Suppression of the GnRH Pulse Generator by Neurokinin B Involves a κ-Opioid Receptor-Dependent Mechanism

P. Grachev, X. F. Li, J. S. Kinsey-Jones, A. L. di Domenico, R. P. Millar, S. L. Lightman, and K. T. O'Byrne

Division of Women's Health (P.G., X.F.L., J.S.K.-J., A.L.D., K.T.O), School of Medicine, King's College London, London, SE1 1UL, United Kingdom; Centre for Integrative Physiology (R.P.M.), University of Edinburgh, Edinburgh, Scotland, EH8 9XD, United Kingdom; Mammal Research Institute (R.P.M.), University of Pretoria, Zoology & Entomology, Pretoria 0001, South Africa; UCT/MRC Research Group for Receptor Biology (R.P.M.), Division of Medical Biochemistry, Faculty of Health Sciences, University of Cape Town, Private Bag X3, Cape Town 7935, South Africa; and Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology (S.L.L.), University of Bristol, Bristol, BS1 3NY, United Kingdom

Neurokinin B (NKB) and its receptor (NK3R) are coexpressed with kisspeptin, Dynorphin A (Dyn), and their receptors [G-protein-coupled receptor-54 (GPR54)] and κ-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 17β-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 (*GNRH1* and *Kiss1r*, respectively), but did not affect the expression of *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. **(*Endocrinology* 153: 4894–4904, 2012)**

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2012-1574 Received May 29, 2012. Accepted July 24, 2012.
First Published Online August 17, 2012

Abbreviations: aCSF, Artificial cerebrospinal fluid; ARC, arcuate nucleus; AVPV, anteroventral periventricular; DMSO, dimethylsulfoxide; Dyn, dynorphin A; E_2, 17β-estradiol; GPR54, G-protein-coupled receptor-54; HPG, hypothalamopituitary-gonadal; icv, intracerebroventricular; KOR, κ-opioid receptor; KNDy, kisspeptin/NKB/Dyn; ME, median eminence; mPOA, medial preoptic area; NKB, neurokinin B; NK3R, NKB receptor; nor-BNI, nor-binaltorphimine; OVX, ovariectomized.

Loss of function mutations in genes encoding neurokinin B (NKB), a member of the tachykinin family of neuropeptides, or its receptor (NK3R) result in hypogonadotropic hypogonadism and infertility (1), which suggests 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 population 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 receptor, GPR54 (G-protein-coupled receptor-54), has been well characterized as a potent stimulator of the hypothalamopituitary-gonadal (HPG) axis, and intra-ARC administration of a GPR54 antagonist suppresses LH pulses in rats (3). Kisspeptin/GPR54 signaling is essential for pubertal onset in humans, and kisspeptin-null mice are infertile and fail to progress through puberty (8, 9), although a recent study shows normal reproductive development and fertility status in kisspeptin neuron-depleted mice (10). KOR agonists have been shown to inhibit LH release (7, 11–14). A dense bilateral network 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) signaling 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. Intravenous administration of the NK3R agonist, senktide, increased LH secretion in agonadal male monkeys (16, 17), and a similar effect has been observed after central senktide injection in gonadal-intact male mice (18, 19), ovary-intact rats (20, 21), and follicular phase sheep (22). These findings may explain the hypogonadotropic hypogonadism phenotype in patients with mutant NKB or NK3R genes. Although the majority of reports in this field document predominantly stimulatory LH responses to senktide administration, recent evidence indicates that activation 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). Furthermore, we and others have shown that the effect of senktide on LH secretion in OVX rats implanted with capsules containing 17β-estradiol (E_2) is inhibitory (21, 23), although one study (20) has provided evidence that conflicts with this notion. In intact rats, senktide induced an increase in LH release during diestrus (20, 21) and proestrus (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, E_2-replaced (OVX + E_2) 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 multiunit activity (MUA) volleys, an electrophysiological correlate 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 neurosecretion 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 neurosecretion 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 generation of the E_2-induced preovulatory LH surge (25) and the control of GnRH neuron excitability (26). Taken together, 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 response to central injection of an NK3R agonist in a range of steroid hormone environments and to establish the functional relationship between NKB, Dyn, and kisspeptin. 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 secretion. We therefore measured the mRNA levels of potential 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-

trolled 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×E_2) or two (OVX + 2×E_2) subcutaneous E_2-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 E_2 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 E_2 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 = 3) was similarly implanted with cardiac catheters to enable collection of blood samples (300 μl) for measurement of diestrous E_2 levels. Blood was sampled this way from each intact animal through three consecutive estrous cycles.

Correct cannula placement in the ARC was confirmed by microscopic inspection of 30-μm brain sections. Only data from animals with correct cannula placement were analyzed. Each animal was used only once.

Effect of senktide on pulsatile LH secretion

On the morning of experimentation, an intra-ARC injection cannula (Plastics One) with extension tubing, preloaded with drug or vehicle [artificial cerebrospinal fluid (aCSF)], was inserted into the guide cannula. The distal end of the tubing, prefilled with aCSF was extended outside of the cage to allow remote microinfusion without disturbing the rat during the experiment. Microinfusion was performed manually over 5 min using a 5-μl syringe (Hamilton, Bonaduz, Switzerland). One of the two cardiac catheters was then attached via the fluid swivel to a computer-controlled automated blood-sampling system, which allows for the intermittent withdrawal of small blood samples (25 μl) every 5 min for 5–6 h without disturbing the rats (28). Once connected, the animals were left undisturbed for 1 h before blood sampling was initiated. After removal of each 25-μl blood sample, an equal volume of heparinized saline (50 U/ml normal saline; CP Pharmaceuticals, Wrexham, UK) was automatically infused into the animal to maintain patency of the catheter and blood volume. Blood samples were frozen at −20 C for later RIA to determine LH concentrations. After 2 h of control blood sampling, senktide (Tocris, Bristol, UK) or vehicle was infused intra-ARC over 5 min. OVX + 1×E_2 rats received a single dose of 0.1, 1, or 10 pmol senktide in 400 nl aCSF, bilaterally on each side (n = 6–9 per treatment group). Control rats (n = 6) received 400 nl aCSF. OVX + 2×E_2 rats were treated with 10 pmol senktide (n = 8) or vehicle (n = 8).

OVX + 1×E_2 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 U50488 on pulsatile LH secretion

OVX + 1×E_2 rats were used in the following experiment. Injection cannulas were preloaded with U50488 [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×E_2 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×E_2 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′-AGCTGCTGCTTCTCC TCTGT-3′; *Kiss1* (antisense): 3′-AGGCTTGCTCTCTGCATACC-5′; *Kiss1r* (sense): 5′-GGTGCT GGGAGACTTCATGT-3′; *Kiss1r* (antisense): 3′-AGTGGCACATGTGGCTTG-5′; *GNRH1* (sense): 5′-GCCGCTGTTGTTCTGTTGACT-3′; *GNRH1* (antisense): 3′-TTCCTCTT CAATCAGAC GTTCC-5′; *HPRT1* (sense): 5′-GCAGACTTTGCTTTCCTTGG-3′; *HPRT1*(antisense): 3′-CGAGA GGTCCTTTTCACCAG-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, Orangeburg, NY) was used to estimate the E_2 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 pretreatment 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 from LH concentrations in the 2-h period preceding treatment and were compared between groups implanted with either one or two E_2 capsules. E_2 levels in OVX + 1×E_2 and OVX + 2×E_2 were calculated from means of E_2 levels detected 10 and 17 d after OVX, because these did not differ significantly ($P > 0.05$, Student's *t* test). E_2 levels in diestrous rats were calculated from means of E_2 levels detected during diestrus in three consecutive estrous cycles. Frequency of epithelial cornification in OVX + 1×E_2 and OVX + 2×E_2 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×E_2 and OVX + 2×E_2 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 E_2, mean LH levels, and vaginal cytology

The circulating levels of E_2 in OVX + 2×E_2 rats were more than double those in OVX + 1×E_2 rats (83.3 ± 14.2 pg/ml *vs.* 35.9 ± 1.7 pg/ml; $P < 0.05$). Although circulating levels of E_2 in OVX + 1×E_2 rats were significantly lower than in diestrous rats (35.9 ± 1.7 pg/ml *vs.* 96.4 ± 28.9 pg/ml; $P < 0.05$), E_2 levels in OVX + 2×E_2 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×E_2 rats than in OVX + 1×E_2 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×E_2 rats than in OVX + 1×E_2 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.* first 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×E_2 (23.3 ± 1.3 min *vs.* 157.0 ± 20.0 min; $P < 0.05$) and OVX + 2×E_2 rats (22.2 ± 0.8 min *vs.* 158.5 ± 8.3 min; $P < 0.05$). In the

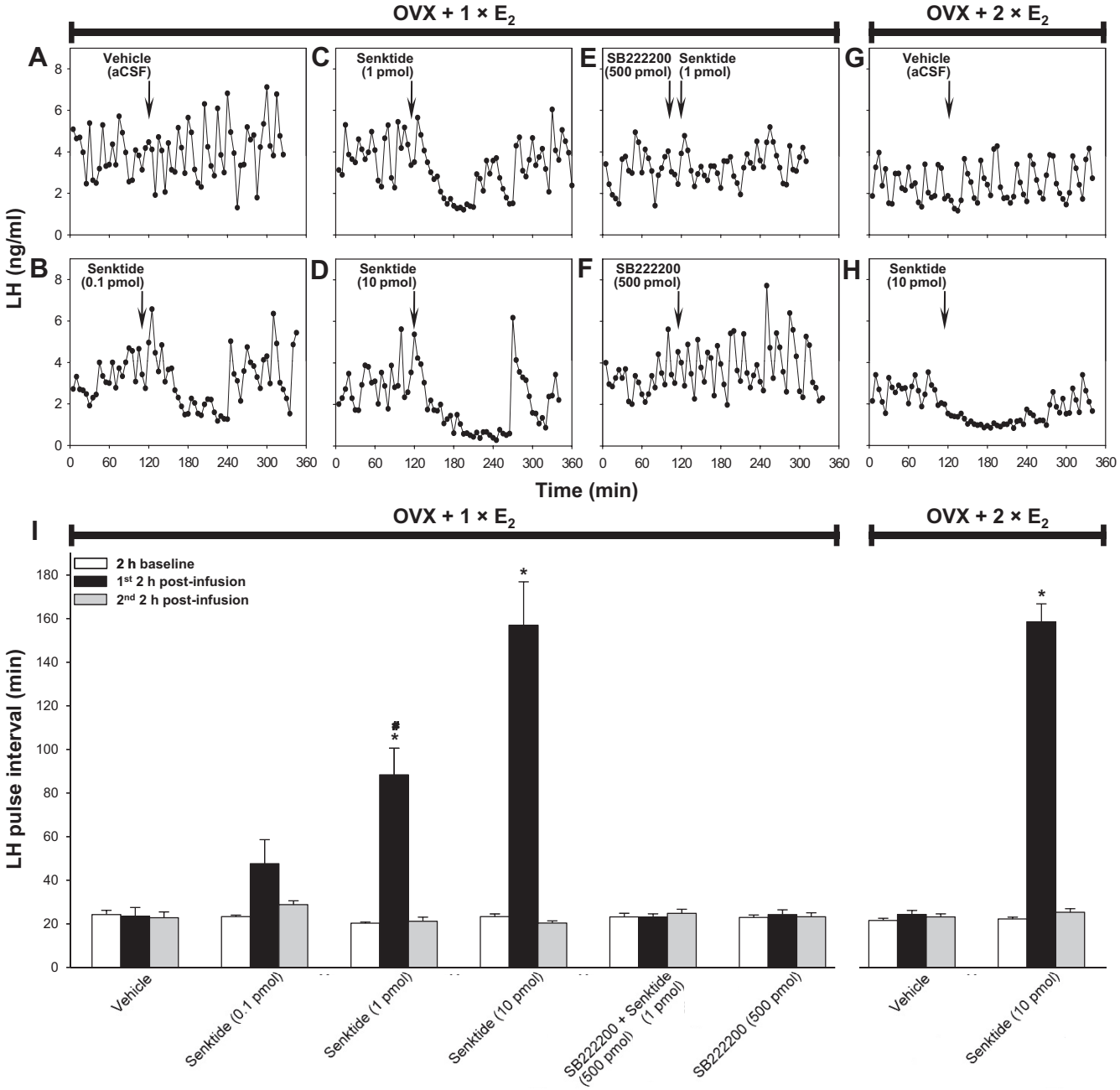

FIG. 1. Effect of NK3R agonist and antagonist on LH pulse frequency. Representative LH profiles demonstrating 1) the effect of intra-ARC administration (↓) of a selective NK3R agonist, senktide (B–D), or vehicle (A), and 2) the effect of intra-ARC administration of senktide after pretreatment with a selective NK3R antagonist, SB222200 (E), or that of SB222200 alone (F), on pulsatile LH secretion in OVX rats implanted with either one (A–F) or two (G and H) E_2 capsules. Central administration of senktide caused a dose-dependent suppression of LH pulses; this effect was blocked by pretreatment with SB222200, as summarized in panel I. *, $P < 0.05$ *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.* same 2-h period within the group treated with both SB222200 and senktide; n = 6–9 per group.

control group, injections of vehicle did not significantly affect the LH pulse frequency in either OVX + 1×E_2 (24.3 ± 1.9 min *vs.* 23.5 ± 4.1 min; $P > 0.05$) or OVX + 2×E_2 (21.5 ± 1.1 min *vs.* 24.3 ± 1.9 min; $P > 0.05$) rats. Furthermore, the LH pulse in both OVX + 1×E_2 and OVX + 2×E_2 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×E_2 rats and 10 pmol senktide in OVX + 2×E_2 rats, respectively. To localize the observed effect of senktide to ARC NK3R, we administered senktide (1 pmol, intra-ARC) to OVX + 1×E_2 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×E_2 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×E_2 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; $P < 0.05$).

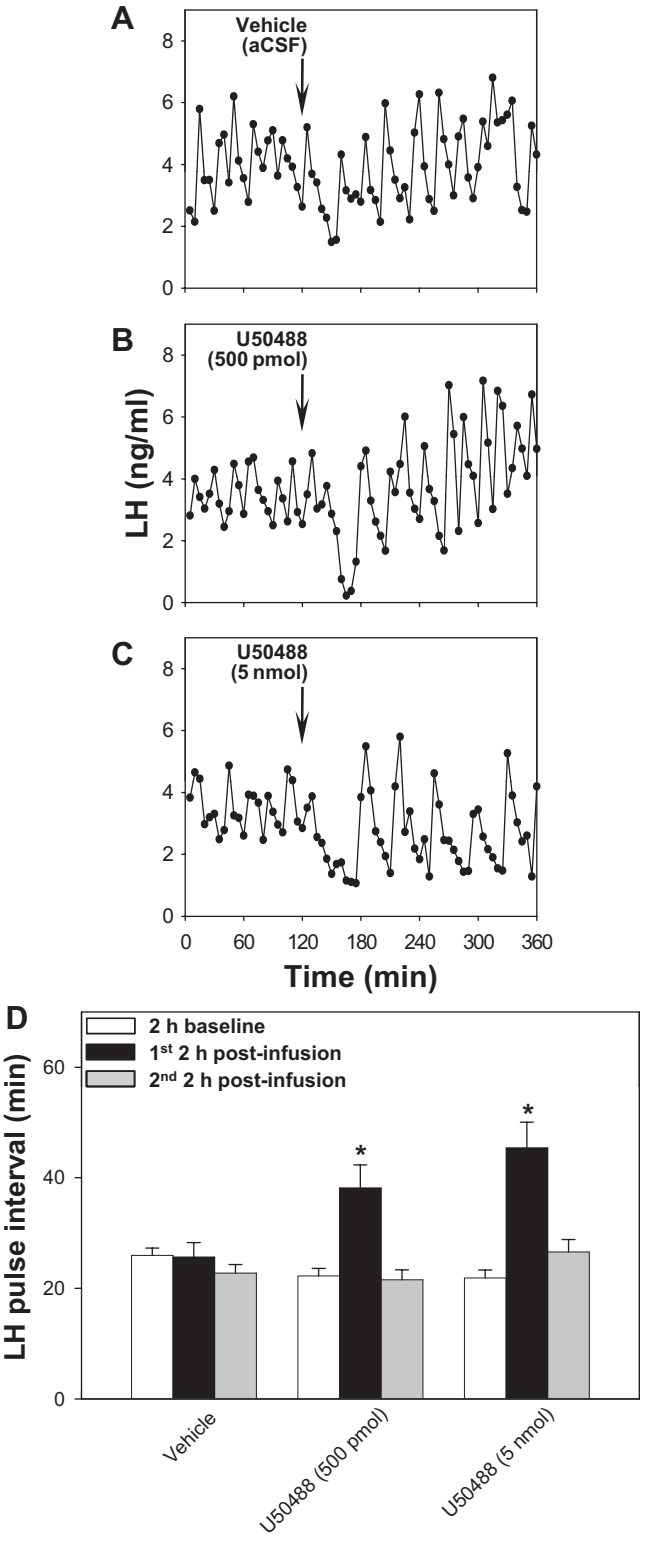

FIG. 2. Effect of KOR agonist on LH pulse frequency. Representative LH profiles demonstrating the effect of intra-ARC administration (↓) of a selective KOR agonist, U50488 (B and C), or that of vehicle (A), on pulsatile LH secretion in OVX rats implanted with a single E_2 capsule. Central administration of U50488 causes a dose-dependent decrease in LH pulse frequency, as summarized in panel D. *, $P < 0.05$ *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; n = 5–10 per group.

Discussion

In the present study we provide the first direct evidence that ARC KNDy neurons regulate the frequency of the

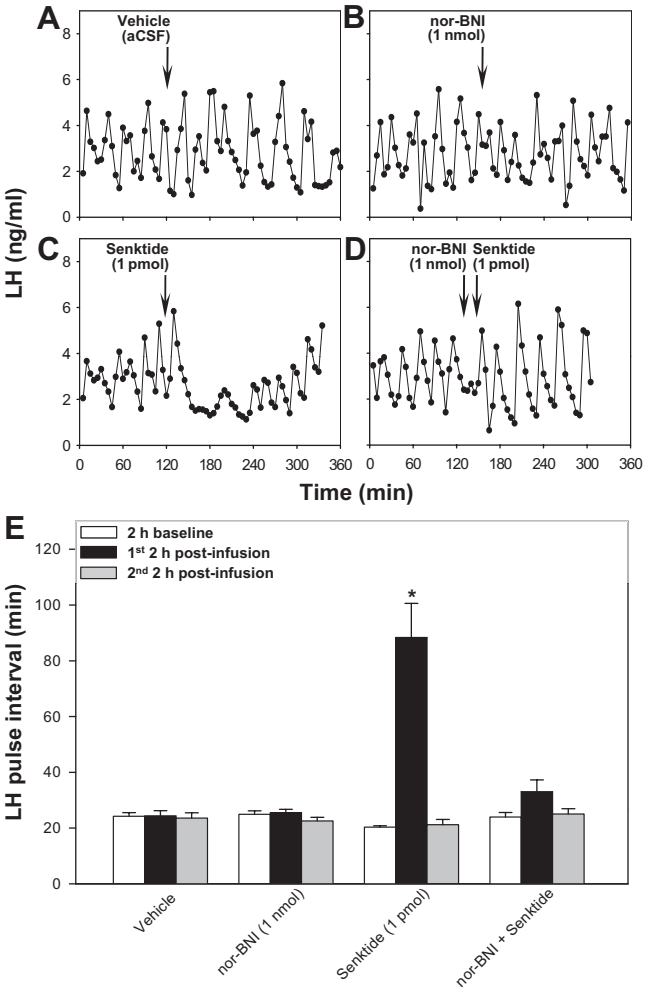

FIG. 3. Effect of NK3R agonist on LH pulse frequency after pretreatment with KOR antagonist. Representative LH profiles demonstrating the effect of intra-ARC administration (↓) of a selective NK3R agonist, senktide, with (D) or without (C) pretreatment with a selective KOR antagonist, nor-BNI, or that of vehicle (A), or nor-BNI alone (B), on pulsatile LH secretion in OVX rats implanted with a single E_2 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.

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 + E_2 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 tanycyte 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 + E_2 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 + E_2) of E_2 (21). In the present study we have confirmed that doubling the E_2 replacement from one to two E_2 capsules, which produced E_2 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 E_2 capsule replacement regimen produced circulating concentrations of E_2 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 E_2 concentrations *in vivo* (37). Nevertheless, implantation of two E_2 capsules (OVX + 2×E_2) more than doubled the circulating E_2 levels observed in OVX + 1×E_2 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×E_2 rats than in OVX + 1×E_2 rats, indicative of higher E_2 levels in these animals. Taken together, these data suggest that the two E_2 replacement regimens produced circulating E_2 levels that are probably within the physiological range observed during the diestrus 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 E_2-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

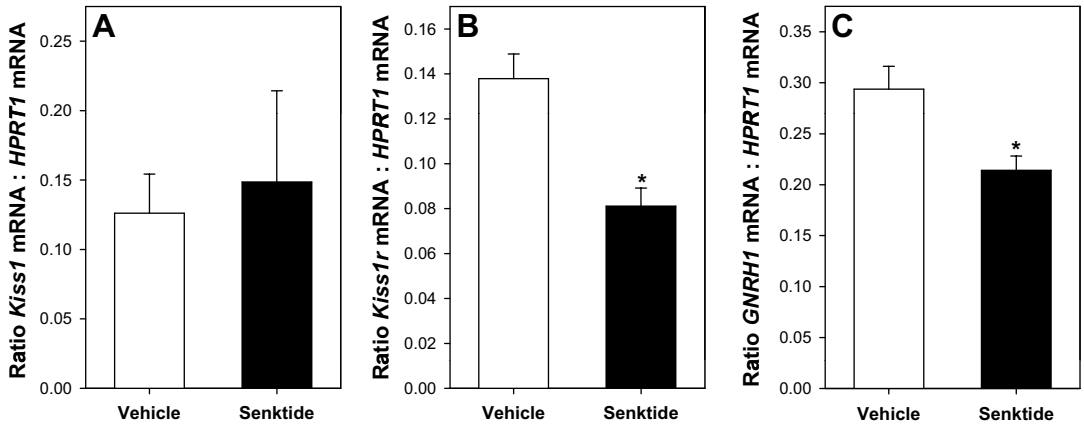

FIG. 4. Effect of NK3R agonist on expression of *Kiss1*, *Kiss1r*, and *GNRH1* mRNA in mPOA/AVPV. Effect of intra-ARC administration of a selective NK3R agonist, senktide, on the expression of *Kiss1* (A), *Kiss1r* (B), or *GNRH1* (C) mRNA within mPOA/AVPV in OVX rats implanted with a single E_2 capsule. *, $P < 0.05$ *vs.* vehicle-treated control group; n = 6–8 per group.

an inhibitory to a stimulatory effect of senktide on LH secretion was evident in OVX animals that received E_2 replacement producing circulating E_2 levels equivalent to the proestrous phase (20). These data suggest that the gonadal steroid milieu, in particular E_2, 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 E_2 replacement or in the presence of low physiological levels of E_2 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 E_2 (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 E_2, as well as a NKB-Dyn (nonkisspeptin) interaction that suppresses LH secretion when E_2 negative-feedback is considerably weaker. Elucidation of the mechanism by which varying E_2 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 E_2; 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.

Acknowledgments

Address all correspondence and requests for reprints to: Professor Kevin O'Byrne, Division of Women's Health, 2.92W Hodgkin Building, King's College London, Guy's Campus, London, SE1 1UL, United Kingdom. E-mail: kevin.o'byrne@kcl.ac.uk

This work was supported by a grant from Biotechnology and Biological Sciences Research Council. P.G. is a recipient of a Medical Research Council Ph.D. Studentship.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO, Cook JR, Ozbek MN, Imamoglu S, Akalin NS, Yuksel B, O'Rahilly S, Semple RK** 2009 TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for neurokinin B in the central control of reproduction. Nat Genet 41:354–358
2. **Knobil E** 1990 The GnRH pulse generator. Am J Obstet Gynecol 163:1721–1727
3. **Li XF, Kinsey-Jones JS, Cheng Y, Knox AM, Lin Y, Petrou NA, Roseweir A, Lightman SL, Milligan SR, Millar RP, O'Byrne KT** 2009 Kisspeptin signalling in the hypothalamic arcuate nucleus regulates GnRH pulse generator frequency in the rat. PLoS One 4:e8334
4. **Burke MC, Letts PA, Krajewski SJ, Rance NE** 2006 Coexpression of dynorphin and neurokinin B immunoreactivity in the rat hypothalamus: morphologic evidence of interrelated function within the arcuate nucleus. J Comp Neurol 498:712–726
5. **Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ** 2007 Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology 148:5752–5760
6. **Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK,**

Steiner RA 2009 Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. J Neurosci 29:11859–11866

7. **Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda K, Steiner RA, Okamura H** 2010 Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. J Neurosci 30: 3124–3132
8. **Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma FJ, Gustafson EL** 2003 The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem Biophys Res Commun 312:1357–1363
9. **Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley Jr WF, Aparicio SA, Colledge WH** 2003 The GPR54 gene as a regulator of puberty. N Engl J Med 349:1614–1627
10. **Mayer C, Boehm U** 2011 Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. Nat Neurosci 14:704–710
11. **Schulz R, Wilhelm A, Pirke KM, Gramsch C, Herz A** 1981 β-Endorphin and dynorphin control serum luteinizing hormone level in immature female rats. Nature 294:757–759
12. **Kinoshita F, Nakai Y, Katakami H, Imura H** 1982 Suppressive effect of dynorphin-(1–13) on luteinizing hormone release in conscious castrated rats. Life Sci 30:1915–1919
13. **Goodman RL, Coolen LM, Anderson GM, Hardy SL, Valent M, Connors JM, Fitzgerald ME, Lehman MN** 2004 Evidence that dynorphin plays a major role in mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep. Endocrinology 145:2959–2967
14. **Bowe JE, Li XF, Kinsey-Jones JS, Paterson S, Brain SD, Lightman SL, O'Byrne KT** 2005 Calcitonin gene-related peptide-induced suppression of luteinizing hormone pulses in the rat: the role of endogenous opioid peptides. J Physiol 566:921–928
15. **Krajewski SJ, Burke MC, Anderson MJ, McMullen NT, Rance NE** 2010 Forebrain projections of arcuate neurokinin B neurons demonstrated by anterograde tract-tracing and monosodium glutamate lesions in the rat. Neuroscience 166:680–697
16. **Ramaswamy S, Seminara SB, Ali B, Ciofi P, Amin NA, Plant TM** 2010 Neurokinin B stimulates GnRH release in the male monkey (*Macaca mulatta*) and is colocalized with kisspeptin in the arcuate nucleus. Endocrinology 151:4494–4503
17. **Ramaswamy S, Seminara SB, Plant TM** 2011 Evidence from the agonadal juvenile male rhesus monkey (*Macaca mulatta*) for the view that the action of neurokinin B to trigger gonadotropin-releasing hormone release is upstream from the kisspeptin receptor. Neuroendocrinology 94:237–245
18. **García-Galiano D, van Ingen Schenau D, Leon S, Krajnc-Franken MA, Manfredi-Lozano M, Romero-Ruiz A, Navarro VM, Gaytan F, van Noort PI, Pinilla L, Blomenröhr M, Tena-Sempere M** 2012 Kisspeptin signaling is indispensable for neurokinin B, but not glutamate, stimulation of gonadotropin secretion in mice. Endocrinology 153:316–328
19. **Navarro VM, Gottsch ML, Wu M, García-Galiano D, Hobbs SJ, Bosch MA, Pinilla L, Clifton DK, Dearth A, Ronnekleiv OK, Braun RE, Palmiter RD, Tena-Sempere M, Alreja M, Steiner RA** 2011 Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. Endocrinology 152:4265–4275
20. **Navarro VM, Castellano JM, McConkey SM, Pineda R, Ruiz-Pino F, Pinilla L, Clifton DK, Tena-Sempere M, Steiner RA** 2011 Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. Am J Physiol Endocrinol Metab 300: E202–E210
21. **Kinsey-Jones JS, Grachev P, Li XF, Lin YS, Milligan SR, Lightman SL, O'Byrne KT** 2012 The inhibitory effects of neurokinin B on GnRH pulse generator frequency in the female rat. Endocrinology 153:307–315
22. **Billings HJ, Connors JM, Altman SN, Hileman SM, Holaskova I, Lehman MN, McManus CJ, Nestor CC, Jacobs BH, Goodman RL** 2010 Neurokinin B acts via the neurokinin-3 receptor in the retrochiasmatic area to stimulate luteinizing hormone secretion in sheep. Endocrinology 151:3836–3846
23. **Sandoval-Guzmán T, Rance NE** 2004 Central injection of senktide, an NK3 receptor agonist, or neuropeptide Y inhibits LH secretion and induces different patterns of Fos expression in the rat hypothalamus. Brain Res 1026:307–312
24. **Krajewski SJ, Anderson MJ, Iles-Shih L, Chen KJ, Urbanski HF, Rance NE** 2005 Morphologic evidence that neurokinin B modulates gonadotropin-releasing hormone secretion via neurokinin 3 receptors in the rat median eminence. J Comp Neurol 489:372–386
25. **Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA** 2006 Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. J Neurosci 26:668766–668794
26. **Liu X, Porteous R, d'Anglemont de Tassigny X, Colledge WH, Millar R, Petersen SL, Herbison AE** 2011 Frequency-dependent recruitment of fast amino acid and slow neuropeptide neurotransmitter release controls gonadotropin-releasing hormone neuron excitability. J Neurosci 31:2421–2430
27. **Paxinos G, Watson C** 1986 The rat brain in stereotaxic coordinates. 2nd ed. London: Academic Press
28. **Cates PS, Forsling ML, O'byrne KT** 1999 Stress-induced suppression of pulsatile luteinising hormone release in the female rat: role of vasopressin. J Neuroendocrinol 11:677–683
29. **Li XF, Mitchell JC, Wood S, Coen CW, Lightman SL, O'Byrne KT** 2003 The effect of oestradiol and progesterone on hypoglycaemic stress-induced suppression of pulsatile luteinizing hormone release and on corticotropin-releasing hormone mRNA expression in the rat. J Neuroendocrinol 15:468–476
30. **Kinsey-Jones JS, Li XF, Knox AM, Wilkinson ES, Zhu XL, Chaudhary AA, Milligan SR, Lightman SL, O'Byrne KT** 2009 Down-regulation of hypothalamic kisspeptin and its receptor, Kiss1r, mRNA expression is associated with stress-induced suppression of luteinising hormone secretion in the female rat. J Neuroendocrinol 21: 20–29
31. **Palkovits M, Brownstein MJ** 1988 Maps and guide to microdissection of the rat brain. New York: Elsevier Science
32. **Monroe SE, Parlow AF, Midgley Jr AR** 1968 Radioimmunoassay for rat luteinizing hormone. Endocrinology 83:1004–1012
33. **Van Cauter E** 1988 Estimating false-positive and false-negative errors in analyses of hormonal pulsatility. Am J Physiol 254:E786–EE794
34. **Rodríguez EM, Blázquez JL, Guerra M** 2010 The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: the former opens to the portal blood and the latter to the cerebrospinal fluid. Peptides 31:757–776
35. **Corander MP, Challis BG, Thompson EL, Jovanovic Z, Loraine Tung YC, Rimmington D, Huhtaniemi IT, Murphy KG, Topaloglu AK, Yeo GS, O'Rahilly S, Dhillo WS, Semple RK, Coll AP** 2010 The effects of neurokinin B upon gonadotrophin release in male rodents. J Neuroendocrinol 22:181–187
36. **Cagampang FR, Maeda KI, Tsukamura H, Ohkura S, Ota K** 1991 Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile LH release in ovariectomized rats. J Endocrinol 129:321–328
37. **Lee JS, Ettinger B, Stanczyk FZ, Vittinghoff E, Hanes V, Cauley JA,**

Chandler W, Settlage J, Beattie MS, Folkerd E, Dowsett M, Grady D, Cummings SR 2006 Comparison of methods to measure low serum estradiol levels in postmenopausal women. J Clin Endocrinol Metab 91:3791–3797

38. **Kalra SP, Kalra PS** 1983 Neural regulation of luteinizing hormone secretion in the rat. Endocr Rev 4:311–351
39. **Rance NE** 2009 Menopause and the human hypothalamus: evidence for the role of kisspeptin/neurokinin B neurons in the regulation of estrogen negative feedback. Peptides 30:111–122
40. **Noritake K Matsuoka T Ohsawa T, Shimomura K, Sanbuissho A, Uenoyama Y, Maeda K, Tsukamura H** 2011 Involvement of neurokinin receptors in the control of pulsatile luteinizing hormone secretion in rats. J Reprod Dev 57:409–415
41. **Amstalden M, Coolen LM, Hemmerle AM, Billings HJ, Connors JM, Goodman RL, Lehman MN** 2010 Neurokinin 3 receptor immunoreactivity in the septal region, preoptic area and hypothalamus of the female sheep: colocalisation in neurokinin B cells of the arcuate nucleus but not in gonadotrophin-releasing hormone neurones. J Neuroendocrinol 22:1–12
42. **Grachev P, Li XF, Lin YS, Hu MH, Elsamani L, Paterson SJ, Millar RP, Lightman SL, O'Byrne KT** 2012 GPR54-dependent stimulation of luteinizing hormone secretion by neurokinin B in prepubertal rats. PLoS One 10.1371/journal.pone.0044344