview of studies on hypothalamic kisspeptin neurons throughout the past two decades. Second, we review studies that have provided direct evidence that ARC kisspeptin neurons serve as the GnRH pulse generator, with a special focus on our gene-modified rat models. Finally, we discuss the mechanism underlying GnRH pulse generation. The knowledge obtained from gene-modified rat models should be clinically important and could be adapted to new tools to improve reproductive performance in livestock by stimulating GnRH/gonadotropin pulses.

Key words : gene-modified rats, GnRH, kisspeptin, LH, pulse generator

Introduction

Development of new technology to improve reproductive performance of domestic animals is certainly required because the conception (pregnancy) rate has decreased in dairy cows around the world^{1,2)}. For example, the conception rate of the first artificial insemination decreased from > 60% to approximately 40% in dairy cows during the past three decades in Japan³⁾. It is estimated that the decrease in the conception rate from 60% to 40% would cause a 1.5-fold increase in the number of cows to maintain production⁴⁾. Thus, the feed, manure, and greenhouse gases would increase accordingly. Therefore, improving the reproductive performance of cows may be warranted in terms of the sustainability of livestock production.

Reproductive function is controlled by hormones released by the hypothalamus-pituitary-gonadal axis in

mammals, including domestic animals. Hypothalamic gonadotropin-releasing hormone (GnRH), pituitary gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]), and gonadal steroid hormones (such as estradiol-17 β , progesterone, and testosterone) work together to promote follicular development, ovulation, and the development of corpora lutea in females and spermatogenesis in males. Of those hormones, tonic (pulsatile) GnRH release is fundamentally important for gonadotropin release and gonadal functions, such as gametogenesis and steroidogenesis. This is based on the fact that pulsatile GnRH treatment has restored circulating gonadotropin concentrations at physiological levels in adult female rhesus monkeys, which lacked endogenous GnRH release by hypothalamic lesions⁵⁾. Indeed, continuous GnRH treatment paradoxically decreased circulating gonadotropin concentrations in such female rhesus monkeys, while the pulsatile GnRH treatment triggered menstrual cycles with cyclic increases in circulating LH levels, and hence circulating estradiol-17 β (indicating follicular development) and progesterone (indicating the development of corpora lutea) levels in immature female rhesus monkeys⁶⁾. These results suggest that the GnRH pulses are needed to maintain pituitary responsiveness to GnRH and the biosynthesis and

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release of gonadotropins from the pituitary gland, thereby controlling ovarian function in female mammals. Therefore, unraveling the mechanism controlling GnRH pulses, that is GnRH pulse generator, is essential for improving reproductive technologies for domestic animals.

The GnRH pulse generator was predicted to be located in the mediobasal hypothalamus (MBH) including the arcuate nucleus (ARC)^{7, 8)} because Halasz and Pupp showed in 1965 that female rats bearing complete isolation of the MBH from the rest of the brain (known as “hypothalamic deafferentation”) exhibited follicular development⁷⁾. GnRH neuronal cell bodies are mainly located in the anterior hypothalamic areas, such as the preoptic area (POA) in rats⁹⁾. The GnRH pulse generator has been considered to be composed of non-GnRH neurons, but the source driving GnRH pulses has long remained unknown. Since the discovery of kisspeptin, originally called metastin¹⁰⁾, as an endogenous ligand of orphan receptor GPR54 in 2001, growing evidence suggests that the ARC population of kisspeptin neurons is a source of the GnRH pulse generator.

In this review, we focused on the indispensable role of ARC kisspeptin neurons as the GnRH pulse generator in mammals. First, we gave a brief overview of studies on hypothalamic kisspeptin neurons during the past two decades. Second, we summarized our studies that provided direct evidence that ARC kisspeptin neurons serve as the GnRH pulse generator, with a special focus on our gene-modified rat models. Finally, we discussed the mechanism underlying GnRH pulse generation and its application in order to improve reproductive performance in livestock.

Hypothalamic kisspeptin neurons

It is now well known that hypothalamic kisspeptin neurons serve as master regulators of reproduction by driving hypothalamic GnRH release and hence pituitary gonadotropin release in mammals^{11, 12, 13)}. The pivotal role of kisspeptin in mammalian reproduction was first discovered in humans : In 2003, two articles demonstrated that loss-of-function mutations in the kisspeptin receptor gene (known as *GPR54*) resulted in hypogonadotropic hypogonadism, which is the absence of puberty onset with a lack of gonadotropin release, in humans^{14, 15)}. Subsequently, the role of the kisspeptin receptor in puberty onset and reproductive functions was verified using gene-modified mice by targeted disruption of the *Gpr54* gene (encoding kisspeptin receptor in rodents)^{15, 16)}. Furthermore, the indispensable role of kisspeptin *per se*

in mammalian reproduction was confirmed in gene-modified mice and rats by targeted disruption of the *Kiss1* gene (encoding kisspeptin in rodents)^{17, 18, 19)} and in patients suffering from hypogonadotropic hypogonadism with inactivating mutations of the *KISS1* gene²⁰⁾.

Kisspeptin profoundly stimulated GnRH/gonadotropin release in mammals : Central or peripheral administration of kisspeptin immediately stimulated gonadotropin release in rodents^{21, 22, 23, 24)}, ruminants²⁵⁾, and primates²⁶⁾. Furthermore, GnRH receptor antagonists blocked the stimulatory effect of kisspeptin on gonadotropin release in rodents^{21, 23, 27)} and primates²⁶⁾, indicating that kisspeptin is a potent secretagogue of GnRH release in mammals. Indeed, *Gpr54* is expressed in most GnRH neurons in mice^{25, 28, 29)} and rats^{21, 30)}.

One major population of hypothalamic kisspeptin neurons is localized in the ARC of mammals such as mice^{31, 32)}, rats^{22, 33)}, sheep^{34, 35)}, goats³⁶⁾, cattle³⁷⁾, pigs^{38, 39, 40)}, and monkeys^{26, 41, 42)}. To date, ARC kisspeptin neurons have been considered as the GnRH pulse generator based on various pieces of some circumstantial evidence : Rhythmic increases in the electrical activity of multiple neurons (known as multiple unit activity [MUA] volleys) around the electrode inserted near the ARC kisspeptin neurons were synchronized with LH pulses in female and male goats^{43, 44)}, and rhythmic Ca²⁺ increase in ARC kisspeptin neurons using green fluorescent protein (GFP)-based Ca calmodulin probe (GCaMP) fiber photometry recordings, synchronized with LH pulses, were detected in *Kiss1-Cre* female and male mice^{45, 46, 47, 48)}. Importantly, Keen *et al.*⁴⁹⁾ demonstrated pulsatile release of kisspeptin, most of which was synchronized with GnRH pulses, at the median eminence of female rhesus monkeys. Notably, most ARC kisspeptin neurons coexpress neurokinin B, a tachykinin family peptide, and dynorphin A, an endogenous opioid peptide, in mice^{50, 51)}, rats^{52, 53)}, sheep⁵⁴⁾, goats⁴⁴⁾, and cattle³⁷⁾. Therefore, ARC kisspeptin neurons are also known as KNDy neurons. In addition, a majority of ARC kisspeptin neurons are also glutamatergic, because about 70 % of ARC kisspeptin neurons expressed Cre in *Slc17a6* (glutamatergic marker gene)-Cre mice⁴⁸⁾.

Another major population of hypothalamic kisspeptin neurons located in the anteroventral periventricular nucleus (AVPV) in rodents or POA in ruminants is considered to serve as the GnRH surge generator to control ovulation in female mammals. Indeed, preovulatory levels of estradiol-17 β evoked AVPV *Kiss1* expression in female rats³³⁾ and *fos* (a marker of neuronal

activation) expression in the AVPV/POA kisspeptin neurons of female rats³³⁾ and goats³⁶⁾. Interestingly, AVPV kisspeptin neurons show an apparent sexual dimorphism in rodents: Male rats and mice show few kisspeptin neurons in the AVPV^{33, 55)}, and male rats never show LH surge even if castrated males are treated with preovulatory levels of estradiol-17 β ⁵⁶⁾.

Identification of the GnRH pulse generator using gene-modified rats

Rats have the advantages of being easier to collect blood samples from frequently, and for being able to withstand various surgeries, because they are approximately 10 times larger than mice, even though their lifespan, sexual maturation, estrous cycle length, gestation period, and litter size are very similar to those of mice. Indeed, it is easier to analyze the pulsatile release of LH in rats by frequent blood sampling compared to mice. In addition, rat embryonic stem (ES) cells were established in the 2010s, making it easier to generate gene-modified rats⁵⁷⁾. Currently, an improvement in gene editing technology is rapidly facilitating the generation of gene-modified rats⁵⁸⁾. Therefore, we generated gene-modified rats to identify the GnRH pulse generator with gene targeting in rat ES cells or embryos.

Restoration of GnRH pulse generation by rescuing KNDy neurons in global *Kiss1* knockout (KO) rats

To evaluate the indispensable role of ARC kisspeptin neurons (= KNDy neurons) in GnRH pulse generation, global *Kiss1* KO rats were first generated by gene targeting using rat ES cells⁵⁷⁾, in which *Kiss1* exons 2 and

3 were replaced with tdTomato cDNA (encoding a red fluorescent protein)¹⁹⁾. Female and male global *Kiss1* KO rats exhibited an absence of puberty onset along with complete suppression of LH and FSH release, resulting in tiny gonads. These results suggest that kisspeptin is indispensable for GnRH release and the pituitary and gonadal functions in rats of both sexes.

To answer the question as to whether ARC KNDy neurons generate GnRH pulses, the “KNDy neuron rescue” experiment was performed using global *Kiss1* KO female rats⁵⁹⁾ (Fig. 1). Briefly, recombinant adeno-associated virus (AAV) vectors carrying CAG-promoter-driven *Kiss1* cDNA were injected in the ARC of global *Kiss1* KO female rats. The rescue rate of ARC KNDy neurons was evaluated using the ratio of ARC *Kiss1*- and *Tac3* (encoding neurokinin B)-co-expressing cells out of the total ARC *Tac3*-expressing cells in AAV-*Kiss1*-treated global *Kiss1* KO rats. Because the rescue rate varied between individuals, the AAV-*Kiss1*-treated global *Kiss1* KO rats were divided into three groups based on their ARC KNDy neuron rescue rate: 20–50% (highly) KNDy neuron-rescued, 3–15% (moderately) KNDy neuron-rescued, and 0–1% (few) KNDy neuron-rescued rats. As a result, LH pulses, an indicator of GnRH pulses, were profoundly restored in the highly KNDy neuron-rescued rats and partly restored in the moderately KNDy neuron-rescued rats along with increases in *Lhb* (encoding β -subunit of LH) mRNA expression and LH content in the pituitary gland. On the other hand, no LH pulses were found in the few KNDy neuron-rescued rats or AAV carrying enhanced GFP (AAV-EGFP)-treated

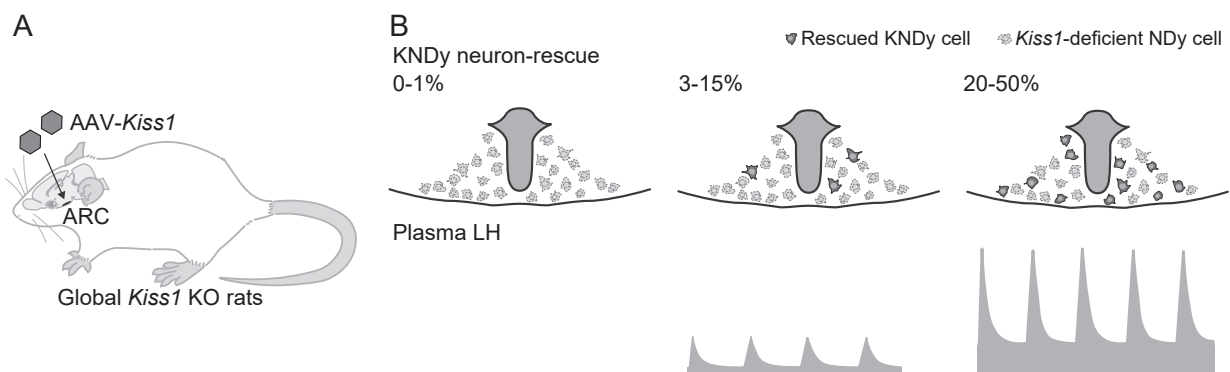


Fig. 1 Recovery of luteinizing hormone (LH) pulses in kisspeptin/neurokinin B/dynorphin A (KNDy) neuron-rescued female rats. (A) Adeno-associated virus (AAV) vectors carrying CAG-promoter-driven *Kiss1* cDNA were injected in the arcuate nucleus (ARC) of global *Kiss1* knockout (KO) female rats with a lack of gonadotropin-releasing hormone (GnRH)/gonadotropin release. (B) LH pulses were restored in ARC KNDy neuron-rescued rats when approximately 5–20% of KNDy neurons were rescued.

global *Kiss1* KO controls. Importantly, many *Kiss1*-positive cells, which were infected with AAV-*Kiss1*, were observed even in the few KNDy neuron-rescued rats, indicating that *Kiss1* expression outside ARC *Tac3* neurons did not restore LH pulses, and that rescuing *Kiss1* expression in the original KNDy neuron could restore GnRH pulse generation in AAV-*Kiss1*-treated global *Kiss1* KO rats. These results provided direct evidence that ARC KNDy neurons serve as the GnRH pulse generator in female rats.

Furthermore, the highly and moderately KNDy neuron-rescued rats showed large preovulatory follicles (known as Graafian follicles) in the ovaries, whereas the few KNDy neuron-rescued rats showed only small follicles in the ovaries, similar to AAV-*EGFP*-treated global *Kiss1* KO controls. In addition, ovarian estrogen synthase (known as aromatase, coded by *Cyp19a1*) mRNA expression increased in the highly and moderately KNDy neuron-rescued rats compared to AAV-*EGFP*-treated global *Kiss1* KO control rats. Therefore, GnRH/LH pulses in the highly and moderately KNDy neuron-rescued rats were enough to promote follicular development. Interestingly, no corpora lutea, which are formed after ovulation, were found in the ovaries of the highly and moderately KNDy neuron-rescued rats. These results indicate that KNDy neuron-rescued rats failed to show LH surge and ovulation, despite the presence of preovulatory follicles in their ovaries. Taken together, KNDy neurons are responsible for GnRH pulse generation but not GnRH surge generation in female rats.

Lack of GnRH pulse generation in ARC-specific *Kiss1* KO rats

To address the question with a different approach, we performed “ARC-specific *Kiss1* KO” experiment using *Kiss1*-floxed female rats⁵⁹⁾ (Fig. 2). *Kiss1*-floxed rats were also generated by gene targeting using rat ES cells⁵⁷⁾, in which two loxP sequences were inserted to delete *Kiss1* exons 2 and 3 using the Cre-loxP system⁵⁹⁾. To induce ARC-specific *Kiss1* KO, recombinant AAV vectors carrying CAG-promoter-driven *Cre* cDNA were injected into the ARC of *Kiss1*-floxed rats. The resultant ARC *Kiss1* KO rate was evaluated based on the number of ARC *Kiss1*-expressing cells in AAV-*Cre*-treated *Kiss1*-floxed rats. Since the KO rate differed individually, the animals were classified into two groups: > 90% (highly) ARC *Kiss1* KO and 60–80% (moderately) ARC *Kiss1* KO rats. As a result, LH pulses were profoundly suppressed in the highly ARC *Kiss1* KO rats, whereas apparent LH pulses were found in the moderately ARC *Kiss1* KO rats, similar to AAV-*EGFP*-treated *Kiss1*-floxed control rats. Thus, the results obtained from the “ARC-specific *Kiss1* KO” experiment also provided direct evidence that ARC kisspeptin neurons play a role as the GnRH pulse generator in female rats.

It was notable that the highly ARC *Kiss1* KO rats exhibited a smaller amplitude of estrogen-induced surge-like LH increase compared to AAV-*EGFP*-treated *Kiss1*-floxed control rats, indicating that the GnRH surge generator functioned in the highly ARC *Kiss1* KO rats. Indeed, preovulatory levels of estradiol-17 β evoked *Kiss1* expression in the AVPV of the

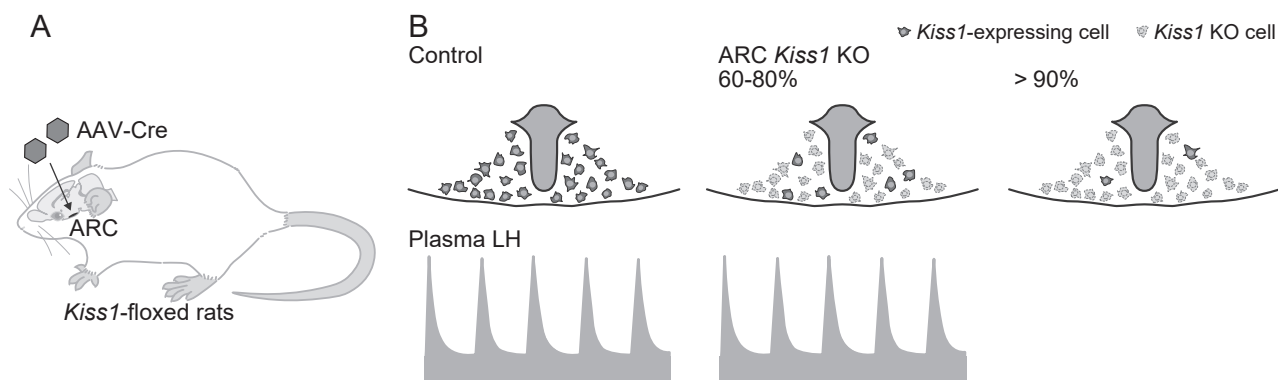


Fig. 2 Suppression of LH pulses in ARC-specific *Kiss1* KO female rats. (A) AAV vectors carrying CAG-promoter-driven *Cre* cDNA were injected into the ARC of *Kiss1*-floxed rats. (B) LH pulses were profoundly suppressed in the > 90% *Kiss1* KO rats, whereas apparent LH pulses were found in the 60–80% *Kiss1* KO rats.

highly ARC *Kiss1* KO rats. The smaller amplitude of the LH surge in the highly ARC *Kiss1* KO rats could be caused by a decrease in LH content and *Lhb* mRNA expression in the pituitary gland in the highly ARC *Kiss1* KO rats with a lack of GnRH pulse generation.

Mechanisms underlying GnRH pulse generation in ARC KNDy neurons

Most ARC KNDy neurons also express the Gq-coupled (stimulatory) neurokinin-3 receptor (NK3R, known as neurokinin B receptor) in mice^{50, 51, 60, 61}, goats⁶², and sheep⁶³. In addition, the Gi-coupled (inhibitory) κ -opioid receptor (KOR, known as dynorphin A receptor) was found in the majority of ARC KNDy neurons in rats^{64, 65} and sheep⁶⁶. Therefore, it was postulated that ARC KNDy neurons could synchronize with each other by stimulatory neurokinin B-NK3R and inhibitory dynorphin A-KOR signaling pathways in an autocrine/paracrine manner^{50, 67, 68}. Indeed, the frequency of the MUA volleys was increased by central administration of neurokinin B, senktide (an NK3R agonist), or nor-binaltorphimine (nor-BNI, a KOR antagonist) in female goats^{44, 62, 69}. In addition, the MUA volley frequency was decreased by central administration of dynorphin A in female goats^{44, 69}. Furthermore, neurokinin B, senktide, or KOR antagonists (nor-BNI and PF-4455242) stimulated LH release in various mammals, such as ewes^{70, 71}, female goats⁷², female rats^{73, 74}, and male mice⁶⁰. Taken together, it is most plausible that neurokinin B acts as an accelerator for ARC KNDy neurons, while dynorphin A serves as a brake for the neurons, leading to the synchronized pulsatile KNDy neuronal activity to generate GnRH pulses. In addition, glutamate stimulated LH release in wild-type female rats, but not *Kiss1* KO rats¹⁹, and Ca^{2+} increase of ARC KNDy neurons in male *Kiss1-Cre* mice, using GCaMP fiber photometry⁷⁵. Furthermore, ionotropic glutamate receptor antagonists (6-Cyano-7-nitroquinoxaline-2,3-dione and D-2-Amino-5-phosphonovaleric acid) blocked basal Ca^{2+} increase and, at least in part, neurokinin B-induced Ca^{2+} increase of ARC KNDy neurons in male mice⁷⁵, suggesting that glutamate-ionotropic glutamate receptor signaling also drives synchronized pulsatile KNDy neuronal activity in rodents.

Recently, we generated KOR-expressing cell-specific *Kiss1* KO rats by crossing *Kiss1*-floxed rats and *Oprk1* (encoding KOR in rats)-*Cre* rats to clarify whether KOR-negative KNDy neurons could maintain fertility and whether KOR-positive KNDy neurons are required

for GnRH/LH pulses⁶⁵. *Oprk1-Cre* rats were generated by gene targeting using AAV and CRISPR/Cas9 system in rat embryos⁵⁸, in which *Cre* cDNA was inserted into the 3'-end of the *Oprk1* gene. *Kiss1*-floxed/*Oprk1-Cre* rats, in which *Kiss1* was deleted in more than 90% ARC KNDy neurons, showed normal puberty onset, as did *Kiss1*-floxed control rats. On the other hand, *Kiss1*-floxed/*Oprk1-Cre* rats showed a one-day longer estrous cycle compared to *Kiss1*-floxed controls. Besides, *Kiss1*-floxed/*Oprk1-Cre* rats were fertile, whereas the animals had fewer pups than *Kiss1*-floxed controls. Furthermore, the OVX *Kiss1*-floxed/*Oprk1-Cre* rats exhibited apparent LH pulses, whereas diestrous levels of estradiol-17 β -treated OVX *Kiss1*-floxed/*Oprk1-Cre* rats showed profound LH pulse disruption. Collectively, these results suggest that KOR-expressing KNDy neurons have a role in maintaining normal GnRH pulse generation, normal length of estrous cycle, and normal pup number in female rats. We envision that the remaining KOR-negative KNDy neurons may synchronize with KOR-positive "*Kiss1*-deficient NDy" neurons in *Kiss1*-floxed/*Oprk1-Cre* rats via mutual contact among KNDy and "*Kiss1*-deficient NDy" neurons through neurokinin B-NK3R signaling, gap junctions, and/or glial connections. This is because Ikegami *et al.*⁵¹ showed that gap junction inhibitors blocked the senktide-induced synchronized activities among mouse *Kiss1*-GFP cells and the neighboring glial cells.

Conclusion and perspective

Our previous studies using gene-modified rats provided direct evidence that ARC KNDy neurons serve as the GnRH pulse generator in female rats. It is plausible that ARC KNDy neurons synchronize with each other through stimulatory neurokinin B-NK3R and glutamate-ionotropic glutamate receptors signaling, and inhibitory dynorphin A-KOR signaling in an autocrine/paracrine manner. The resultant kisspeptin released from KNDy neurons stimulates GnRH release in a pulsatile manner (Fig. 3). NK3R agonists or KOR antagonists may enhance GnRH/gonadotropin pulses in livestock with central reproductive dysfunction. Importantly, our studies using gene-modified rats have suggested functional redundancy of KNDy neurons: a small number of KNDy neurons is sufficient to generate GnRH/gonadotropin pulses⁵⁹ and fertility⁶⁵ in female rats. Therefore, full restoration of KNDy neurons is not necessarily required to restore GnRH pulse generation, tonic gonadotropin secretion, and consequent follicular

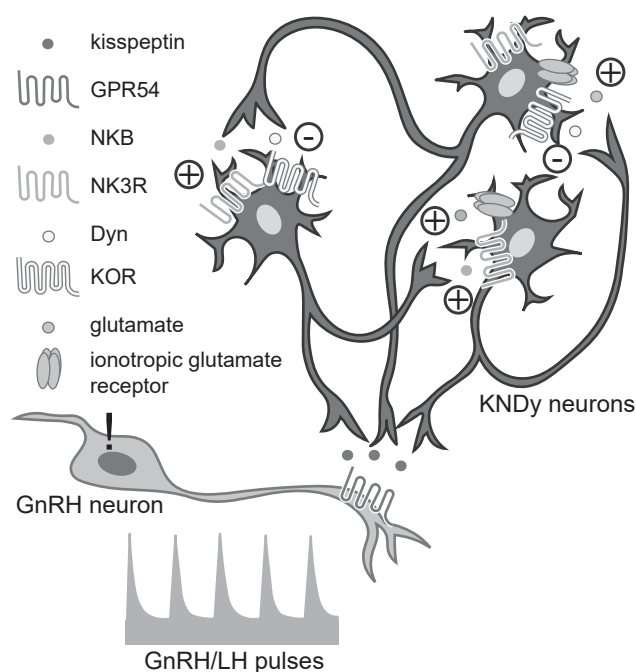


Fig. 3 Schematic illustration showing the possible mechanism underlying GnRH/LH pulse generation in rats. It is plausible that ARC KNDy neurons synchronize with each other through the stimulatory neurokinin B-neurokinin-3 receptor (NK3R) and glutamate-ionotropic glutamate receptor signaling, and the inhibitory dynorphin A- κ -opioid receptor (KOR) signaling in an autocrine/paracrine manner. The resultant kisspeptin released by KNDy neurons stimulates GnRH release in a pulsatile manner.

development in livestock with reproductive dysfunction. The knowledge obtained from the gene-modified rat models should be clinically important and could be adapted as a new set of tools for improving reproductive performance in livestock by stimulating the GnRH/gonadotropin pulse generation.

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遺伝子改変ラットを用いた弓状核キスペプチンニューロンの 性腺刺激ホルモン放出ホルモンパルスジェネレーターとしての役割解明

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(応用動物科学コース)

世界において、乳牛の受胎率（妊娠率）が低下しており、家畜の繁殖成績向上のための効果的な治療法が必要とされている。家畜を含む哺乳類において、生殖機能は視床下部－下垂体－性腺軸から分泌されるホルモンによって制御されている。これらのホルモンのうち、性腺刺激ホルモン放出ホルモン（GnRH）のパルス状分泌（GnRH パルス）は、性腺刺激ホルモン分泌と性腺機能に本質的に重要である。したがって、GnRH パルスを制御するメカニズム（GnRH パルスジェネレーター）を解明することは、家畜の生殖技術を向上させるために不可欠である。本総説では、著者らの遺伝子改変ラットモデルを用いた弓状核キスペプチンニューロン（KNDy ニューロンとしても知られる）が GnRH パルスジェネレーターの本体であることの直接的な証拠を示した研究を中心として、過去20年間の研究を概説した。また、KNDy ニューロンが分泌するニューロキニン B、グルタミン酸、ダイノルフィン A が KNDy ニューロンの神経活動を同期させ、GnRH パルスを発生させるメカニズムについて論じた。遺伝子改変ラットモデルから得られた知識は、GnRH/性腺刺激ホルモンパルスを刺激して、家畜の繁殖能力を向上させる新規繁殖促進剤開発に寄与すると期待できる。

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