Continuous acceleration of neural activity of the GnRH pulse generator during chronic peripheral infusion of neurokinin 3 receptor agonist in goats

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Abstract. Secretion of pulsatile gonadotropin-releasing hormone (GnRH) is essential for reproduction. Kisspeptin neurons in the arcuate nucleus (ARC), which coexpress neurokinin B (NKB) and its receptor (NK3R), are believed to be components of the GnRH pulse generator that regulates pulsatile GnRH secretion. We examined the effects of peripheral infusion of senktide, an NK3R selective agonist, on GnRH pulse generator activity by monitoring multiple unit activity (MUA) in the goat ARC. Previous studies have shown that characteristic increases in MUA (MUA volleys) reflect GnRH pulse generator activity. Senktide was infused intravenously or intravaginally for 2 h while recording MUA. Both infusions significantly increased the MUA volley frequency compared with the control. These results demonstrate that peripherally administered senktide acts centrally to sustainably accelerate the neural activity of the GnRH pulse generator throughout the infusion period. This suggests the possibility of practical applications of NK3R agonists for improving reproductive activity in farm animals.

Key words: Drug delivery, GnRH pulse generator, Intravaginal administration, Kisspeptin, Neurokinin B

Mammalian reproductive function is regulated by the gonadotropin-releasing hormone (GnRH) pulse generator, which regulates intermittent GnRH secretion into portal vessels and commands the secretion of pulsatile luteinizing hormone (LH) into peripheral circulation. Multiple unit activity (MUA) recording from the mediobasal hypothalamus in monkeys [1], rats [2], and goats [3, 4] has demonstrated that GnRH pulse generator activity can be monitored as episodic bursts (MUA volleys), which are followed by LH pulses, in the peripheral circulation.

Kisspeptin plays an essential role in controlling the release of GnRH [5, 6]. Kisspeptin-expressing neurons (kisspeptin neurons) are exclusively localized in the medial preoptic area (the anteroventral periventricular nucleus in rodents) and arcuate nucleus (ARC) [7, 8]. The latter group of neurons coexpress several neuropeptides, including neurokinin B (NKB) and dynorphin A (Dyn) [9–11]. Therefore, kisspeptin neurons in the ARC are known as KNDy neurons.

We previously reported that MUA volleys can be detected through electrodes inserted in close proximity of KNDy neurons, suggesting that KNDy neurons are candidates for investigating the intrinsic GnRH pulse generator [10, 12, 13]. In addition, NKB plays a stimulatory role in KNDy neurons. NKB administration in the close vicinity of KNDy neurons immediately accelerates neural activity in goats, as reflected by MUA volleys [14], indicating that KNDy neurons are directly stimulated by NK3R agonists. Intracerebroventricular administration of an NK3R agonist induces c-Fos expression in KNDy neurons in rats [15] and sheep [16]; incidentally, most KNDy neurons coexpress NK3R in goats [14]. These findings suggest that NKB acts directly on KNDy neurons, thereby stimulating the GnRH pulse generator activity in mammals. Thus, NK3R agonists may increase the frequency of GnRH/LH pulses and downstream ovarian activity in domestic mammals.

We have previously shown that peripheral bolus administration of NK3R agonists has a positive effect on GnRH pulse generator activity [17]. However, no study has reported the effects of chronic peripheral administration of NK3R agonists on the neural activity of GnRH pulse generator in ruminants. In this study, an NK3R agonist, senktide, was infused intravenously (IV) and intravaginally to evaluate the effects of chronic administration of the agonist on the activity of the GnRH pulse generator. The intravaginal route of administration was examined to extend our findings to future practical applications in the field.

MUA volleys were continuously observed in all three ovariectomized (OVX) goats. IV administration of saline had no effect on the occurrence of MUA volleys (Fig. 1A, upper panel). The number of MUA volleys during vehicle infusion was 4.00 ± 0.577. In the case of senktide IV administration (10 nmol), the number of MUA volleys was higher than that in the vehicle group (Fig. 1A, middle panel). The numbers of MUA volleys during 10 nmol and 40 nmol senktide infusion for 120 min were 10.67 ± 0.667 and 15.00 ± 1.00 (Fig. 1A, lower panel), respectively. There were significant differences between vehicle vs. 10 nmol senktide, vehicle vs. 40 nmol senktide, and 10 nmol senktide vs. 40 nmol senktide infusions (one-way ANOVA, Tukey’s post hoc test, P < 0.05; Fig. 2A). No significant difference was detected in the number of MUA volleys between the first and second hour of infusion of the vehicle or the two doses of senktide (data not shown).

In OVX+ estradiol (E2) goats, the frequency of MUA volleys decreased due to the inhibitory effect of E2 on GnRH pulse generator activity (Fig. 1B, upper panel), as reported previously [18]. IV administration of saline had no effect on the occurrence of MUA volleys (Fig. 1B, upper panel). In contrast, IV administration of senktide evoked MUA volleys (Fig. 1B, middle panel). There were...
significant differences in the number of MUA volleys between vehicle vs. 10 nmol senktide and vehicle vs. 40 nmol senktide infusions (one-way ANOVA, Tukey’s post hoc test, \( P < 0.05; \) Fig. 2B). No significant difference was detected in the number of MUA volleys between the 10 and 40 nmol senktide infusions (\( P = 0.378; \) Fig. 2B). Furthermore, no significant difference was detected in the number of MUA volleys between the first and second hour of senktide infusion (data not shown).

MUA volleys were continuously observed in three goats. Intravaginal saline administration did not affect the occurrence of MUA volleys (Fig. 1C, upper panel). The number of MUA volleys during vehicle infusion was \( 4.333 \pm 0.333 \) (\( n = 3 \)). This result was comparable to that observed following the IV infusion of the vehicle into OVX goats (Fig. 1C, upper panel). The number of MUA volleys during the senktide infusion (300 nmol for 120 min) was \( 8.667 \pm 0.333 \) (\( n = 3 \)). A significant difference in the number of MUA volleys was detected between the vehicle and 300 nmol senktide infusions (paired \( t \)-test, \( P = 0.0059; \) Fig. 2C). Intravaginal infusion of senktide for 120 min had a stimulatory effect on MUA. However, no significant difference was detected in the number of MUA volleys between the first and second hour of infusion (data not shown).

In this study, the peripheral infusion of senktide, an NK3R agonist, stimulated the neural activity of the GnRH pulse generator in goats. Episodic rises in MUA (MUA volleys), which reflect GnRH pulse generator activity, were repeatedly observed during the infusion period. The activation of KNDy neurons by senktide administration resulted in the occurrence of high-frequency MUA volleys with short intervolley intervals rather than a single sustained rise in MUA during the infusion period. This result is consistent with those previously demonstrated using an intracerebroventricular (icv) injection of NKB [10]. A single icv injection of NKB evoked multiple MUA volleys with very short intervolley intervals. These results suggest that the stimulatory effect of an NK3R agonist on KNDy neurons is intermittently extinguished by an endogenous inhibitory drive, although the underlying mechanism remains unclear.

We demonstrated that the number of MUA volleys during the first and second hour of infusion was comparable, indicating that the stimulatory effect of senktide on GnRH pulse generator activity was continuous and stable for at least the 2 h infusion period. In general, the sustained effect of drug administration on neurons is attenuated by a process called desensitization. For example, the continuous administration of a kisspeptin receptor agonist on GnRH neurons suppresses GnRH/LH secretion [18]. In this report, desensitization of GnRH neuronal responses to a kisspeptin receptor agonist was observed during chronic administration of the agonist for several days. As we cannot rule out the occurrence of desensitization to an NK3R agonist under a prolonged administration period, further investigation is warranted.

In this study, we monitored only MUA volleys. Previous studies have indicated that MUA volleys are exclusively followed by the pulsatile secretion of LH in both OVX and OVX+E2 goats [10], as well as in intact females [4]. Endo et al. [19] reported that continuous senktide administration in goats during the follicular phase evoked LH release immediately after injection, although discrete LH pulses were not detected. Continuous administration of senktide (300 nmol/ min) initially induces a gradual increase in LH secretion, whereas
To reduce the amount of senktide loss, the chronic administration of senktide using the intravaginal drug delivery system described above should be further evaluated to assess its efficacy using MUA. Moreover, senktide is degraded in vivo by neutral endopeptidase (NEP) [23]. Modified substances that resist NEP have shown prolonged stimulatory effects on the GnRH pulse generator following a single IV administration in OVX goats using the MUA recording technique [24]. These findings indicate that senktide modification can improve metabolic stability with potent NK3R agonist activity. Novel agonists with stable structures should be employed to administer NK3R agonists intravaginally and sustainably stimulate the GnRH pulse generator.

In conclusion, chronic infusion of senktide, a selective NK3R agonist, continuously stimulated the neural activity of the GnRH pulse generator in goats. Although the intravaginal route of administration required a larger amount of senktide compared with the IV route, a stimulatory effect of the agonist on the GnRH pulse generator was similarly observed with either route of administration. These findings suggest that administration of an NK3R agonist to activate the GnRH pulse generator may be useful for enhancing the reproductive function of farm animals.

**Methods**

We used seven adult female Shiba goats (4–8 years; weighing 18.0–25.0 kg). The goats were ovariectomized at least three months before MUA electrode implantation. The animals were maintained on a standard pelleted diet and dry hay, and had free access to water and supplemental minerals. All animal experiments were performed in accordance with guidelines approved by the Animal Ethics Committee of the Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization (No. 21C112ILGS).

OVX goats were anesthetized using halothane and stereotaxically implanted with an array of bilateral recording electrodes. According to our previous study, MUA volleys are recorded only when the recording electrodes are implanted in the vicinity of KNDy neurons [10, 14]; therefore, in this study, the electrodes were aimed at a cluster of KNDy neurons concentrated in the posterior part of the ARC. The recording electrodes consisted of six Teflon-insulated silicone elastomer devices that were used to facilitate synchronized estrus in farm ruminants, including cows, sheep, and goats. Therefore, in this study, we assessed the effects of the intravaginal infusion of senktide on neural activity of the GnRH pulse generator in goats. Although the intravaginal route of administration was not the most efficient method, it allowed us to administer senktide continuously and sustainably. This method may be useful for enhancing the reproductive function of farm animals.
Three O VX goats were subcutaneously implanted with a silicone tubing (inner diameter, 3 mm; outer diameter, 5 mm; length, 20 mm; Dow Corning, Midland, MI, USA) filled with crystalline E 
2 (Sigma-Aldrich, St. Louis, MO, USA) (OVX+E 2). This implant has been shown to maintain plasma E 2 concentrations in the luteal phase (4–8 pg/mL) in goats [25]. Experiments were performed 1–2 weeks after implantation.

To determine the timing of control or senktide (Cat. # AS-22887, AnaSpec Inc., Fremont, CA, USA) administration, the spontaneous onset of MUA volleys was recorded for 2 h on the day of the experiment. To analyze spontaneously occurring MUA volleys, the mean value and standard deviation (SD) of MUA counts/20 sec during the control periods were determined. The start of a “volley” was defined when the count exceeded twice the SD of the mean value at a given time. The MUA signals were analyzed as spikes/20 sec.

Four goats (#603, 613, and 616 with OVX and #613, 616, and 619 with OVX+E 2 conditions) were used in this experiment. A microinjection pump (model ESP-32, Eicom, Kyoto, Japan) was used to administer the vehicle (0.04 N NaHCO 3 in saline) or senktide (10 nmol and 40 nmol), at a rate of 30 µl/min for 120 min using a microinjection pump. The starting point of the infusion was completely random regardless of the timing of the MUA volleys. MUA was monitored during administration as well as 1 h before and after administration. Finally, the number of MUA volleys during infusion was counted and compared between vehicle and senktide infusions.

Three goats (#G164, 205, and 903) were used in this experiment. Prior to the experiment, the MUA was recorded for several hours. The tip of the silicone tube was connected to a sponge (approximately 5 cm long and 2 × 2 cm in height and width) to prevent the tube from falling out of the vagina during administration. A sponge was inserted into the vagina immediately before vehicle or senktide administration. The vehicle (0.04 N NaHCO 3 in saline) or senktide (300 nmol in vehicle) was administered at a rate of 30 µl/min for 120 min using a microinjection pump. The starting point of the infusion was completely random regardless of the timing of the MUA volleys. MUA was monitored during administration as well as 1 h before and after administration. Finally, the number of MUA volleys during infusion was counted and compared between vehicle and senktide infusions.

The effects of senktide or vehicle infusion on MUA during continuous administration were analyzed using a one-way ANOVA, followed by Tukey’s post hoc tests. A paired t-test was performed for data obtained after intravaginal senktide or vehicle administration. The number of MUA volleys during the first and second hour of senktide infusion was compared and analyzed using a paired t-test. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA), and the results with P < 0.05 were considered statistically significant. Data obtained from the three goats are shown as the mean ± SEM.

Conflict of interests: The authors have no conflicts of interest to declare.

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