Suppression of the GnRH Pulse Generator by Neurokinin B Involves a \(\kappa\)-Opioid Receptor-Dependent Mechanism


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Neurokinin B (NKB) and its receptor (NK3R) are coexpressed with kisspeptin, Dynorphin A (Dyn), and their receptors [G-protein-coupled receptor-54 (GPR54)] and \(\kappa\)-opioid receptor (KOR), respectively within kisspeptin/NKB/Dyn (KNDy) neurons in the hypothalamic arcuate nucleus (ARC), the proposed site of the GnRH pulse generator. Much previous research has employed intracerebroventricular (icv) administration of KNDy agonists and antagonists to address the functions of KNDy neurons. We performed a series of in vivo neuropharmacological experiments aiming to determine the role of NKB/NK3R signaling in modulating the GnRH pulse generator and elucidate the interaction between KNDy neuropeptide signaling systems, targeting our interventions to ARC KNDy neurons. First, we investigated the effect of intra-ARC administration of the selective NK3R agonist, senktide, on pulsatile LH secretion using a frequent automated serial sampling method to obtain blood samples from freely moving ovariectomized 17\(\beta\)-estradiol-replaced rats. Our results show that senktide suppresses LH pulses in a dose-dependent manner. Intra-ARC administration of U50488, a selective KOR agonist, also caused a dose-dependent, albeit more modest, decrease in LH pulse frequency. Thus we tested the hypothesis that Dyn/KOR signaling localized to the ARC mediates the senktide-induced suppression of the LH pulse by profiling pulsatile LH secretion in response to senktide in rats pretreated with nor-binaltorphimine, a selective KOR antagonist. We show that nor-binaltorphimine blocks the senktide-induced suppression of pulsatile LH secretion but does not affect LH pulse frequency per se. In order to address the effects of acute activation of ARC NK3R, we quantified (using quantitative RT-PCR) changes in mRNA levels of KNDy-associated genes in hypothalamic micropunches following intra-ARC administration of senktide. Senktide down-regulated expression of genes encoding GnRH and GPR54 (\textit{GNRH1} and \textit{Kiss1r}, respectively), but did not affect the expression of \textit{Kiss1} (which encodes kisspeptin). We conclude that NKB suppresses the GnRH pulse generator in a KOR-dependent fashion and regulates gene expression in GnRH neurons. (\textit{Endocrinology} 153: 4894–4904, 2012)
oss of function mutations in genes encoding neuro-
kinin B (NKB), a member of the tachykinin family of
neuropeptides, or its receptor (NK3R) result in hypogo-
nadotropic hypogonadism and infertility (1), which sug-
gests that NKB plays a key role in pulsatile release of
gnRH. The arcuate nucleus (ARC), the proposed site of
the GnRH pulse generator (2, 3), contains a major pop-
ulation of NKB neurons (4), which coexpress kisspeptin
and the κ-opioid receptor (KOR) ligand, dynorphin A
(Dyn) (3, 5–7). Kisspeptin, acting through its putative re-
cipient, GPR54 (G-protein-coupled receptor-54), has been
well characterized as a potent stimulator of the hypo-
thalamopituitary-gonadal (HPG) axis, and intra-ARC ad-
mnistration of a GPR54 antagonist suppresses LH pulses
in rats (3). Kisspeptin/GPR54 signaling is essential for pu-
bertal onset in humans, and kisspeptin-null mice are
infertile and fail to progress through puberty (8, 9),
although a recent study shows normal reproductive de-
vlopment and fertility status in kisspeptin neuron-de-
deleted mice (10). KOR agonists have been shown to
inhibit LH release (7, 11–14). A dense bilateral net-
work of NKB/Dyn axons and close apposition of NKB/Dyn
cell bodies and dendrites have been described within the
ARC (4). Moreover, kisspeptin neurons have been shown
to express NK3R (15) and KOR (4, 6). These data suggest
interplay between the kisspeptin/NKB/Dyn (KNDy) signal-
ing systems, although the mechanisms involved remain largely unknown.

Studies examining the effects of NK3R agonists on LH
secretion have produced conflicting data, with results
varying with animal model and gonadal status. Intrave-
nous administration of the NK3R agonist, senktide, in-
creased LH secretion in agonadal male monkeys (16, 17),
and a similar effect has been observed after central senk-
tide injection in gonadal-intact male mice (18, 19), ovari-
intact rats (20, 21), and follicular phase sheep (22). These
findings may explain the hypogonadotropic hypogonad-
ism phenotype in patients with mutant NKB or NK3R
genes. Although the majority of reports in this field doc-
ument predominantly stimulatory LH responses to senk-
tide administration, recent evidence indicates that activa-
tion of central NK3R inhibits LH secretion under certain
conditions. Intracerebroventricular (icv) administration
of senktide robustly reduced the plasma concentration of
LH in the ovariectomized (OVX) rat (20, 21). Further-
more, we and others have shown that the effect of senktide
on LH secretion in OVX rats implanted with capsules
containing 17β-estradiol (E2) is inhibitory (21, 23), al-
though one study (20) has provided evidence that conflicts
with this notion. In intact rats, senktide induced an in-
crease in LH release during diestrus (20, 21) and proestrous
(20) phases of the estrous cycle. Also, although senktide
stimulates LH secretion in male mice (18, 19), its effect
on LH secretion in OVX females is inhibitory (6). In contrast,
senktide had no measurable effect on LH secretion in the
OVX, E2-replaced (OVX + E2) mouse (6). In the ewe, icv
administration of senktide increased LH concentrations in
the follicular phase, but had no effect in the luteal phase
(22), and in OVX goats, icv injection of senktide inhibited
LH secretion, but increased the frequency of ARC multi-
unit activity (MUA) volleys, an electrophysiological cor-
relate of the GnRH pulse generator (7). Taken together,
this evidence explains why a consensus on the involvement
of NKB/NK3R signaling in the regulation of GnRH neu-
rosecretion has, to date, not been reached.

A dense bilateral plexus of NKB/Dyn neurons projects
to the median eminence (ME), where they form numerous
close appositions to termini of GnRH axons (4, 15, 24).
Therefore, NKB neurons might influence GnRH neuro-
secretion directly through NK3R in the ME. However,
ARC NKB neurons also project to the medial preoptic area
(mPOA), where GnRH perikarya are located, as well as to
the anteroventral periventricular (AVPV) nucleus (15),
which contains kisspeptin neurons involved in the gener-
ation of the E2-induced preovulatory LH surge (23) and
the control of GnRH neuron excitability (26). Taken to-
gether, these data provide the basis for the hypothesis that
NKB neurons originating in the ARC modulate GnRH
secretion via multiple neural pathways.

The purpose of the present study was to examine the
dynamics of pulsatile LH secretion in female rats in re-
sponse to central injection of an NK3R agonist in a range
of steroid hormone environments and to establish the
functional relationship between NKB, Dyn, and kisspept-
in. First, we tested the hypothesis that activation of ARC
NK3R inhibits pulsatile GnRH secretion by assessing the
effect of localized intra-ARC microinfusion of senktide on
LH pulse frequency. We then determined whether NKB
and Dyn tones endogenous to the ARC sustain episodic
LH release and the etiology of the functional hierarchy
between NKB and Dyn in their inhibitory influence on the
GnRH pulse generator. Finally, we postulated that ARC
NKB neurons projecting to other hypothalamic regions
may alter gene expression in effector neurons as part of the
mechanism by which NKB mediates its effects on LH se-
cretion. We therefore measured the mRNA levels of po-
tential target genes in mPOA, including the AVPV.

Materials and Methods

Animals and surgical procedures

Adult female Sprague Dawley rats (200–250 g) obtained
from Charles River (Margate, UK), were housed under con-
controlled conditions (12-h light, 12-h dark cycle; lights on 0700 h; temperature, 22 ± 2 °C; food and water ad libitum). All animal procedures were undertaken in accordance with the 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; Pharmacia and Upjohn, Crawley, UK) and xylazine (Rompun, 10 mg/kg, ip; Bayer, Leverkusen, Germany).

Two weeks before experiments took place rats were bilaterally OVX and implanted with either one (OVX + 1×E2) or two (OVX + 2×E2) subcutaneous E2-filled SILASTIC (Dow Corning Corp., Midland, MI) capsules, as described previously (21). At the time of ovariectomy, all rats were also fitted with a bilateral guide cannula (22 gauge; Plastics One, Roanoke, VA) directed toward the ARC, the stereotaxic coordinates for implantation being 0.5 mm lateral, 2.8 mm posterior to bregma, and 9.4 mm below the surface of the dura (27). The guide cannula was secured using dental cement (Dental Filling, Swindon, UK) and fitted with a dummy cannula (Plastics One) to maintain patency (28). A stainless steel slotted screw (Instec Laboratories, Boulder, CO) was affixed to the surface of the skull posterior to the guide cannula using dental cement. After a 10-d recovery period, the rats were fitted with two custom-made jugular catheters, which were exteriorized at the back of the head and enclosed within a 30-cm metal spring tether (Instec Laboratories) secured to the slotted screw (29). The distal end of the tether was attached to a two-channel fluid swivel (Instec Laboratories), which allowed the rat freedom to move around the enclosure (29). Experimentation commenced 3 d later.

Blood samples (300 μl) for the measurement of plasma E2 were obtained manually via the jugular catheters. One sample was obtained on the day of iv cannulation (10 d after OVX), and a further blood sample was collected on the day animals were killed (17 d after OVX). Plasma was stored at −20 C for later RIA to determine E2 concentrations. Vaginal cytology was assessed every 2 d. An additional group of ovary-intact rats monitored daily for normal ovarian cyclicity by means of vaginal cytology (n = 8) or vehicle (n = 10). Injections were administered over 5 min after 2 h of control blood sampling.

Effect of US0488 on pulsatile LH secretion

OVX + 1×E2 rats were used in the following experiment. Injection cannulas were preloaded with US0488 [500 pmol in 400 nl (n = 3) or 5 nmol in 400 nl (n = 6); Tocris] or vehicle (400 nl aCSF; n = 10). Injections were administered over 5 min after 2 h of control blood sampling.

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

OVX + 1×E2 rats were used in the following experiment. Senktide (1 pmol in 400 nl aCSF) and the selective NK3R antagonist, SB222200 [500 pmol in 400 nl aCSF/15% dimethylsulfoxide (DMSO)/20% cyclodextrin; Tocris], were preloaded into the microinjection cannulas, with 200 nl air separating SB222200 from senktide. SB222200 was administered over 5 min after 1 h 45 min of control blood sampling, followed by senktide 15 min later. Negative control rats (n = 3) received 400 nl aCSF/15% DMSO/20% cyclodextrin, whereas positive controls (n = 5) received 500 pmol SB222200 in 400 nl aCSF/15% DMSO/20% cyclodextrin.

Effect of senktide on pulsatile LH secretion

OVX + 1×E2 rats were used in the following experiment. Injection cannulas were preloaded with US0488 [500 pmol in 400 nl aCSF (n = 5) or 5 nmol in 400 nl aCSF (n = 6); Tocris] or vehicle (400 nl aCSF; n = 10). Injections were administered over 5 min after 2 h of control blood sampling.

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

OVX + 1×E2 rats were used in the following experiment. Senktide (1 pmol in 400 nl aCSF) and the selective KOR antagonist, nor-binaltorphimine (nor-BNI, 1 nmol in 400 nl aCSF; Tocris), were preloaded into microinjection cannulas, with 200 nl air separating nor-BNI from senktide. Nor-BNI was administered over 5 min after 1 h 45 min of control blood sampling, followed by senktide 15 min later (n = 7). Negative control rats (n = 7) received 400 nl aCSF, whereas positive controls (n = 8) received 1 nmol nor-BNI in 400 nl aCSF.

Effect of senktide on mPOA expression of Kiss1, Kiss1r, and GNRH1 mRNA

OVX + 1×E2 rats were used in the following experiment. To investigate the effect of intra-ARC administration of senktide on hypothalamic Kiss1/Kiss1r and GNRH1 gene expression, injection cannulas were preloaded with senktide or aCSF as described above. Animals were left undisturbed overnight. On the morning of experimentation rats received a single dose of 10 pmol senktide in 400 nl aCSF (n = 8). Control rats (n = 6) received 400 nl aCSF. Rats were decapitated 6 h after drug administration; brains were then collected and snap frozen on dry ice. Brains were stored at −80 C. The rationale for the collection of brain tissue at 6 h after treatment for examining expression of Kiss1, Kiss1r, and GNRH1 mRNA was based on our previous experience examining the effects of stress and CRF on the expression levels of these genes (30).
Tissue collections and quantitative RT-PCR (qRT-PCR)

Expression of Kiss1, Kiss1r and GNRH1 mRNA was determined by a real-time qRT-PCR in the mPOA. Brain sections (300 μm) were cut on a cryostat (Bright, Cambridgeshire, UK), and bilateral punches (1 mm diameter) of the mPOA, which included the AVPV, were taken from bregma +0.2 to −0.4 mm (27) following the micropunch method of Palkovits (31). Total RNA was extracted from the microdissected mPOA tissue for each rat using TRI reagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer’s protocol. RT was then carried out using the reverse transcriptase Superscript II (Invitrogen, Carlsbad, CA) and random primer following the manufacturer’s instructions.

For the qRT-PCR, the primers used were: Kiss1 (sense): 5’-AGCTGCTGCTTCTC TCTGT-3’; Kiss1 (antisense): 3’-AGCTGCTCCTGTGACATACC-5’; Kiss1r (sense): 5’-GGGTGCTGGGAGACTTCATGT-3’; Kiss1r (antisense): 3’-AGTGCGCATGTGGCTTG-5’; GNRH1 (sense): 5’-GCCGCTGTGTTCTCCTGACT-3’; GNRH1 (antisense): 3’-TTCCCTCTTCAATCGAGGTTCCTTGACAGC-5’; HPRT1 (sense): 5’-GCCGCTGTGTTCTCCTGACT-3’; HPRT1 (antisense): 3’-CGAGA GGTCTCCTTGACAGC-5’. The primer pairs selected for Kiss1, Kiss1r, and GNRH1 detection were designed as described previously (30). Rotorgene 6000 (QIAGEN, Crawley, UK) was used for real-time quantitative analysis of Kiss1, Kiss1r, and GNRH1 mRNA expression as described previously (30). Quantification of Kiss1/Kiss1r/ GNRH1 mRNA was carried out for each sample with HPRT1 mRNA quantified as a reference gene against a separate standard curve of samples containing known concentrations of HPRT1 mRNA product. Values are expressed as ratios of Kiss1/Kiss1r/ GNRH1 mRNA to HPRT1 mRNA PCR product.

RIA for hormone measurement

A double-antibody RIA supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) supplied by the NIDDK (Bethesda, MD) was used to determine LH concentrations in the 25-μl whole-blood samples (32). Referenced preparation was rLH-RP-3. The sensitivity of the assay was 0.093 ng/ml. The intraassay variation was 7.89%, and the interassay variation was 9.41%.

A double-antibody RIA (ImmuChem; MP Biomedicals, Orangebury, NY) was used to estimate the E2 content of the plasma samples following the manufacturer’s protocol. Total plasma steroids were not extracted before analysis. The sensitivity of the assay was 7.2 pg/ml. The intraassay variation was 10.2%, and the interassay variation was 11.9%.

Data analysis

Detection of LH pulses was established through the use of the algorithm ULTRA (33). The effect of intra-ARC infusion of KNDy agonists and antagonists 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 period duration (in minutes) was divided 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, alone at the same time points, as well as to the mean pulse interval during the 2-h pre-treatment period. Values given in the text and figures represent mean ± SEM. Statistical significance was tested using one-way ANOVA and Duncan’s New Multiple Range post hoc test. P < 0.05 was considered statistically significant.

Mean basal LH levels were calculated for LH concentrations in the 2-h period preceding treatment and were compared between groups implanted with either one or two E2 capsules. E2 levels in OVX + 1×E2 and OVX + 2×E2 were calculated from means of E2 levels detected 10 and 17 d after OVX, because these did not differ significantly (P > 0.05, Student’s t test). E2 levels in diestrous rats were calculated from means of E2 levels detected during diestrus in three consecutive estrous cycles. Frequency of epithelial cornification in OVX and OVX + 2×E2 rats was calculated as a function of total vaginal smears obtained, and expressed as a percentage. Mean epithelial cornification frequencies were calculated for the OVX + 1×E2 and OVX + 2×E2 groups. The significance of the effect of senktide on Kiss1, Kiss1r, and GNRH1 mRNA expression was compared with control animals injected with aCSF. Values given in the text and figures represent mean ± SEM. Statistical significance was tested using Student’s t test. P < 0.05 was considered statistically significant.

Results

Plasma E2, mean LH levels, and vaginal cytology

The circulating levels of E2 in OVX + 2×E2 rats were more than double those in OVX + 1×E2 rats (83.3 ± 14.2 pg/ml vs. 35.9 ± 1.7 pg/ml; P < 0.05). Although circulating levels of E2 in OVX + 1×E2 rats were significantly lower than in diestrous rats (35.9 ± 1.7 pg/ml vs. 96.4 ± 28.9 pg/ml; P < 0.05), E2 levels in OVX + 2×E2 rats were not significantly different from those in diestrous rats (83.3 ± 14.2 pg/ml vs. 96.4 ± 28.9 pg/ml; P > 0.05). Mean basal LH levels were significantly (36%) lower in OVX + 2×E2 rats than in OVX + 1×E2 rats (3.42 ± 0.34 ng/ml vs. 2.51 ± 0.27 ng/ml; P < 0.05). Cornification of vaginal epithelia was significantly more prevalent in OVX + 2×E2 rats than in OVX + 1×E2 rats (62.3 ± 8.9% vs. 36.3 ± 16.5%; P < 0.05).

Effect of senktide on pulsatile LH secretion

Direct intra-ARC administration of senktide caused a dose-dependent inhibition of LH pulses (Fig. 1). Injections of 0.1 pmol senktide doubled the duration of the LH pulse interval (2 h preinjection vs. 1st 2 h postinjection, 23.3 ± 0.7 min vs. 47.6 ± 11.0 min; P > 0.05), whereas 1 pmol senktide injections caused a more than 4-fold prolongation of the LH pulse interval (20.3 ± 0.5 min vs. 88.3 ± 12.2 min; P < 0.05). Senktide (10 pmol) suppressed LH pulses for more than 2 h in both OVX + 1×E2 (23.3 ± 1.3 min vs. 157.0 ± 20.0 min; P < 0.05) and OVX + 2×E2 rats (22.2 ± 0.8 min vs. 158.5 ± 8.3 min; P < 0.05). In the
control group, injections of vehicle did not significantly affect the LH pulse frequency in either OVX + 1×E₂ (24.3 ± 1.9 min vs. 23.5 ± 4.1 min; P > 0.05) or OVX + 2×E₂ (21.5 ± 1.1 min vs. 24.3 ± 1.9 min; P > 0.05) rats. Furthermore, the LH pulse in both OVX + 1×E₂ and OVX + 2×E₂ rats had a tendency to recover after suppression due to senktide, with pulse intervals returning to 28.8 ± 1.8 min 21.2 ± 1.9 min, 20.4 ± 1.0 min and 25.3 ± 1.6 min within the second 2-h period after treatment with 0.1 pmol, 1 pmol, and 10 pmol senktide in OVX + 1×E₂ rats and 10 pmol senktide in OVX + 2×E₂ rats, respectively. To localize the observed effect of senktide to ARC NK3R, we administered senktide (1 pmol, intra-ARC) to OVX + 1×E₂ animals pretreated with SB222200 (500
pmol, intra-ARC). SB222200 completely blocked the inhibitory effect of senktide on LH secretion (23.2 ± 1.7 min vs. 23.1 ± 1.5 min; P > 0.05), without affecting LH pulse frequency when administered alone (23.0 ± 1.1 min vs. 24.3 ± 2.2 min; P > 0.05).

Effect of U50488 on pulsatile LH secretion
To investigate whether Dyn and NKB suppress the HPG axis in a similar fashion, we administered U50488 (500 pmol or 5 nmol) intra-ARC to OVX + 1×E2 rats. U50488 decreased the LH pulse frequency in a dose-dependent fashion (Fig. 2). Whereas 500 pmol increased the duration of the LH pulse interval by 72% (2 h preinjection vs. first 2 h after injection, 22.2 ± 1.4 min vs. 38.2 ± 4.2 min; P < 0.05), 5 nmol more than doubled the basal LH pulse interval duration (21.9 ± 1.4 min vs. 45.4 ± 4.6 min; P < 0.05). The LH pulse had a tendency to recover gradually toward basal frequency within 2 h of administration of 500 pmol U50488, whereas LH pulse frequency recovery in rats treated with 5 nmol U50488 was comparatively slower.

Effect of senktide on pulsatile LH secretion in the presence of nor-BNI
To investigate the interaction between NKB and Dyn, we administered senktide (1 pmol, intra-ARC) to OVX + 1×E2 rats pretreated (intra-ARC) with 1 nmol nor-BNI (Fig. 3). Nor-BNI blocked the senktide-dependent LH pulse suppression (first 2 h after injection, senktide vs. senktide + nor-BNI, 88.3 ± 12.2 min vs. 33.1 ± 4.2 min; P < 0.05). Neither vehicle nor nor-BNI alone had an effect on LH pulse frequency (2 h preinjection vs. first 2 h after injection, 24.2 ± 1.3 min vs. 24.4 ± 1.9 min, and 24.9 ± 1.2 min vs. 25.5 ± 1.2 min, respectively; P > 0.05).

Effect of senktide on mPOA/AVPV expression of Kiss1, Kiss1r, and GNRH1 mRNA
We measured Kiss1, Kiss1r, and GNRH1 mRNA expression using qRT-PCR in mPOA/AVPV punches microdissected from rats treated (intra-ARC) with vehicle or 10 pmol senktide (Fig. 4). Senktide did not have a significant effect on Kiss1 expression (vehicle vs. senktide, 0.13 ± 0.03 vs. 0.15 ± 0.07; P > 0.05). By contrast, senktide significantly inhibited the expression of Kiss1r (vehicle vs. senktide, 0.13 ± 0.01 vs. 0.08 ± 0.01; P < 0.05) and GNRH1 (vehicle vs. senktide, 0.29 ± 0.02 vs. 0.21 ± 0.01; P < 0.05).

Discussion
In the present study we provide the first direct evidence that ARC KNDy neurons regulate the frequency of the
GnRH pulse generator. We have demonstrated that intra-ARC administration of senktide resulted in a dose-dependent reduction of LH pulse frequency in the OVX + E2 rat. We (21) and others (23) have previously reported that icv administered NK3R agonists have a suppressive effect on LH secretion in this animal model; however, this is the first evidence to show that this effect is indeed due to agonism local to ARC NK3R. These findings are consistent with the observation that icv administration of senktide prolongs the interval between MUA volleys recorded from the ARC in OVX rats (21), which consolidates the notion that NKB modulates LH secretion through altering GnRH pulse generator frequency. Although we cannot formally rule out drug diffusion from the intended infusion site in the ARC to the ME, such an effect is improbable because, at least in rodents, a barrier of tanyocyte processes bound by tight and adherens junctions separates the intercellular milieu of the ARC from that of the ME (34). Interestingly, neither iv administration of 0.65 or 65 nmol/kg senktide to OVX + E2 rats (Grachev P., X. F. Li, and K. T. O’Byrne, unpublished observation), nor ip administration of NKB to male mice (35), affected LH secretion; thus the ME is unlikely to mediate the effects of central NK3R agonism on the HPG axis. Additionally, injections of senktide administered via misplaced cannulas (dorsal or lateral with respect to the ARC) did not affect LH secretion, suggesting that other adjacent hypothalamic nuclei are also not implicated in the reported response to senktide.

Our previous data indicate that icv administration of senktide suppressed pulsatile LH secretion with similar dynamics in the absence (OVX) and presence (OVX + E2) of E2 (21). In the present study we have confirmed that doubling the E2 replacement from one to two E2 capsules, which produced E2 levels indistinguishable from those measured in the diestrus phase of the estrous cycle, does not affect the suppression of LH secretion due to activation of ARC NK3R. Interestingly, the single E2 capsule replacement regimen produced circulating concentrations of E2 comparable to those previously reported in diestrous rats (36). This difference may be explained by the high variability and limited accuracy of current commercially available RIA kits to measure E2 concentrations in vivo (37). Nevertheless, implantation of two E2 capsules (OVX + 2×E2) more than doubled the circulating E2 levels observed in OVX + 1×E2 animals and produced a significant decrease (36%) in mean basal LH levels. Further, assessment of vaginal cytology confirmed more prevalent epithelial cell cornification in OVX + 2×E2 rats than in OVX + 1×E2 rats, indicative of higher E2 levels in these animals. Taken together, these data suggest that the two E2 replacement regimens produced circulating E2 levels that are probably within the physiological range observed during the diestrous phase of the estrous cycle.

Although NK3R agonists consistently stimulate LH secretion in gonadal intact mice (18, 19), rats (20, 21), and follicular phase ewes (22), and consistently suppress LH secretion in OVX mice (6), rats (20, 21, 23), and goats (7), there are inconsistencies in the response to activation of NK3R in OVX E2-replaced animals ranging from no effect in mice (6) and goats (7) to inhibition (21, 23) or stimulation (20) in rats. Interestingly, in the rat the switch from

FIG. 3. Effect of NK3R agonist on LH pulse frequency after pretreatment with KOR antagonist. Representative LH profiles demonstrating the effect of intra-ARC administration (↓) of a selective NK3R agonist, senktide, with (D) or without (C) pretreatment with a selective KOR antagonist, nor-BNI, or that of vehicle (A), or nor-BNI alone (B), on pulsatile LH secretion in OVX rats implanted with a single E2 capsule. Central administration of senktide resulted in a suppression of LH pulses; this effect was attenuated by pretreatment with nor-BNI, as summarized in panel E. *, 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, and vs. the same 2-h period within the group treated with both, nor-BNI and senktide; n = 7–8 per group.
an inhibitory to a stimulatory effect of senktide on LH secretion was evident in OVX animals that received E2 replacement producing circulating E2 levels equivalent to the proestrous phase (20). These data suggest that the gonadal steroid milieu, in particular E2, is critical in determining the response of the GnRH pulse generator to NK3R activation. Although the physiological relevance of the inhibitory LH response to NK3R activation in OVX animals in the absence of E2 replacement or in the presence of low physiological levels of E2 remain to be established, there is a precedence from many signaling molecules exerting an inhibitory influence on the GnRH pulse generator in OVX animals that is robustly reversed by treatment with E2 (38). Indeed there may be important physiological (e.g. menopause) or pathological (e.g. amenorrhea, infertility, etc.) hypoestrogenic states that may uncover the importance of the inhibitory effects of NKB/NK3R signaling on the HPG axis.

We provide the first evidence that ARC KOR agonism also decreases LH pulse frequency in a dose-dependent fashion, although with considerably lower potency than activation of ARC NK3R by senktide. There is a substantial body of morphological evidence that confirms a high degree of coexpression between NKB and Dyn within ARC KNDy neurons in a variety of mammalian species, including mouse (6), rat (4), ewe (3), and goat (7), as well as in humans and nonhuman primates (39). The same neurons have also been shown to express NK3R and KOR (4, 6). These observations reveal the feasibility of autocrine, juxtacrine, and/or paracrine signaling mechanisms by which NKB may recruit Dyn to activate KOR, and thus suppress the GnRH pulse generator and, therefore, pulsatile LH secretion. Indeed, our results showed that intra-ARC pretreatment with nor-BNI robustly blocked the senktide-induced inhibition of LH pulses. From this it can be inferred that the inhibitory effect of NKB on LH secretion is dependent on Dyn/KOR signaling. We have recently shown this to be the case through similar experiments employing icv administration of pharmacological agents (21), although by localizing drug administration we demonstrate here that the NKB-Dyn interaction, which results in the suppression of pulsatile LH secretion, is occurring within the ARC.

Antagonism of ARC NK3R had no effect on LH secretion, which is commensurate with recent findings that subtype-specific antagonists of neurokinin (NK) receptors (NK1R, NK2R, and NK3R) administered singly icv did not affect LH secretion in OVX rats (40). Antagonism of KOR in the ARC did not affect LH pulse frequency either. This concurs with our previous reports of a lack of effect on pulsatile LH secretion after icv delivery of nor-BNI (14, 21). It has, however, been demonstrated that icv administration of nor-BNI increases LH pulse frequency and net LH secretion, as well as the frequency of ARC MUA volleys in OVX goats (7). Species differences may be accountable for this disparity of data. In contrast to the goat, the OVX rat appears to be devoid of endogenous NKB or Dyn tones, which are apparently not essential for physiological pulsatile LH release, at least under these experimental conditions.

We used qRT-PCR to quantify the relative levels of mRNA expression of several genes known to be associated with the regulation of the HPG axis, in the preoptic region, after intra-ARC administration of senktide. Our results show that senktide down-regulated GNRH1 expression, which implies that ARC NKB/Dyn neurons may suppress GnRH secretion directly or indirectly by inhibiting GNRH1 transcription. Furthermore, expression of Kiss1r mRNA in the mPOA/AVPV was suppressed by intra-ARC senktide treatment, suggesting another mechanism of NKB-driven inhibition of GnRH secretion. Our results also indicate that intra-ARC injection of senktide did not
affect the expression of Kiss1 mRNA in this region of the hypothalamus, suggesting that ARC KNDy projections to the mPOA (15) may target GnRH neurons, rather than kisspeptin neurons of the AVPV. These are the first findings that hint at multiple mechanisms by which NKB exerts its effects on the HPG axis.

The effects of ARC NK3R agonism on gene expression in the mPOA are unlikely to be mediated by NKB at the level of GnRH somata because there is no discernible effect of senktide on the firing rate of GnRH neurons (19), and they apparently lack NK3R expression (19, 41), although low levels of NK3R staining have been reported in GnRH somata within the organum vasculosum of the lamina terminalis in rats (24). It is important to appreciate, however, that the down-regulation of GNRH1 mRNA in response to senktide may, in fact, be secondary to the decrease in LH pulse frequency and, consequently, diminished activity of GnRH neurons and peptide release. An additional caveat concerns the limitation of the time point used for gene expression analysis to explain the observed effects on LH secretion, with a need for future experiments to quantify gene expression in tissue collected at earlier time points concomitant with the period of LH pulse suppression. Future studies will also need to address changes in gene expression within the ARC and ME in response to intra-ARC administration of NK3R agonists to fully characterize the roles of hypothalamic NKB/NK3R signaling. Unfortunately, brain sections containing these loci were used for the confirmation of correct cannula placement and were therefore unsuitable for gene expression analysis. Previous studies in the rat (21) and monkey (17, 18), aiming to elucidate the functional relationship between NKB and kisspeptin in the context of their effects on the GnRH pulse generator, have shown that, because the kisspeptin-induced rise in LH levels is unaffected by senktide, an interaction between these neuropeptides is unlikely, and that they perhaps control different attributes of the GnRH pulse generator. The suppression of mPOA Kiss1r mRNA expression by senktide demonstrates an indirect interaction between the NKB/NK3R and kisspeptin/GPR54 signaling systems.

We have recently reported that icv senktide administration robustly elicits single LH pulses in a GPR54-dependent fashion in ovary-intact prepubertal rats (42). Furthermore, neither augmentation nor restriction of the Dyn tone affected the senktide-induced LH pulses (42). These findings, together with those presented herewith, somewhat deconvolute the current argument of stimulatory vs. inhibitory effects of central NK3R activation on the HPG axis, by providing direct evidence for a NKB-kisspeptin (non-Dyn) interaction involved in stimulating LH secretion under stronger negative-feedback effects of E2, as well as a NKB-Dyn (nonkisspeptin) interaction that suppresses LH secretion when E2 negative-feedback is considerably weaker. Elucidation of the mechanism by which varying E2 levels modulate the apparently biphasic NKB/NK3R signaling system will require further studies.

The present study enhances the current understanding of the roles of NKB/NK3R signaling in the regulation of the GnRH pulse generator by demonstrating that 1) local activation of ARC NK3R suppresses pulsatile LH secretion in OVX rats replaced with a range of low doses of E2; and 2) this effect is dependent upon Dyn/KOR signaling in the ARC; 3) endogenous NKB and Dyn tones do not sustain pulsatile LH secretion under these conditions; and 4) agonism of ARC NK3R brings about long-term suppression of Kiss1r and GNRH1 mRNA expression in GnRH somata of the mPOA.

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