The Inhibitory Effects of Neurokinin B on GnRH Pulse Generator Frequency in the Female Rat

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Neurokinin B (NKB) and its receptor (neurokinin-3 receptor) are coexpressed with kisspeptin and dynorphin A (Dyn) within neurons of the hypothalamic arcuate nucleus, the suggested site of the GnRH pulse generator. It is thought that these neuropeptides interact to regulate gonadotropin secretion. Using the ovariectomized (OVX) and OVX 17β-estradiol-replaced rat models, we have carried out a series of *in vivo* neuropharmacological and electrophysiological experiments to elucidate the hierarchy between the kisspeptin, NKB, and Dyn signaling systems. Rats were implanted with intracerebroventricular cannulae and cardiac catheters for frequent (every 5 min) automated serial blood sampling. Freely moving rats were bled for 6 h, with intracerebroventricular injections taking place after a 2-h control bleeding period. A further group of OVX rats was implanted with intra-arcuate electrodes for the recording of multiunit activity volleys, which coincide invariably with LH pulses. Intracerebroventricular administration of the selective neurokinin-3 receptor agonist, senktide (100–600 pmol), caused a dose-dependent suppression of LH pulses and multiunit activity volleys. The effects of senktide did not differ between OVX and 17β-estradiol-replaced OVX animals. Pretreatment with a selective Dyn receptor (κ opioid receptor) antagonist, norbinaltorphimine (6.8 nmol), blocked the senktide-induced inhibition of pulsatile LH secretion. Intracerebroventricular injection of senktide did not affect the rise in LH concentrations after administration of kisspeptin (1 nmol), and neither did kisspeptin preclude the senktide-induced suppression of LH pulses. These data show that NKB suppresses the frequency of the GnRH pulse generator in a Dyn/κ opioid receptor-dependent fashion. **(*Endocrinology* 153: 307–315, 2012)**

Neurokinin B (NKB) is a member of the tachykinin family of excitatory neuropeptides that is essential for reproductive development. Humans with inactivating mutations in genes encoding NKB (*TAC3*) or its cognate G protein-coupled receptor, neurokinin-3 receptor [NK3R (*TACR3*)], display a hypogonadotropic hypogonadism phenotype, characterized by low circulating levels of gonadal steroids and LH, delayed puberty, and infertility (1). NKB-expressing neurons are particularly numerous in the hypothalamic arcuate (ARC) nucleus (2), which has been postulated as the site of the GnRH pulse generator (3). Most coexpress NK3R, kisspeptin, and its receptor [G protein-coupled receptor 54 (GPR54)], as well as an inhibitory endogenous opioid peptide, dynorphin A (Dyn), which binds the κ opioid receptor (KOR) (2, 4).

Animal studies addressing the effects of NKB/NK3R signaling on the functions of the hypothalamo-pituitary gonadal (HPG) axis have amassed a great deal of largely conflicting evidence. Sandoval-Guzmán and Rance (5) first reported a robust inhibition of LH secretion in the

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Abbreviations: aCSF, Artificial cerebrospinal fluid; ARC, arcuate; AUC, area under the curve; Dyn, dynorphin A; E_2, 17β-estradiol; GPR54, G protein-coupled receptor 54; HPG, hypothalamo-pituitary gonadal; icv, intracerebroventricular; KOR, κ opioid receptor; Kp-10, kisspeptin-10; MUA, multiunit activity; NK3R, neurokinin-3 receptor; NKB, neurokinin B; nor-BNI, norbinaltorphimine; OVX, ovariectomized; P_4, progesterone.

ovariectomized (OVX) rat treated with low levels of 17β-estradiol (E_2) after central administration of a selective NK3R agonist, senktide. A similar effect was reported in the OVX mouse (6). Subsequently, senktide injections have been shown to stimulate LH secretion in castrated male macaques (7) and gonadal intact ewes (8), whereas in the female rat, either an inhibition or a stimulation of LH secretion has been reported, dependent on the gonadal status and the resulting steroid milieu (9). These data describe either a net increase or decrease in circulating LH levels in response to the activation of NK3R. However, they do not address the role of NKB in the regulation of pulsatile GnRH/LH secretion, about which little is currently known.

Multiunit activity (MUA) recorded in the ARC provides an electrophysiological correlate of GnRH pulse generator activity. Coordinated bursts of neuronal firing, referred to as MUA volleys, coincide invariably with LH pulses in the macaque (10), rat (11, 12), and goat (4, 13). Thus, MUA volleys, like serial analysis of GnRH/LH pulses, serve as a powerful tool for studying the effects of neuropeptides on the HPG axis. It has recently been shown that intracerebroventricular (icv) kisspeptin-10 (Kp-10) administration does not affect MUA volleys in the OVX + E_2 rat (11) and OVX goat (12), whereas NKB increases and Dyn decreases MUA volley frequency (4). Intriguingly, the increase in MUA volley frequency in response to senktide is associated with an inhibition of LH secretion (4). In light of the apparent species differences in the response of the HPG axis to NK3R activation, we explored the effect of icv senktide treatment on MUA and pulsatile LH secretion in the female rat, both in the presence and absence of E_2 and progesterone (P_4). We also investigated the effects of central senktide administration on overall LH secretion in gonadal intact female rats.

Although the role of NKB remains controversial, it is now widely accepted that kisspeptin treatment causes raised plasma LH concentrations and that Dyn suppresses LH release. However the interaction between these modulatory neuropeptides remains to be elucidated. Corander *et al.* (14) provide evidence to suggest a complex interaction between kisspeptin and NKB in the modulation of GnRH secretion: in gonadal intact male mice, NKB coadministered icv with the potent GPR54 agonist, Kp-10, increases LH levels above those observed with Kp-10 alone; however, NKB abrogates kisspeptin-mediated GnRH release from hypothalamic explants. It is postulated that the NKB/NK3R, kisspeptin/GPR54, and Dyn/KOR signaling systems form a hierarchical mode of action within ARC neural networks and that NKB may act upstream of kisspeptin and Dyn to regulate pulsatile LH secretion. We tested this hypothesis by evaluating the effect of administration of senktide on LH pulse frequency after pretreatment of OVX + E_2 rats with pharmacological modulators of Dyn/KOR and kisspeptin/GPR54 signaling.

Materials and Methods

Animals and surgical procedures

Adult female Sprague Dawley rats (200–250 g), obtained from Charles River (Margate, UK), were housed under controlled conditions (12-h light, 12-h dark cycle, lights on 0700 h, temperature 22 ± 2 C) and provided with food and water *ad libitum*. All animal procedures were undertaken in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986, and were approved by the King's College London Ethical Review Panel Committee. All surgical procedures were carried out under anesthesia induced by ketamine [Vetalar, 100 mg/kg, ip (Pfizer, Sandwich, UK)] and xylazine [Rompun, 10 mg/kg, ip (Bayer, Newbury, UK)]. Each of the rats was used in the study only once.

Bilaterally OVX rats were divided into three groups: no steroid replacement, E_2-replaced, and E_2 + P_4-replaced. Animals in the OXV + E_2 group were implanted with a silicone capsule [inner diameter, 1.57 mm; outer diameter, 3.18 mm (Sani-Tech West, Oxnard, CA)] filled to a length of 25 mm with E_2 (Sigma-Aldrich, Poole, UK) dissolved to a concentration of 20 μg/ml in arachis oil (Sigma-Aldrich). The E_2-containing capsules produced circulating concentrations of E_2 within the range observed during the diestrous phase of the estrous cycle (~35.8 ± 1.2 pg/ml) as previously described (15). A further group of rats was OVX and implanted with E_2 capsules as described above, as well as P_4 capsules, as described previously (16). Silicone capsules [inner diameter 3.11 mm; outer diameter 3.92 mm (Sani-Tech West)] filled to a length of 30 mm with crystalline P_4 (Sigma-Aldrich) were implanted sc, producing concentrations of P_4 in the circulation within the range observed during the diestrous phase of the estrous cycle (~16.7 ± 1.5 ng/ml) as previously described (15). Animals were segregated into OVX, OVX + E_2, and OVX + E_2 + P_4 groups to investigate the effect of various circulating gonadal steroid levels on the response of the hypothalamic GnRH neural system to senktide treatment, thereby eliminating the impact of a fluctuating steroid milieu on the expression of endogenous neuropeptides and their receptors (9) and, in addition, to maintain continuity with standard experimental paradigms common among similar experiments (11, 17).

Rats were implanted with unilateral guide cannulae (22 gauge; Plastics One, Roanoke, VA) directed at the left lateral ventricle for microinfusion of pharmacological agents, the coordinates for implantation being 1.2 mm lateral, 0.3 mm posterior to bregma, and 5.4 mm below the surface of the dura (18). The guide cannulae were secured using dental cement (Simplex Rapid; Kemdent, Swindon, UK) and fitted with dummy cannulae (Plastics One) to maintain patency. All brain cannulae were implanted at the time of OVX. Correct cannula placement was confirmed by injection of 1 μl of artificial cerebrospinal fluid (aCSF) through the internal cannula and the observation of meniscus movement.

After bilateral OVX, the animals were housed singly and allowed 10 d of recovery before being fitted with two indwelling cardiac catheters via the jugular veins, to facilitate serial blood sampling. The catheters were exteriorized at the back of the head and secured to a cranial attachment; the rats were fitted with a 30-cm-long metal spring tether (Instec Laboratories, Boulder, CO). The distal end of the tether was attached to a fluid swivel (Instec Laboratories), which allowed the rats freedom to move around the enclosure. Experimentation commenced 3 d after cardiac catheter implantation.

A separate group of OVX rats was fitted with an array of nine recording electrodes chronically implanted in the mediobasal hypothalamus as described previously (12). The coordinates for electrode implantation were zero midline, 3.0 mm posterior to bregma, and 10.0 mm below the surface of the dura (18). Unilateral guide cannulae aimed at the lateral ventricle were fitted on the day of electrode implantation as described above, although angled 21° from a frontal approach to clear the electrode assembly.

Effect of senktide on pulsatile LH secretion

OVX, OVX + E_2, or OVX + E_2 + P_4 rats were attached via one of the two cardiac catheters to a computer-controlled automated blood sampling system, which allows for the intermittent withdrawal of small blood samples (25 μl) without disturbing the animals. Once connected, animals were left undisturbed for 1 h before sampling commenced. Blood sampling commenced between 1000 and 1100 h, when samples were collected every 5 min for 6 h for LH measurement. After removal of each 25-μl blood sample, an equal volume of heparinized saline (50 U heparin sodium/ml normal saline; Wockhardt, Wrexham, UK) was automatically infused into the animal to maintain patency of the catheter and blood volume. Immediately before injection, cannulae (Plastics One) with extension tubing preloaded with 100 pmol (n = 5–7 per group) or 600 pmol (n = 5 per group) senktide (Tocris Bioscience, Bristol, UK) in 4 μl of vehicle (aCSF), or 4 μl of vehicle (n = 4–6 per group) were inserted into the guide cannulae, extending 1 mm beyond its tip to reach the lateral ventricle. The distal end of the tubing was extended outside of the cage to allow remote infusion without disturbing the rat during the experiment. After 2 h of control blood sampling, treatments were given by icv injection over 5 min. Senktide doses were similar to, or lower than, those used in previous studies (5). Blood sampling continued throughout the experiment. Blood samples were frozen at −20 C for later assay to determine LH concentrations.

Estrous cyclicity was monitored in the gonadal intact group by examining daily vaginal cytology. On the morning of the experiment, the phase of the estrous cycle was confirmed, and only animals in the diestrous phase of the cycle were included. Animals were connected to the automated blood sampling system as described above, and after 2 h of control blood sampling, 600 pmol senktide was infused into the lateral ventricles over 5 min (n = 6). Control rats (n = 4) received 4 μl of aCSF icv.

Effect of senktide on pulsatile LH secretion in the presence of a KOR agonist

Senktide (100 pmol in 4 μl of aCSF) and the selective KOR antagonist, norbinaltorphimine (nor-BNI) (6.8 nmol in 4 μl of aCSF; Tocris Bioscience), were preloaded into microinjection cannulae, with 1 μl of air separating nor-BNI from senktide. OVX rats were administered icv with nor-BNI over 5 min after 1 h 50 min of control blood sampling, followed by senktide 10 min later (n = 9). Data were compared with that from the 100 pmol senktide (OVX) group (n = 5), as per above dose-response experiment.

Effect of a GPR54 agonist on pulsatile LH secretion in the presence of senktide

Senktide (100 pmol in 4 μl of aCSF) and the potent GPR54 agonist, Kp-10 (1 nmol in 4 μl of aCSF; Sigma-Aldrich), were preloaded into microinjection cannulae, with 1 μl of air separating Kp-10 from senktide. OVX + E_2 rats were administered with senktide over 5 min after 1 h 50 min of control blood sampling, followed by Kp-10 10 min later (n = 5). Negative control rats (n = 3) received 400 nl of aCSF, whereas positive controls received 1 nmol Kp-10 in 400 nl of aCSF (n = 3) or 100 pmol senktide in 400 nl of aCSF (n = 3).

Effect of senktide on hypothalamic MUA volleys

After a 10-d period of recovery from electrode implantation, hypothalamic MUA was recorded in OVX rats as described previously (12). Briefly, the electrodes are connected to high-impedance field effect transistors (model E201; Vishay-Siliconix, Bracknell, UK) using a miniconnector (Augat 8058-IG34; Augat, Attleboro, MA) plugged into the socket on the animal's head. The signals were then passed through high-impedance input modules (model HIP511E; Grass Instruments, Quincy, MA) to a bank of amplifiers to provide amplification (×50,000) and filtering (300–1000 Hz bandwidth). MUA discharge frequencies after amplitude discrimination (Brainwaves; DataWave Technologies, Loveland, CO) were processed as activity rates, in 1-min bins, using a data acquisition system (Brainwaves; DataWave Technologies). The discrimination level on the window discriminator was set to give 700-1500 spikes/min. After a control period, during which at least three MUA volleys were observed, senktide was given icv over 5 min (100 or 600 pmol in 4 μl of aCSF), and electrophysiological recording was continued for an additional 5 h (n = 4 per group). Control animals (n = 2) were administered with aCSF (4 μl, icv) as vehicle for senktide, and MUA recording was carried out as described above.

RIA for LH measurement

A double-antibody RIA (19) supplied by the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) was used to determine LH concentrations in the 25-μl whole-blood samples. Referenced preparation was rLH-RP-3. The sensitivity of the assay was 0.093 ng/ml. The intraassay variation was 8.96%, and the interassay variation was 9.01%.

Data analysis

Verification of LH pulses was established by means of the algorithm ULTRA (20). Two intraassay coefficients of variation of the LH RIA were used as the reference threshold for pulse detection. The effect of treatments on pulsatile LH secretion was analyzed by comparing the mean LH pulse interval in the 2-h period preceding treatment, and two consecutive 2-h posttreatment periods. The duration (in minutes) of the 2-h pretreatment, and the first and second 2-h posttreatment periods, was divided

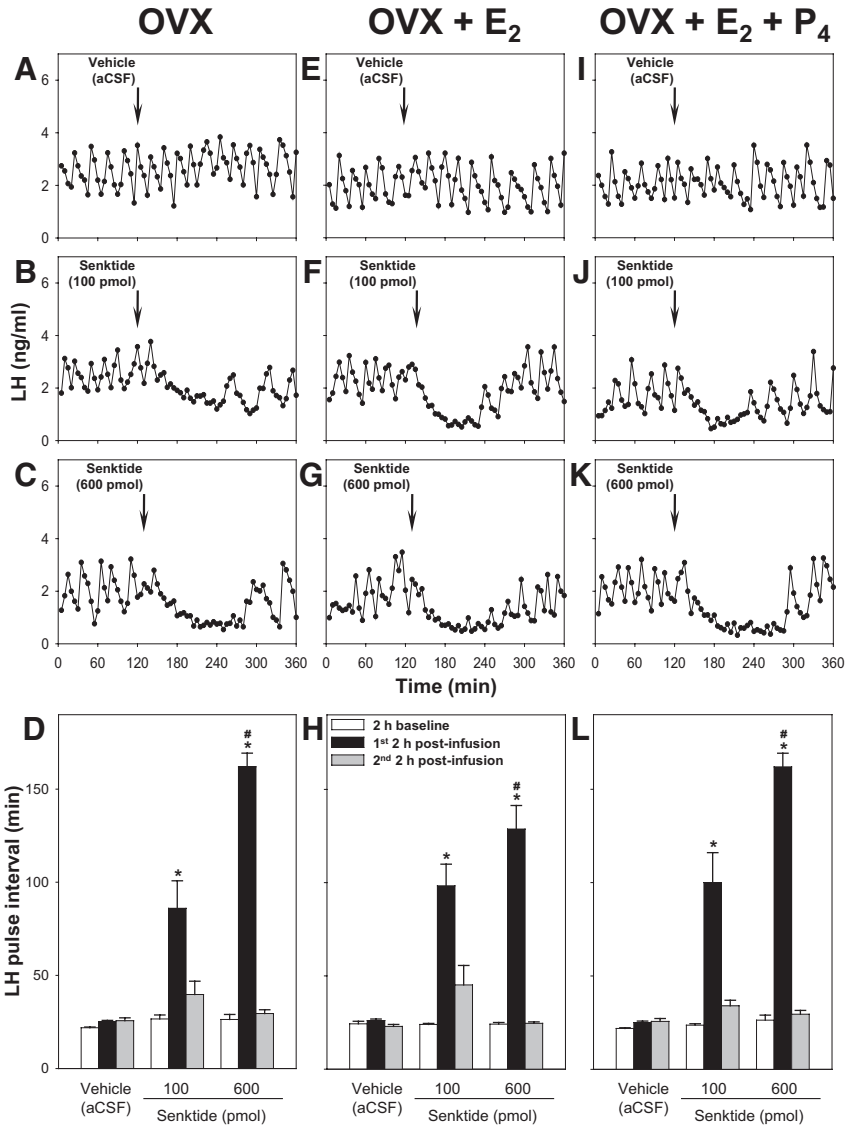

FIG. 1. Effect of senktide on LH pulse frequency. Representative LH profiles illustrating the effect of icv administration (↓) of a selective neurokinin-3 receptor agonist, senktide (B, C, F, G, J, and K), or vehicle [aCSF (A, E, and I)] in OVX (A–C), OVX E_2-replaced (E–G), and OVX E_2- and P_4-replaced (I–K) rats. Central administration of senktide resulted in a dose-dependent suppression of LH pulses regardless of the steroidal milieu, as summarized in D, H, and L. *, $P < 0.05$ *vs.* 2-h baseline control period within the same treatment group, as well as *vs.* the same 2-h period within the vehicle-treated group; #, $P < 0.05$ *vs.* the same 2-h period within the group treated with 100 pmol senktide (n = 4–6 per group).

by the number of LH pulses detected in each of these periods to give the appropriate LH pulse interval. When there were no LH pulses evident during the first 2-h posttreatment period, the LH pulse interval assigned to this period was taken as the interval from the onset of treatment to the first LH pulse in the second 2-h posttreatment period. The significance of the effect of treatments on LH pulse intervals was compared with control animals injected with aCSF, or other appropriate vehicle, at the same time points, as well as to the mean pulse interval during the 2-h pretreatment period. The effect of senktide on LH secretion in gonadal intact rats was calculated by comparing the area under the LH profile [area under the curve (AUC)] in the 1-h period immediately after injection of senktide with that in the 1-h baseline (pretreatment) period immediately before the time of injection within the same treatment group, using SigmaPlot version 11 (Systat Software, San Jose, CA) and were presented as percent of baseline AUC. The effect of senktide on MUA volley frequency was analyzed by comparing the mean time from administration to the next detectable volley (first volley posttreatment) between treatment groups. The effect of sequential administration of senktide and Kp-10 on LH secretion was calculated by comparing the AUC (expressed per hour) in the 2-h pretreatment period with that in the first hour posttreatment, the second 2-h posttreatment, and the fourth hour posttreatment periods; data were binned this way to optimally represent baseline, effect of Kp-10, effect of senktide, and LH pulse recovery, respectively. Values given in the text and figures represent mean ± SEM. Statistical significance was tested using one-way ANOVA and Duncan's *post hoc* (new multiple range) test, unless otherwise indicated. $P < 0.05$ was considered statistically significant.

Results

Effect of senktide on pulsatile LH secretion

Central administration of senktide caused a dose-dependent inhibition of LH pulses in all three groups (OVX, OVX + E_2, and OVX + E_2 + P_4) (Fig. 1). In the OVX group, icv injections of 100 pmol senktide prolonged the LH pulse interval more than 3-fold (preinjection *vs.* postinjection, 26.6 ± 2.2 *vs.* 86 ± 14.7 min; $P < 0.05$), and 600 pmol, 6-fold (preinjection *vs.* postinjection, 26.3 ± 2.7 *vs.* 162 ± 7.4 min; $P < 0.05$) compared with controls, in which treatments with vehicle did not significantly affect the LH pulse frequency (preinjection *vs.* postinjection, 21.9 ± 0.4 *vs.* 25.1 ± 0.8 min; $P > 0.05$). A similar response to senktide was observed in OVX + E_2 and OVX + E_2 + P_4 animals, with 100 pmol injections causing a 4-fold increase in LH pulse interval duration (preinjection *vs.* postinjection, in OVX + E_2 and OVX + E_2 + P_4 rats, 23.9 ± 0.6 *vs.* 98.3 ± 11.6 min and 23.6 ± 0.8 *vs.* 100 ± 16.1 min, respectively; $P < 0.05$); 600 pmol caused a 5-fold LH pulse interval prolongation in OVX + E_2 rats (preinjection *vs.* postinjection, 24.1 ± 10.9 *vs.* 128.8 ± 12.7 min; $P < 0.05$) and a 6-fold prolongation in OVX + E_2 + P_4 rats (preinjection *vs.* postinjection, 26.3 ± 2.7 *vs.* 162 ± 7.4 min; $P < 0.05$).

In each group, 600 pmol senktide had a significantly ($P < 0.05$) stronger inhibitory effect on pulsatile LH secretion than 100 pmol. Vehicle injection had no effect on pulsatile LH secretion in either group (LH pulse interval duration preinjection *vs.* postinjection, in OVX + E_2 and OVX + E_2 + P_4 rats, 24.3 ± 1.4 *vs.* 26.1 ± 0.8 min and 21.9 ± 0.4 *vs.* 25.1 ± 1.5 min, respectively; $P > 0.05$). Furthermore, in all three groups, the LH pulse had a tendency to recover after prolonged pulse suppression due to senktide, with pulse intervals in the OVX, OVX + E_2, and OVX + E_2 + P_4 groups returning to 39.8 ± 7.1, 45.1 ± 10.4, and 34 ± 3 min, respectively, 2 h after treatment with 100 pmol senktide, and to 29.5 ± 2.0, 24.6 ± 0.8, and 29.5 ± 2 min, respectively, 2 h after treatment with 600 pmol senktide.

Interestingly, senktide administered icv to gonadal intact rats in the diestrous phase of the estrous cycle stimulated LH secretion (Fig. 2). Infusion of 600 pmol senktide increased the concentration of circulating LH (percent of baseline AUC after senktide treatment *vs.* that after treatment with vehicle, 145.6 ± 7.4 *vs.* 98.2 ± 5.6%; $P < 0.05$). Senktide increased the mean LH concentration from 0.25 ± 0.01 ng/ml to 0.37 ± 0.02 ng/ml, whereas there was no change in LH concentrations due to vehicle treatment (mean LH concentration preinfusion *vs.* postinfusion of vehicle, 0.26 ± 0.03 *vs.* 0.28 ± 0.04 ng/ml).

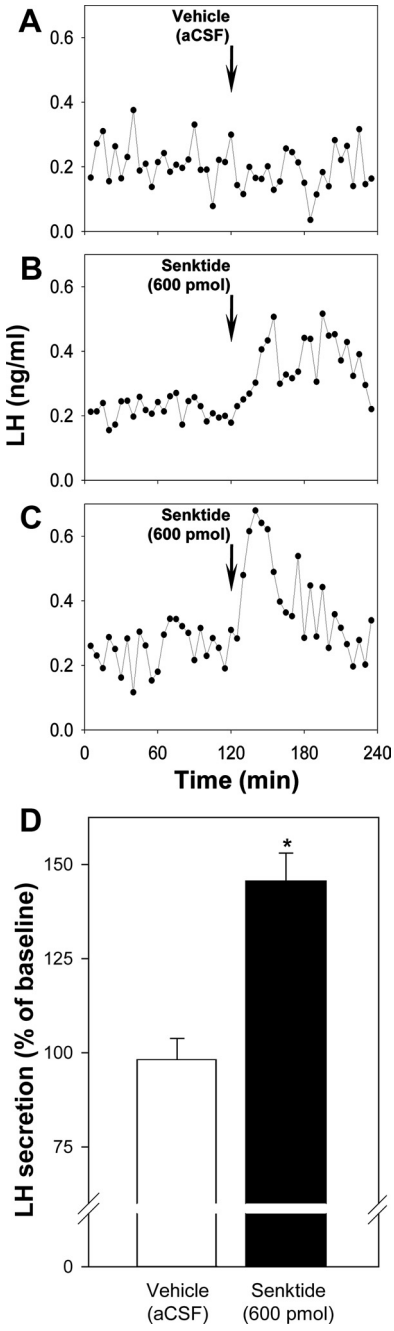

FIG. 2. Effect of senktide on LH secretion in diestrous rats. Representative LH profiles illustrating the effect of icv administration (↓) of a selective neurokinin-3 receptor agonist, senktide (B and C), or vehicle (aCSF) (A) on LH secretion in gonadal intact rats in the diestrus phase of the estrous cycle. Infusion of senktide induced LH secretion, resulting in increased net LH levels after treatment, as summarized in D. *, $P < 0.05$ *vs.* vehicle-treated group (n = 4–6).

Effect of senktide on hypothalamic MUA volleys

Intracerebroventricular administration of senktide caused a dose-dependent suppression of MUA volleys in OVX animals (Fig. 3). Regular MUA volleys were recorded in animals treated with vehicle (MUA volley intervals preinjection *vs.* postinjection, 21.7 ± 1.1 *vs.* 21.1 ± 0.6 min, respectively; $P > 0.05$), as well as during the preinjection periods in the senktide-treated animals (mean MUA volley intervals, 22.4 ± 1.5 min). A period during which no MUA volleys could be detected followed injections of senktide, the length of which was proportional to the dose, whereas no inhibition of MUA volleys was observed after the administration of vehicle (time to first volley posttreatment with vehicle *vs.* 100 pmol senktide *vs.* 600 pmol senktide, 22.1 ± 1.4 *vs.* 90.1 ± 15.6 *vs.* 173.6 ± 10.1 min, respectively; $P < 0.05$).

Effect of senktide on pulsatile LH secretion in the presence of a KOR antagonist

To investigate the interaction between NKB and Dyn, we administered senktide (100 pmol, icv) to rats pretreated (icv) with 6.8 nmol nor-BNI (Fig. 4). Nor-BNI blocked the senktide-induced suppression of pulsatile LH secretion (LH pulse interval in first 2-h postinfusion period in senktide-treated animals *vs.* that in animals pretreated with nor-BNI, 86 ± 14.7 *vs.* 42.2 ± 6 min; $P < 0.05$).

Effect of senktide on pulsatile LH secretion in the presence of a GPR54 agonist

To investigate the interaction between NKB and kisspeptin, we administered Kp-10 to rats pretreated with sen-

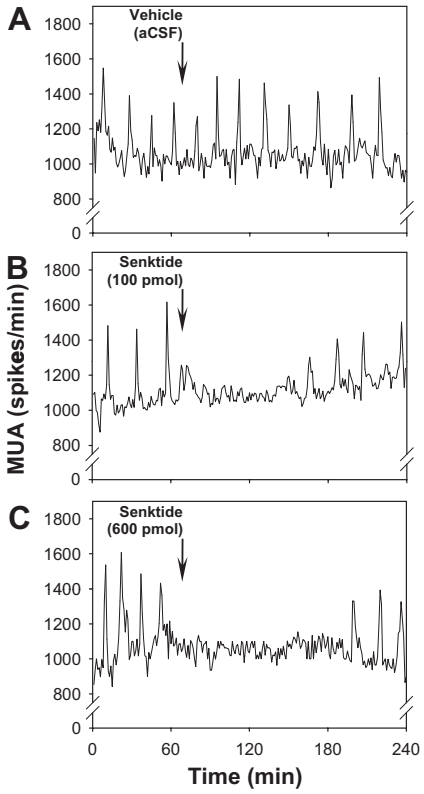

FIG. 3. Effect of senktide on MUA volley frequency in OVX rats. MUA profiles demonstrating the effect icv administration (↓) of a selective neurokinin-3 receptor agonist, senktide (B and C), or vehicle (aCSF) (A) on the electrophysiological correlate of the GnRH pulse generator in OVX rats (n = 2–4 per group).

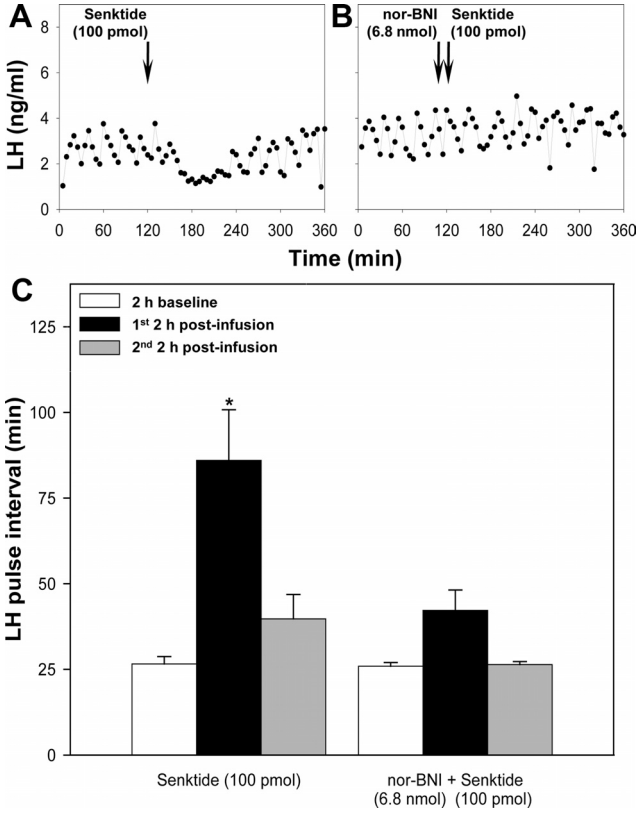

FIG. 4. Effect of senktide on LH secretion in OVX rats after pretreatment with KOR antagonist. Representative LH profiles demonstrating the effect of icv administration (↓) of a selective NK3R agonist, senktide, with (B) or without (A) pretreatment with a selective KOR antagonist, norbinaltorphimine (nor-BNI), on LH secretion in OVX rats. The senktide-induced suppression of LH pulses is blocked by pretreatment with nor-BNI, as summarized in C. *, $P < 0.05$ *vs.* 2-h baseline control period within the same treatment group and *vs.* same 2-h period within the group treated with both nor-BNI and senktide (n = 5–9 per group).

ktide (Fig. 5). In the first hour posttreatment, Kp-10, administered alone, doubled LH secretion compared with vehicle-treated controls (AUC in first h after Kp-10 injection *vs.* that after injection of vehicle, 197 ± 49.7 *vs.* 102.4 ± 9.7 ng/ml · h; $P < 0.05$), whereas single injections of senktide did not significantly influence baseline LH levels (AUC in first hour postinfusion of senktide *vs.* baseline AUC in the same treatment group, 93.4 ± 6.2 *vs.* 110.6 ± 12 ng/ml · h; $P > 0.05$) during this time period. However, in the second and third hour posttreatment, senktide decreased LH secretion 2-fold (AUC in second and third h after senktide injection *vs.* AUC in the same time period in the vehicle-treated group, 46.6 ± 5 *vs.* 103.6 ± 8.2 ng/ml · h; $P < 0.05$), whereas the effect of Kp-10 diminished in this period (AUC in second and third h after Kp-10 injection *vs.* baseline AUC in the same treatment group, 116.5 ± 17.3 *vs.* 102.4 ± 9.7 ng/ml · h; $P > 0.05$). Profiles of LH secretion from animals administered with Kp-10 after pretreatment with senktide demonstrated both the short-term stimulatory and the long-term inhibitory responses to the two agents (baseline AUC *vs.* AUC in first hour posttreatment *vs.* AUC in second and third hour posttreatment, 99 ± 13 *vs.* 208.3 ± 17.9 *vs.* 56.4 ± 3.7 ng/ml · h; $P < 0.05$). In all treatment groups, LH concentrations returned to levels similar to those observed at baseline during the fourth hour posttreatment (AUC in fourth hour postinfusion *vs.* baseline AUC in the same treatment group in vehicle-, Kp-10-, senktide-, and senktide + Kp-10-treated groups: 107.8 ± 7.5 *vs.* 105.4 ± 5.9 ng/ml · h, 102.8 ± 11.8 *vs.* 101.5 ± 1.2 ng/ml · h, 131.1 ± 30.6 *vs.* 110.6 ± 12 ng/ml · h, and 80.6 ± 7.3 *vs.* 99 ± 13 ng/ml · h, respectively).

Discussion

The results of this study demonstrate that central administration of a selective NK3R agonist potently suppressed the frequency of pulsatile LH secretion in the rat in a dose-dependent fashion. They support and extend the findings from several studies that report an inhibitory effect of senktide treatment on circulating LH concentrations in the rat (5) and mouse (6). However, although these previous studies addressed net changes in LH levels after senktide in-

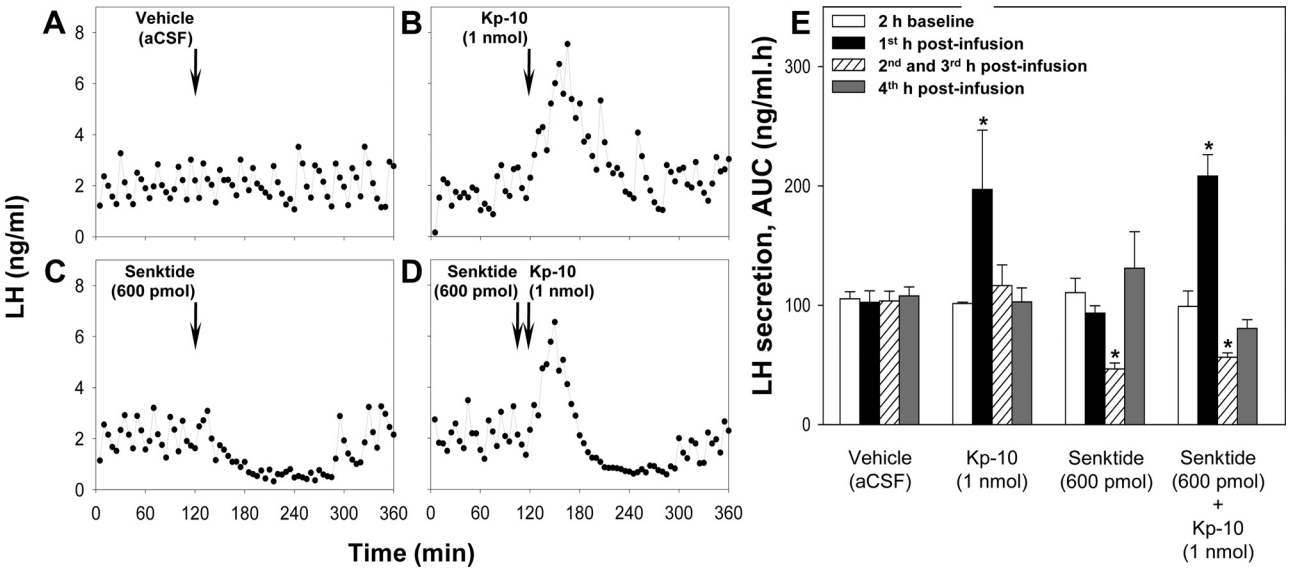

FIG. 5. Effect of GPR54 agonist on LH secretion in ovariectomized estradiol-replaced (OVX + E_2) rats after pretreatment with senktide. Representative LH profiles demonstrating the effect of icv administration (↓) of a potent agonist of GPR54, kisspeptin-10 (Kp-10), with (D) or without (B) pretreatment with the selective neurokinin-3 receptor agonist, senktide, or that of vehicle (aCSF) (A)], or senktide alone (C), on LH secretion in OVX + E_2 rats. E, Summary of experiment. *, $P < 0.05$ *vs.* baseline control period within the same treatment group as well as *vs.* the same period within the vehicle-treated group, paired two-tailed Student's *t* test (n = 3–5 per group).

jection, in the present study, we explored the patterns of pulsatile LH secretion. Our results show that senktide decreased the frequency of LH pulses in a dose-dependent fashion.

We also report that in OVX rats replacement of gonadal steroids did not modulate the effect of senktide on pulsatile LH secretion. The duration of the period after senktide injection devoid of LH pulses was similar in OVX and OVX + E_2 rats. Furthermore, we observed a comparable response to senktide in OVX + E_2 + P_4 rats, suggesting that P_4 does not modulate the effects of NKB on GnRH pulse generator frequency. This is in disagreement with a previous report, in which senktide had an inhibitory effect on LH secretion in OVX rats but stimulated LH release in the OVX + E_2 animals (9). Although there is strong evidence to suggest that NKB neurons are involved in the negative feedback effect of E_2 on gonadotropin release (21), currently, little is known about the ways in which E_2 modulates the actions of NKB. Estrogen receptor α is coexpressed with NKB within ARC kisspeptin neurons (2), and expression of mRNA encoding NKB in the rat ARC is influenced by circulating concentrations of E_2 (9, 22). Indeed, we have replicated the increase in LH secretion after senktide administration to gonadal intact diestrous female rats, as previously reported (9). However, we infer from these experiments that OVX + E_2 rats are not physiologically equivalent to gonadal intact diestrous rats, even if similar circulating E_2 levels are achieved. There are likely to be important differences between the two animal models, which may bring about differential mechanisms that mediate the stimulatory and inhibitory effects of NK3R agonism reported here. On the other hand, the source of the controversy generated by conflicting studies reporting that icv senktide injections administered to OVX + E_2 rats cause elevated LH levels (9) and others showing an inhibition of LH secretion (present study and Ref. 5) may be differences in circulating concentrations of E_2 after steroid replacement resulting from disparities in the design of implantable E_2 capsules between laboratories. Although the E_2 capsules used in our laboratory give rise to higher circulating E_2 levels than those reported by Navarro *et al.* (9), basal LH levels are unaffected by the implantation of OVX rats with these E_2 capsules, as previously reported (15), whereas the capsules used by Navarro *et al.* (9) reduce the basal LH concentrations more than 2.5-fold (despite reportedly producing lower circulating concentrations of E_2), which signifies a stronger physiologic negative feedback effect of E_2 on LH secretion in that study.

Although these findings strongly suggest that NKB modulates the GnRH pulse generator, the most definitive evidence for this was derived from our demonstration that senktide dose dependently inhibits hypothalamic MUA volleys. This is in contrast with data from similar experiments performed in the OVX goat, which also described suppressed LH secretion after injection of senktide, despite being accompanied by a robust rise in MUA volley frequency (4). Species differences have long confounded this field of research. Indeed, there are variations in the localization of NK3R and efficacy of pharmacological

agents between species (23), although differences in the nature, dose, and route of administration of NK3R agonists have also been know to produce disparate results.

Nor-BNI, a selective KOR antagonist, blocked the inhibitory effect of senktide on pulsatile LH secretion. Although Dyn has been well established to inhibit LH secretion (24, 25), this is the first evidence to suggest that the suppression of LH secretion by NKB is Dyn/KOR dependent. Indeed, the patterns of MUA and pulsatile LH release after central administration of Dyn to the OVX goat (4) are strikingly similar to those after icv senktide injection in the OVX rat reported in the present study, and agonists of NK3R and KOR are equally effective at suppressing LH secretion in the mouse (6). Furthermore, we have previously shown that icv administration of nor-BNI alone has no effect on LH pulse frequency (25). It is therefore highly plausible that NKB exerts its suppressive effects on LH secretion by activating auto- and/or paracrine Dyn/KOR signaling cascades within ARC. However, the cellular and molecular mechanisms that NKB employs in modulating opioid activity remain to be elucidated.

The coexpression of NKB and kisspeptin in ARC neurons has sparked a great deal on speculation on the subject of functional interaction between the two neuropeptides in their influence of gonadotropin release (26). We report here that Kp-10 administered icv to OVX + E_2 rats pretreated with senktide did not preclude the ensuing inhibition of pulsatile LH secretion, and neither was the rise in LH levels after Kp-10 injection affected by senktide. Because kisspeptin is able to elicit LH secretion, albeit in a nonpulsatile fashion (11), during the potent suppression of the GnRH pulse generator by senktide, it is likely that the two neuropeptides govern different attributes of the GnRH pulse generator. Although we cannot formally rule out the possibility that the presence of Kp-10 delayed the senktide-induced suppression of pulsatile LH secretion, these data do not advocate an interaction between kisspeptin and NKB. Interplay between ARC kisspeptin and NKB has been suggested in light of marked activation of ARC kisspeptin neurons after icv treatment of OVX + E_2 rats with senktide (9). Previous studies addressing the relationship between NKB and kisspeptin in rodents report that NKB augments the stimulatory effect of kisspeptin on LH secretion, when coadministered icv to gonadal intact male mice (14). However, the same report also features data from a study in which kisspeptin and NKB were applied *ex vivo* to hypothalamic explants from male rats, the results of which suggest rather that NKB abrogates the increase in GnRH secretion associated with kisspeptin (14). The notion of either a synergistic or antagonistic relationship between NKB and kisspeptin is not supported by data presented here, which suggest that, at least under these experimental conditions, neither of the two neuropeptides affects the response of the GnRH pulse generator to the other. Multiple differences in the experimental design are likely to explain the inconsistency of this data with the observations reported here. Given the immediate and transient nature of the effect of kisspeptin on LH secretion, a frequent blood sampling approach is probably better suited to studying the effects of kisspeptin and NKB on LH secretion *in vivo*. In contrast, a study that did address the effects of coadministration of senktide and Kp-10 on pulsatile LH secretion, through experiments in agonadal juvenile male macaques, did provide similar evidence against the interaction between the two neuropeptides (7). Although senktide elicits LH pulses in this animal model, and thus has a stimulatory effect on LH secretion, neither senktide, nor a selective NK3R antagonist (SB222200) affected the kisspeptin-induced rise in LH secretion (7). In summary, data from our and others' experiments employing frequent blood sampling strategies to study the dynamics of pulsatile LH secretion after coadministration of NK3R and GPR54 agonists and antagonists suggest no immediate interaction between NKB and kisspeptin.

The results of this study demonstrate that NKB has an inhibitory effect on hypothalamic MUA volleys and the associated pulsatile LH secretion in OVX rats, both in the presence and absence of E_2, suggesting that NKB suppresses the frequency of the GnRH pulse generator. We provide data that evidence this inhibitory action to be dependent on Dyn/KOR signaling. On the other hand, we confirm that NKB stimulates LH secretion in gonadal intact diestrous rats. Finally, data from our *in vivo* neuropharmacological studies serve as first evidence in the rodent to suggest that kisspeptin and NKB may not interact in modulating the frequency of the GnRH pulse generator but rather control different features of the modulatory input to GnRH neurons.

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