

## Neurokinin B Signaling in the Female Rat: a Novel Link Between Stress and Reproduction

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Acute systemic stress disrupts reproductive function by inhibiting pulsatile gonadotropin secretion. The underlying mechanism involves stress-induced suppression of the GnRH pulse generator, the functional unit of which is considered to be the hypothalamic arcuate nucleus kisspeptin/neurokinin B/dynorphin A neurons. Agonists of the neurokinin B (NKB) receptor (NK3R) have been shown to suppress the GnRH pulse generator, in a dynorphin A (Dyn)-dependent fashion, under hypoestrogenic conditions, and Dyn has been well documented to mediate several stress-related central regulatory functions. We hypothesized that the NKB/Dyn signaling cascade is required for stress-induced suppression of the GnRH pulse generator. To investigate this ovariectomized rats, iv administered with *Escherichia coli* lipopolysaccharide (LPS) following intracerebroventricular pretreatment with NK3R or  $\kappa$ -opioid receptor (Dyn receptor) antagonists, were subjected to frequent blood sampling for hormone analysis. Antagonism of NK3R, but not  $\kappa$ -opioid receptor, blocked the suppressive effect of LPS challenge on LH pulse frequency. Neither antagonist affected LPS-induced corticosterone secretion. Hypothalamic arcuate nucleus NKB neurons project to the paraventricular nucleus, the major hypothalamic source of the stress-related neuropeptides CRH and arginine vasopressin (AVP), which have been implicated in the stress-induced suppression of the hypothalamic-pituitary-gonadal axis. A separate group of ovariectomized rats was, therefore, used to address the potential involvement of central CRH and/or AVP signaling in the suppression of LH pulsatility induced by intracerebroventricular administration of a selective NK3R agonist, senktide. Neither AVP nor CRH receptor antagonists affected the senktide-induced suppression of the LH pulse; however, antagonism of type 2 CRH receptors attenuated the accompanying elevation of corticosterone levels. These data indicate that the suppression of the GnRH pulse generator by acute systemic stress requires hypothalamic NKB/NK3R signaling and that any involvement of CRH therewith is functionally upstream of NKB. (*Endocrinology* 155: 2589–2601, 2014)

It is widely recognized that exposure to stress profoundly impacts reproductive function. Various stress paradigms have been shown to delay pubertal onset (1–3) and suppress gonadotropin secretion (4–8) in a host of exper-

imental animals. The mechanism by which the suppressive effects of stress are relayed to the GnRH pulse generator are currently unknown, although previous studies have strongly implicated the involvement of the CRH signaling

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Abbreviations: aCSF, artificial cerebrospinal fluid; ARC, arcuate nucleus; AUC, area under the curve; AVP, arginine vasopressin; AVPR1A, AVP type 1A receptor; BNST, bed nucleus of the stria terminalis; CORT, corticosterone; DMSO, dimethyl sulfoxide; dpAVP, [deamino-Pen<sub>1</sub>, O-Me-Tyr<sub>2</sub>, Arg<sub>3</sub>]-vasopressin; Dyn, dynorphin A; E<sub>2</sub>, estradiol; GABA,  $\gamma$ -aminobutyric acid; HPA, hypothalamo-pituitary adrenal; icv, intracerebroventricular; KNDy, kisspeptin/neurokinin B/dynorphin A; KOR,  $\kappa$ -opioid receptor; LPS, lipopolysaccharide; mPOA, medial preoptic area; NK3R, neurokinin type 3 receptor; NKB, neurokinin B; nor-BNI, nornalorphimine; OVX, ovariectomized; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus.

system (9): the activation of either of the 2 subtypes of CRH receptor, CRHR1 and CRHR2, by their endogenous ligands, CRH and the related urocortin neuropeptides (Ucn, UcnII, and UcnIII) (10). Major populations of CRH neurons are found within the parvocellular portion of the paraventricular nucleus of the hypothalamus (PVN), central and medial nuclei of the amygdala, and the bed nucleus of the stria terminalis (BNST) (11, 12), all of which play a key role in the regulation of the hypothalamo-pituitary adrenal (HPA) axis during stress and have been implicated in the stress-induced suppression of the GnRH pulse generator (13–15).

Another neuropeptide involved in the central mechanisms underlying the stress response is arginine vasopressin (AVP). AVP is colocalized with CRH in parvocellular neurons of the PVN (16–18), and further major populations of AVP neurons are found resident within the magnocellular PVN and supraoptic nucleus (SON) (19). Acting through the 1A subtype of its putative receptor (AVPR1A; also abbreviated as  $V_{1A}$ ), AVP strongly potentiates CRH-induced secretion of ACTH from the posterior pituitary (18, 20, 21) and independently induces a moderate direct increase in ACTH release by adenohipophysal corticotropes (22, 23). Furthermore, like CRH, AVP is involved in the stress-induced suppression of LH secretion (24).

Despite evidence that CRH can directly suppress GnRH neurosecretion in rodents (25), substantiated by the demonstration of synaptic connections between CRH-immunolabeled axon terminals and GnRH dendrites in the rat medial preoptic area (mPOA) (26), stress-induced suppression of the GnRH pulse generator appears to predominantly involve multiple indirect signaling pathways. Indeed, tract-tracing studies in the rat have failed to detect projections of major (PVN, amygdala, BNST) CRH neuron populations to GnRH perikarya (27). Furthermore, mouse GnRH neurons apparently do not express CRHR2 (28), which has been implicated in the suppression of gonadotropin release in response to acute systemic stress (9).

The neuropeptides kisspeptin and neurokinin B (NKB), that are coexpressed with the endogenous opioid peptide dynorphin A (Dyn) and other signaling molecules, within kisspeptin/neurokinin B/dynorphin A (KNDy) neurons of the arcuate nucleus (ARC), are critical mediators of pulsatile GnRH neurosecretion. KNDy neurons project to GnRH neurons and numerous other hypothalamic targets (29). Each of the KNDy neuropeptides has been implicated in regulating pulsatile LH secretion. Although the stimulatory effect of kisspeptin (30) and inhibitory effect of Dyn (31) on the GnRH pulse generator are widely accepted, activation of the NKB receptor (neurokinin type 3

receptor; NK3R) in laboratory rodents has variable effects. We have recently shown that senktide, a NK3R agonist, suppresses the endocrine (LH pulse) (32, 33), and electrophysiological (hypothalamic multiunit activity volleys) (32) correlates of the GnRH pulse generator in ovariectomized (OVX) rats but stimulates LH secretion in ovary-intact prepubertal (34) and adult (32) rats. Furthermore, the suppressive effects of senktide on the LH pulse are abolished by intra-ARC pretreatment with a Dyn receptor ( $\kappa$ -opioid receptor [KOR]) antagonist (32, 33), whereas senktide-induced LH pulses are prevented by a kisspeptin receptor (GPR54, also Kiss1R) antagonist (34), suggesting that local (ARC) NKB release is functionally upstream of subsequent biphasic regulation of the GnRH pulse generator, which is modulated by estradiol ( $E_2$ )-negative feedback.

The mechanism of NKB-induced kisspeptin/GPR54-dependent stimulation of pulsatile GnRH neurosecretion (34–36) is relatively straightforward. However, elucidation of the mechanism underlying Dyn/KOR-dependent GnRH pulse generator suppression following acute activation of ARC NK3R (33) presents a considerable challenge. Conclusive *in vivo* evidence for a direct inhibitory effect of NKB on GnRH neurons is currently lacking, and although Dyn/KOR signaling is indispensable for senktide-induced suppression of LH secretion, a dynorphinergic mechanism of action on GnRH neurons is also unlikely due to the apparent absence of KOR/GnRH coexpression (37, 38). NK3R (39, 40) and KOR (41–44) are abundantly expressed, however, within several hypothalamic and limbic nuclei that receive projections from ARC NKB/Dyn neurons, notably the PVN, amygdala, and BNST. Therefore the inhibitory effects of ARC NKB and Dyn signaling on GnRH neurosecretion are either reliant on local suppression of kisspeptinergic stimulation of GnRH neurons or are mediated by an indirect mechanism involving another neurotransmitter/neuropeptide-signaling system.

In the present series of studies we interrogated the latter hypothesis and postulated that CRH- and/or AVP-signaling systems inhibit GnRH secretion following activation of ARC NKB neurons. A substantial body of evidence supports this hypothesis. First, both the parvocellular and magnocellular neuronal populations of the PVN are innervated by projections of ARC NKB neurons (45), and further non-NKB-immunoreactive fibers projecting from the ARC to the SON have been reported (45). Also, magnocellular neurons of the PVN and SON exhibit abundant expression of NK3R (46–49). These have been shown to be involved in regulating pituitary ACTH release through AVP secretion into the median eminence (50). Furthermore, NK3R mRNA and immunoreactivity have been de-

tected within the BNST (46), which also receives input from ARC NKB neurons (45). The observations that intracerebroventricular (icv) administration of senktide stimulates expression of c-Fos within these regions (51–53) and increases systemic release of AVP from the posterior pituitary through a mechanism dependent on the activation of NK3R expressed by AVP neurons (54, 55), therefore, are not surprising.

Taken together these data suggest the CRH and/or AVP signaling systems as potential candidates for relaying the inhibitory signal generated by ARC NKB neurons to GnRH neurons and thus provide grounds for a possible mechanism for senktide-induced suppression of pulsatile LH secretion. To investigate this, OVX rats were administered (icv) with senktide following pretreatment with antagonists of CRH or AVP receptors, and blood was collected for detailed profiling of pulsatile LH secretion and any changes in circulating corticosterone (CORT) levels. It is known that CRHR1 and CRHR2 are differentially involved in stress-induced suppression of pulsatile LH secretion (9), and knockout of CRHR1 (56, 57) or CRHR2 (58, 59) in mice has opposite effects on stress-induced HPA axis activation and anxiety-like behavior; thus a range of subtype-selective and nonselective CRH receptor antagonists were used in this study.

## Materials and Methods

### Animals

Adult female Sprague Dawley rats (200–250 g) obtained from Harlan were housed under controlled conditions (12-hour light, 12-hour darkness, with lights on at 7:00 AM; controlled ambient temperature  $22 \pm 2^\circ\text{C}$ ) and provided with standard laboratory chow (RM1; SDS Diets) and water ad libitum. Animals were group housed (maximum 4 per enclosure) prior to surgery and housed individually following surgery and during experimentation. 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.

### Surgical procedures

All surgical procedures were carried out under general anesthesia induced by ketamine (100 mg/kg ip; Pharmacia and Upjohn) coadministered with xylazine (Rompun; 10 mg/kg ip; Bayer); supplementary injections of ketamine (10 mg, ip) were administered to maintain anesthesia as required. Each rat was injected sc with 0.4 mL/kg Duphamox LA antibiotic suspension (200 mg/mL procaine benzylpenicillin, 250 mg/mL dihydrostreptomycin-sulfate; Fort Dodge Animal Health) prior to surgery. Following each surgical procedure, animals were allowed to recover from anesthesia on a heated pad until fully conscious. Postoperative analgesia was provided by means of sc adminis-

tration of carprofen (Rimadyl; 4.4 mg/kg; Pfizer Animal Health) daily for 3 days.

Two weeks before experiments took place rats were bilaterally OVX and implanted with unilateral guide cannulas (22 gauge; Plastics One), stereotaxically targeted toward the left lateral cerebral ventricle for microinfusion of pharmacologic agents. The stereotaxic coordinates for implantation were 1.5 mm lateral, 0.6 mm posterior to bregma, and 4 mm below the surface of the dura (60). Correct cannula placement was confirmed by the observation of gravitational meniscus movement upon insertion of an internal injection cannula (Plastics One) with extension tubing preloaded with artificial cerebrospinal fluid (CSF) (aCSF; see Appendix). A 20-mm stainless steel slotted screw (Instec Laboratories) was affixed to the surface of the skull posterior to the guide cannula, and both were secured using dental cement (Simplex Rapid; Dental Filling). The icv guide cannulas were then fitted with dummy cannulas (Plastics One) to maintain patency. Following a 10-day recovery period, the rats were implanted with 2 custom-made cardiac catheters via the internal jugular veins to enable simultaneous automated serial blood sampling for profiling of LH levels and manual withdrawal of blood for determination of CORT levels (61). The catheters were exteriorized at the back of the head and enclosed within a 30-cm long lightweight metal spring tether (Instec Laboratories) secured to the slotted screw cranial attachment. The distal end of the tether was attached to a dummy swivel (Instec Laboratories), allowing the rat to move freely. After surgery animals were housed singly. Experimentation commenced after a further 3 days' recovery from surgery.

### Experimental design

On the morning of experimentation, an icv injection cannula with extension tubing, preloaded with a combination of drug solutions, was inserted into the guide cannula. The distal end of the tubing was filled with aCSF. The remainder of the tubing was filled with sterile water, with 5  $\mu\text{L}$  air separating the water and aCSF, which allowed the progress of injections to be monitored. The tubing was extended outside the cage and connected via one channel of a 2-channel fluid swivel (Instec Laboratories) to a 25- $\mu\text{L}$  syringe (Hamilton) prefilled with sterile water, to allow remote microinfusion without disturbing the rats during the experiment. One of the 2 cardiac catheters was then attached via the second channel of the fluid swivel to a computer-controlled automated blood sampling system, which enables the intermittent withdrawal of small blood samples (25  $\mu\text{L}$ ) every 5 minutes for 5–6 hours without disturbing the rats. After removal of each 25- $\mu\text{L}$  blood sample, an equal volume of heparinized saline (50 U/mL normal saline; CP Pharmaceuticals) was automatically infused to maintain patency of the catheter and blood volume. Once connected the animals were left undisturbed for 1 hour before blood sampling was initiated (between 9:00 and 11:00 AM). After 1 hour 45 minutes of control blood sampling, icv administration of drug treatments commenced. Additional blood (50  $\mu\text{L}$ ) was sampled manually at +60, +150, +180, and +240 minutes (relative to the time when the automated blood sampling was initiated) via the second cardiac catheter, and each withdrawal was followed by the administration (iv) of 50  $\mu\text{L}$  heparinized saline. Plasma obtained by centrifugation of manually withdrawn blood samples was frozen at  $-20^\circ\text{C}$  and later assayed to determine concentrations of CORT by means of RIA. Automatically sampled blood was

frozen at  $-20^{\circ}\text{C}$  and later assayed to determine LH concentrations by means of RIA.

### Experiment 1: Effects of icv administration of dimethyl sulfoxide (DMSO) on pulsatile LH secretion

To rule out any effects of vehicles containing DMSO (Sigma-Aldrich) on pulsatile LH secretion, and to thereby justify the omission of individual control groups in subsequent experiments, rats were injected icv with  $4\ \mu\text{L}$  aCSF ( $n = 6$ ) or 10% DMSO in aCSF ( $n = 4$ ) or 100% DMSO ( $n = 4$ ) at 120 minutes relative to the start of the 6-hour bleeding procedure.

### Experiment 2a: Effects of central NK3R or KOR antagonism on lipopolysaccharide (LPS)-induced suppression of pulsatile LH secretion

The selective NK3R antagonist, SB222200 (Tocris), was dissolved in DMSO and diluted with aCSF, the final injected solution containing 15% DMSO. The selective KOR antagonist, norbinaltorphimine (nor-BNI; Tocris), was dissolved in sterile water and diluted with aCSF. A separate group of animals was pretreated (icv) with 3 nmol SB222200 ( $n = 4$ ) or 6.8 nmol nor-BNI ( $n = 6$ ) in  $4\ \mu\text{L}$  corresponding vehicle, or  $4\ \mu\text{L}$  aCSF ( $n = 6$ ) 100 minutes following the initiation of the bleeding procedure, and 20 minutes later was administered with  $25\ \mu\text{g}/\text{kg}$  LPS (Sigma-Aldrich) in 0.3 mL saline via the cardiac catheter not used for blood withdrawal.

### Experiment 2b: Effects of central NK3R or KOR antagonism on LPS-induced CORT secretion

Additional blood samples were collected during Experiment 2a to allow the quantitation of changes in plasma CORT levels following systemic LPS treatment, as well as the determination of the effect of SB222200 or nor-BNI pretreatment on this parameter. To determine whether any observed changes in CORT levels might be attributable to the discomfort of iv infusion or any related stressor, a group of negative controls ( $n = 6$ ) was pretreated with  $4\ \mu\text{L}$  aCSF and 20 minutes later was administered with 0.3 mL saline, iv.

### Experiment 3a: Effect of AVPR1A or CRH receptor antagonism on senktide-induced suppression of pulsatile LH secretion

Pretreatment regimen consisted of 3 consecutive icv injections of  $25\ \mu\text{g}$  selective AVPR1A antagonist, [deamino-Pen<sup>1</sup>, O-Me-Tyr<sup>2</sup>, Arg<sup>8</sup>]-vasopressin (dpAVP; Tocris;  $n = 12$ );  $5\ \mu\text{g}$  nonselective CRH receptor antagonist, astressin (Tocris;  $n = 17$ );  $25\ \mu\text{g}$  selective CRHR1 antagonist, CP-154526 (Tocris;  $n = 9$ );  $25\ \mu\text{g}$  selective CRHR2 antagonist, astressin-2B (Tocris;  $n = 4$ ); or aCSF ( $n = 10$ ); each was administered over 5 minutes, 20 minutes apart, with a further icv injection of 100 pmol senktide (Tocris), administered 15 minutes following the first injection. Controls received triple icv injections of dpAVP ( $n = 5$ ), astressin ( $n = 5$ ), CP-154526 ( $n = 3$ ), or astressin-2B ( $n = 3$ ) at the stated doses, with aCSF being administered at 120 minutes instead of senktide. Each injection was given in a volume of  $4\ \mu\text{L}$  vehicle, with  $2\ \mu\text{L}$  air separating the treatments. dpAVP and astressin were dissolved in aCSF. CP-154526 and astressin-2B were dis-

solved in DMSO and diluted with aCSF, the final concentrations of DMSO being 2 and 20%, respectively.

### Experiment 3b: Effect of AVPR1A or CRH receptor antagonism on senktide-induced CORT secretion

Additional blood samples were collected during *Experiment 3a* to allow the quantitation of changes in plasma CORT levels following icv administration of senktide, as well as the determination of the effect of AVPR1A or CRH receptor antagonist pretreatment on this parameter. A further group of rats ( $n = 5$ ) received a single icv injection of 6.8 nmol nor-BNI in  $4\ \mu\text{L}$  aCSF and 20 minutes later was administered icv with senktide. To determine whether any observed changes in CORT levels might be attributable to the potential increase in ventricular pressure due to the administration of a substantial volume into the lateral ventricle or any related effect, a group of negative controls ( $n = 3$ ) received 4 consecutive injections of  $4\ \mu\text{L}$  aCSF timed as in other treatment groups.

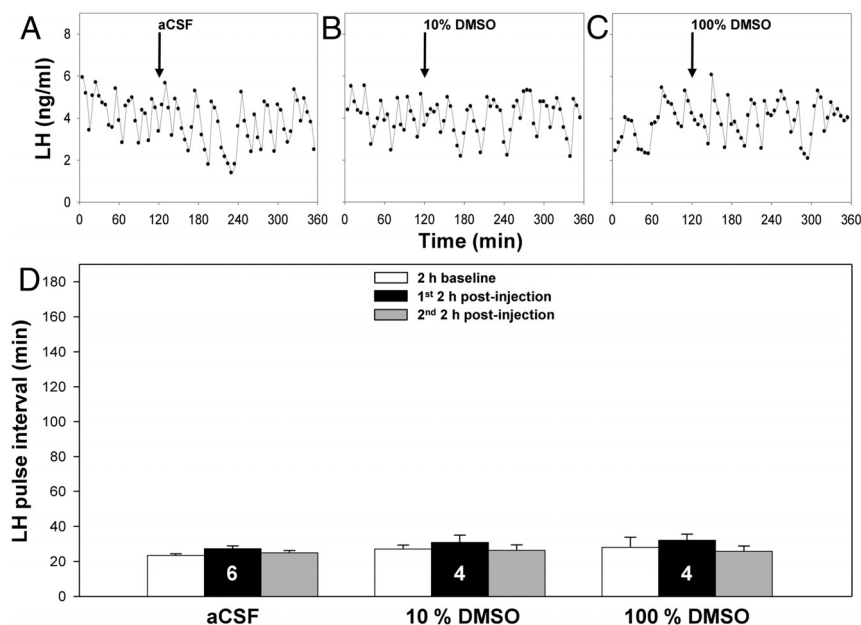
### Hormone RIA

A double-antibody RIA supplied by the National Hormone and Peptide Program (Torrance, CA) was used to determine LH concentrations in the  $25\text{-}\mu\text{L}$  whole-blood samples. The sensitivity of the assay was  $0.093\ \text{ng}/\text{mL}$ . The inter- and intraassay variations were 6.8% and 8.0%, respectively.

A double-antibody RIA kit (ImmuChem; MP Biomedicals) was used to estimate the CORT content of plasma samples ( $5\ \mu\text{L}$ ) following the manufacturer's protocol. The sensitivity of the assay was  $7.7\ \text{ng}/\text{mL}$ . The intraassay variation was 7.3%, and the interassay variation was 6.9%.

### Data analysis

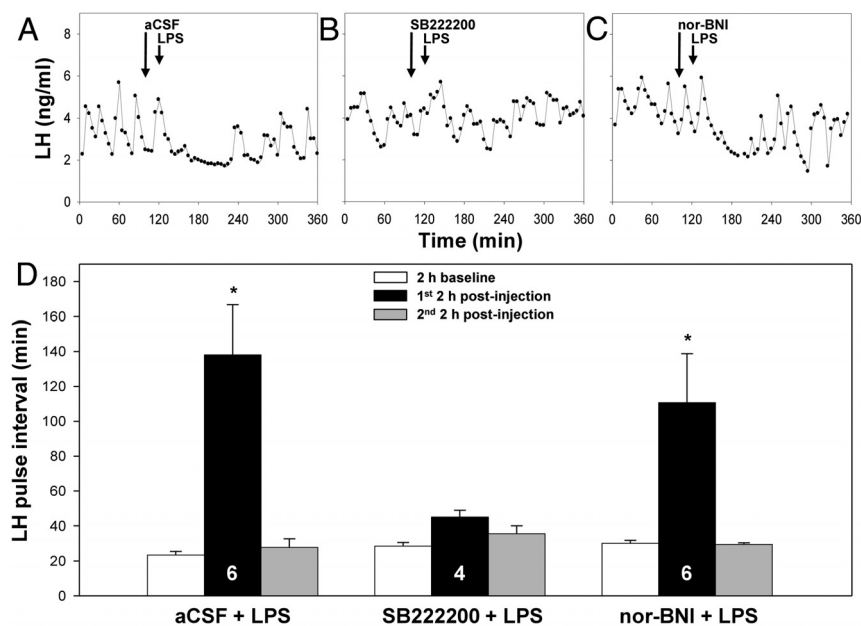
Detection of LH pulses was facilitated by the use of the algorithm ULTRA (62). Two intraassay coefficients of variation ( $2 \times c_v$ ) of the LH RIA were used as the reference threshold for pulse detection. The effect of systemic LPS challenge or icv infusion of senktide (with antagonist pretreatment) on pulsatile LH secretion was analyzed by comparing the mean LH pulse interval in the 2-hour period preceding treatment, and 2 consecutive 2-hour 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-hour 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-hour posttreatment period. The significance of the effect of treatments on LH pulse intervals was compared with the positive control (aCSF + Senktide) group at the same time points, as well as with the mean pulse interval during the 2-hour pretreatment period in the same group. Data from the positive control replicates assigned to the individual experiments were subsequently combined into a single group and mean  $\pm$  SEM values for this treatment group were reproduced for reference in *Experiment 2a* and *Experiment 3a* subsections of *Results*. CORT levels were expressed as percent of the +60 (baseline) time point in order to insulate the observed trends from the confounding effects of variability in absolute CORT concentrations between treatment groups and individual replicates. Values given in the text and figures represent mean  $\pm$  SEM.



**Figure 1.** Effects of icv administration of DMSO on pulsatile LH secretion. Representative LH profiles demonstrating the lack of effect of icv administration of 4  $\mu$ L aCSF (A), 10% DMSO (B), or 100% DMSO (C), on pulsatile LH secretion in OVX rats. The histogram (D) summarizes the experimental means, variability (SEM error bars), and treatment group sizes (white on black bars).

Statistical significance was tested using one-way ANOVA with Bonferroni post hoc test.  $P < .05$  was considered statistically significant.

results of this experiment are summarized in the histogram (Figure 1D).



**Figure 2.** Effects of central NK3R or KOR antagonism on LPS-induced suppression of pulsatile LH secretion. Representative LH profiles demonstrating the effect of iv administration of LPS following icv pretreatment with vehicle (aCSF; panel A), a selective NK3R antagonist (SB222200; panel B), or a selective KOR antagonist (nor-BNI; panel C), on pulsatile LH secretion in OVX rats. The icv administration of these antagonists, at the same doses as here, has previously been shown to be without effect on LH secretion in rats (32, 92). LPS increased the duration of the LH pulse interval (A). Pretreatment with SB222200 (B), but not with nor-BNI (C), blocked the LPS-induced suppression of LH pulses (B), as summarized in panel D. Treatment group sizes are indicated in white within black bars (D). \*,  $P < .05$  vs 2-hour baseline control period within the same treatment group.

## Results

### Experiment 1: Effects of icv administration of DMSO on pulsatile LH secretion

The effect of the icv administration of DMSO, at various concentrations, on the LH pulse was investigated (Figure 1) in order to establish whether a different vehicle control group is necessary for each experimental group treated with a drug solution containing DMSO in subsequent experiments. DMSO, 10% (Figure 1 B), and 100% DMSO (Figure 1 C), like that of aCSF (Figure 1 A), did not alter the frequency of the LH pulse (LH pulse interval in first 2 hours postinjection in aCSF group vs that in 10% DMSO group vs that in 100% DMSO group;  $27.3 \pm 1.6$  minutes vs  $30.9 \pm 4.2$  minutes vs  $32.1 \pm 3.6$  minutes;  $P > .05$ ). The

### Experiment 2a: Effects of central NK3R or KOR antagonism on LPS-induced suppression of pulsatile LH secretion

In order to investigate the roles of hypothalamic NKB/NK3R and Dyn/KOR signaling systems in the stress-induced suppression of the GnRH pulse generator, we administered LPS to rats pretreated with SB222200 or nor-BNI, respectively (Figure 2). LPS significantly decreased LH pulse frequency in controls pretreated with aCSF, a transient effect that saw recovery to basal LH pulse frequency within the second 2 hours postinjection (Figure 2 A; LH pulse interval preinjection vs first 2 hours postinjection vs second 2 hours postinjection;  $23.4 \pm 2.1$  minute vs  $138.1 \pm 28.6$  minutes vs  $27.8 \pm 4.3$  minutes;  $P < .05$ ). Pretreatment with SB222200 blocked the LPS-induced suppression of the LH pulse (Figure 2B; LH pulse inter-

val in first 2 hours postinjection in aCSF + LPS group vs that in SB222200 + LPS group;  $138.1 \pm 28.6$  minutes vs  $45.1 \pm 3.8$  minutes;  $P < .05$ ). nor-BNI pretreatment did not significantly affect the suppression of the LH pulse induced by LPS (Figure 2C; LH pulse interval in first 2 hours postinjection in aCSF + LPS group vs that in nor-BNI + LPS group;  $138.1 \pm 28.6$  minutes vs  $110.7 \pm 28.1$  minute;  $P > .05$ ). The results of this experiment are summarized in the histogram (Figure 2D).

### Experiment 2b: Effects of central NK3R or KOR antagonism on LPS-induced CORT secretion

Plasma CORT levels were greatly elevated by LPS challenge (area under the curve [AUC], aCSF + saline vs aCSF + LPS;  $234.9 \pm 28.5$  vs  $935.8 \pm 70.5$ ;  $P < .05$ ), with a more than 6-fold increase in the first 30 minutes following administration, and remained elevated for at least 1½ hours thereafter (Figure 3). Neither SB222200 nor nor-BNI pretreatment was effective at altering the extent of CORT secretion stimulated by LPS (AUC, aCSF + LPS vs SB222200 + LPS vs nor-BNI + LPS;  $935.8 \pm 70.5$  vs  $822.1 \pm 58.2$  vs  $945.5 \pm 97.6$ ).

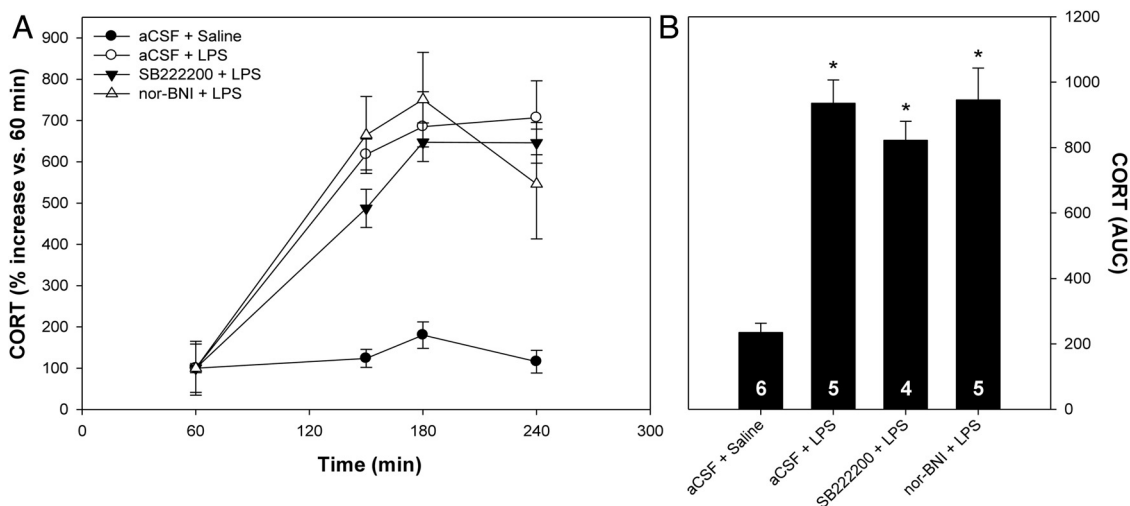
### Experiment 3a: Effect of AVPR1A or CRH receptor antagonism on senktide-induced suppression of pulsatile LH secretion

Senktide potently suppressed pulsatile LH secretion immediately following icv administration for a period of at least 2 hours, with a subsequent gradual recovery of the LH pulse seen in the second 2 hours postadministration (Figure 4, A and B, and Figure 5, A and B; LH pulse interval preinjection vs first 2 hours postinjection vs second 2

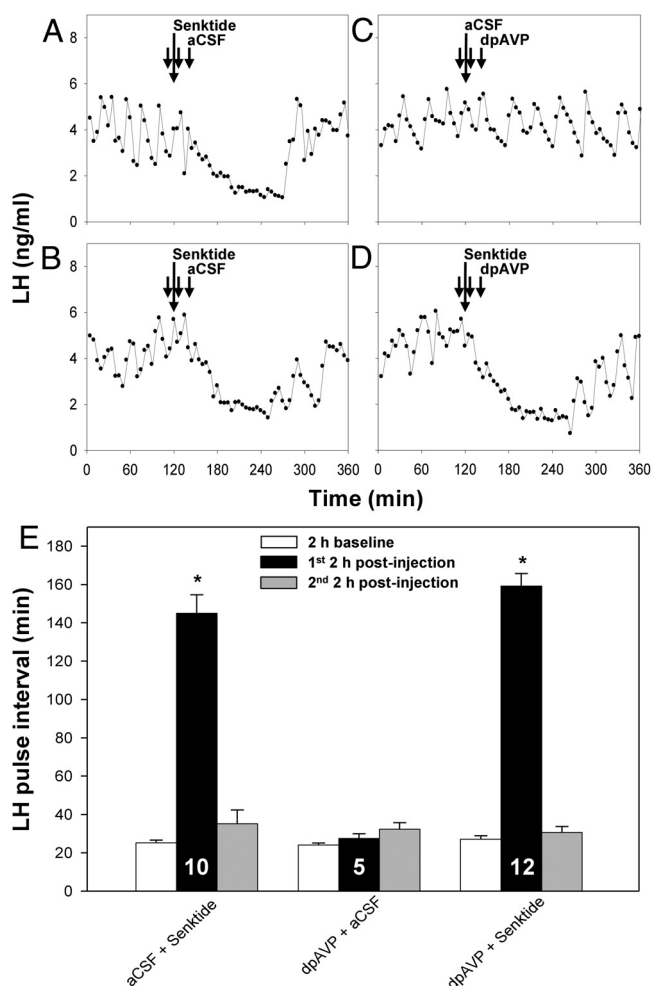
hours postinjection;  $25.2 \pm 1.5$  minutes vs  $145 \pm 9.6$  minutes vs  $35.2 \pm 7.2$  minutes;  $P < .05$ ). Senktide-induced suppression of LH secretion was neither altered by pretreatment with 3 consecutive injections of dpAVP (Figure 4D; LH pulse interval preinjection vs first 2 hours postinjection vs second 2 hours postinjection;  $27.2 \pm 1.7$  minutes vs  $159.2 \pm 6.5$  minutes vs  $30.6 \pm 3.1$  minute;  $P < .05$ ) nor that of either of the 3 CRH receptor antagonists, astressin (Figure 5D; LH pulse interval preinjection vs first 2 hours postinjection vs second 2 hours postinjection;  $28.3 \pm 2.8$  minutes vs  $161.6 \pm 11.7$  minutes vs  $30.8 \pm 3$  minutes;  $P < .05$ ), CP-152526 (Figure 5 F; LH pulse interval preinjection vs first 2 hours postinjection vs second 2 hours postinjection;  $29.6 \pm 2$  minutes vs  $132.5 \pm 6$  minutes vs  $28 \pm 1.5$  minutes;  $P < .05$ ) and astressin-2B (Figure 5H; LH pulse interval preinjection vs first 2 hours postinjection vs second 2 hours postinjection;  $30.1 \pm 4.7$  minutes vs  $156.3 \pm 9.9$  minutes vs  $30.6 \pm 3.7$  minutes;  $P < .05$ ). None of the pretreatment regimens affected the basal LH pulse when administered with aCSF instead of senktide. Histograms summarizing these experiments are provided (Figure 4E and Figure 5I) to enable comparison of the effects of AVPR1A and CRH receptor antagonists on pulsatile LH secretion in the presence of senktide.

### Experiment 3b: Effect of AVPR1A or CRH receptor antagonism on senktide-induced CORT secretion

Senktide induced a significant increase in plasma CORT levels (AUC, aCSF + aCSF vs aCSF + senktide;  $195.8 \pm 26.5$  vs  $640.8 \pm 82.3$ ;  $P < .05$ ), which peaked 30–60 minutes following administration and subsequently declined (Figure 6). This increase was completely



**Figure 3.** Effects of central NK3R or KOR antagonism on LPS-induced CORT secretion. Increase in plasma CORT levels (% vs baseline; panel A) in response to iv administration of LPS following pretreatment with vehicle (aCSF), a selective NK3R antagonist (SB222200), or a selective KOR antagonist (nor-BNI) in OVX rats. As depicted in the histogram showing mean AUC values for the experiment (B), the LPS-induced increase in CORT levels was not significantly affected by SB222200 or nor-BNI pretreatment. Number of replicates in each treatment group is indicated in white on the black bars in panel B. \*,  $P < .05$  vs negative control (aCSF + saline) group.



**Figure 4.** Effect of an AVPR1A antagonist on the senktide-induced suppression of pulsatile LH secretion. Representative LH profiles demonstrating the effect of icv administration of a selective NK3R agonist, senktide (panels A, B, and D), or vehicle (C), following pretreatment with the selective AVPR1A antagonist, dpAVP (panels C and D), or vehicle (panels A and B), on pulsatile LH secretion in OVX rats. The prolonged suppression of the LH pulse by senktide was unaffected by the intermittent administration of dpAVP, as summarized in panel E. Number of replicates in each treatment group is indicated in white on the black bars in panel E. \*,  $P < .05$  vs 2-hour baseline control period within the same treatment group, as well as vs the same 2-hour period within the vehicle-treated group.

blocked by pretreatment with nor-BNI (AUC, nor-BNI + senktide;  $194.8 \pm 19.1$ ), and partially ( $P > .05$ ) attenuated by pretreatment with either astressin (AUC, astressin-2B + senktide;  $379.8 \pm 176.4$ ) or astressin-2B (AUC, astressin-2B + senktide;  $376.6 \pm 87$ ). Neither dpAVP nor CP-154526 pretreatment altered the extent of senktide-induced CORT secretion (AUC, dpAVP + senktide and CP-154526 + senktide;  $758.6 \pm 109.8$  and  $783.6 \pm 40.2$ , respectively).

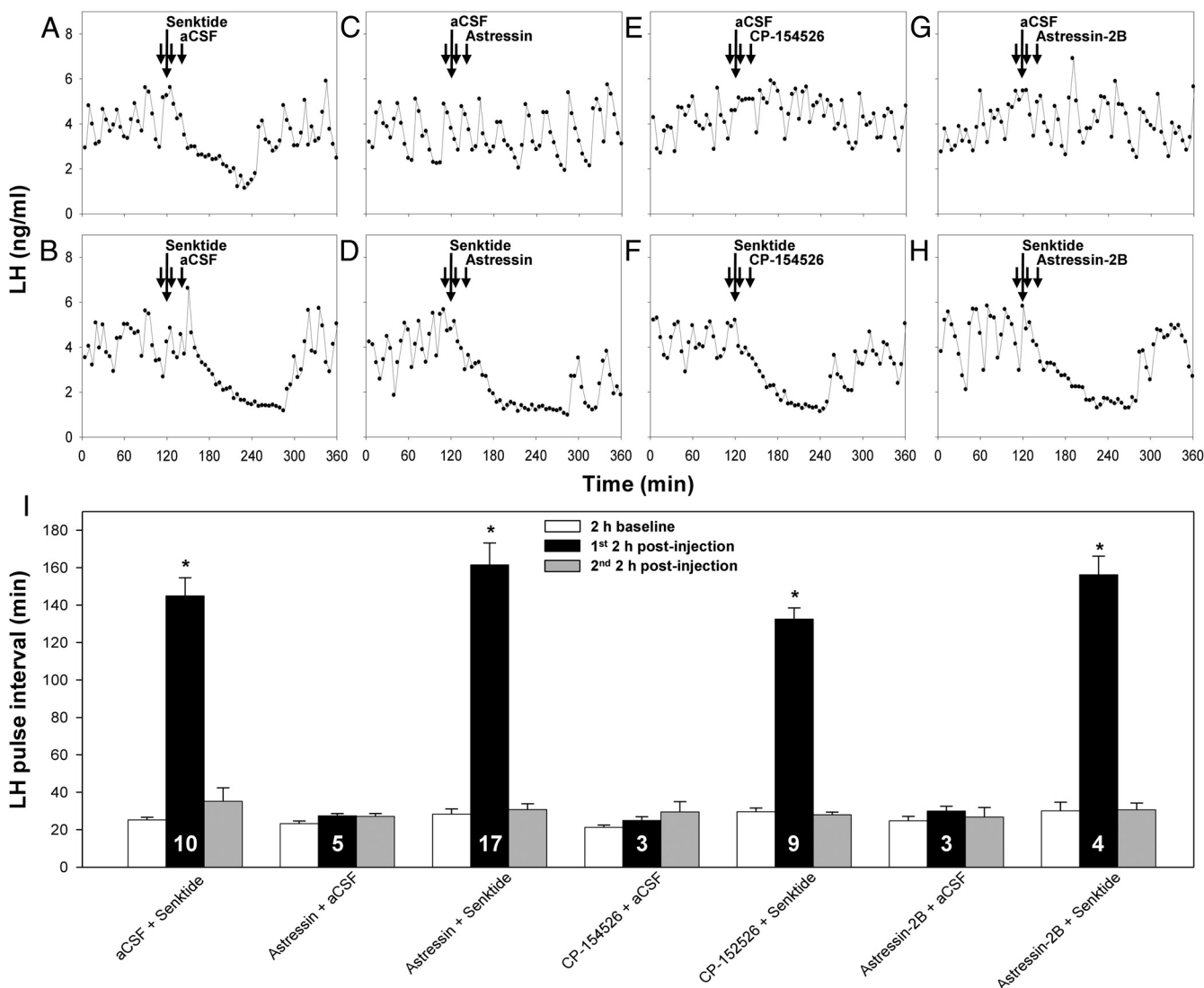
## Discussion

Intracerebroventricular administration of the NK3R antagonist, SB222200, blocked the suppression of pulsatile

LH secretion in OVX rats subjected to acute LPS stress. This blockade is attributable to the antagonistic properties of SB222200, rather than to any action of the vehicle, which contained 20% DMSO, because, like that of aCSF, icv administration of 4  $\mu$ L 10 or 100% DMSO did not affect LH secretion. Indeed, 2  $\mu$ L 100% DMSO has previously been shown to have no effect on the dynamics of pulsatile LH secretion in OVX rats (63). Because subsequent experiments featured drugs administered in a range of concentrations of DMSO, to simplify comparison between controls and drug treatments, we considered it prudent to pool the controls into a single group administered with aCSF in addition to other treatments.

The CRH signaling system is a well-established mediator of the suppression of the GnRH pulse generator induced by exposure to stress. Because astressin-2B had the same influence on the suppressive effects of LPS stress on the LH pulse (9) as SB222200, an interaction between the NKB/NK3R- and CRH/CRHR2-signaling systems may be responsible for the stress-induced suppression of the GnRH pulse generator, which is independent of subsequent adrenocortical activation (64). Furthermore, we have recently shown that intra-mPOA administration of a  $\gamma$ -aminobutyric acid (GABA) receptor type A antagonist also blocked the LPS-induced suppression of pulsatile LH secretion in OVX rats (65). In contrast, the inhibitory effect of restraint, a psychogenic stress paradigm, on LH pulse frequency was unaffected by the competitive selective NK3R antagonist, (D-Pro<sup>2</sup>, D-Trp<sup>6,8</sup>, Nle<sup>10</sup>)-NKB (Lin Y.S., X.F. Li, and K.T. O'Byrne, unpublished observations, 2012), or the selective antagonists of CRHR2 (9) or GABA receptor type A (65), but fully blocked by iv administration of a CRHR1 antagonist (9) or a GABA receptor type B antagonist administered intra-mPOA (65). Thus, evidently, distinct neural networks mediate the suppressive effects of different acute stressors on pulsatile LH secretion, with the mechanisms for LPS- and restraint-induced suppression of the LH pulse differentially involving hypothalamic NKB/NK3R-, GABA-, and CRH-signaling components.

Because of the apparent involvement of NKB/NK3R signaling in the mechanism of LPS-induced suppression of pulsatile LH secretion, and numerous lines of evidence suggesting interplay between KNDy neurons and CRH or AVP neurons of the PVN, we investigated whether NKB inhibition of pulsatile LH secretion in the OVX rat is the result of the involvement of central stress pathways. Unanesthetized, freely moving rats received repeated icv injections of CRH or AVP receptor antagonists (before and after icv senktide or vehicle infusion) to ensure effective and widespread receptor blockade. Neither antagonist affected basal pulsatile LH secretion when administered



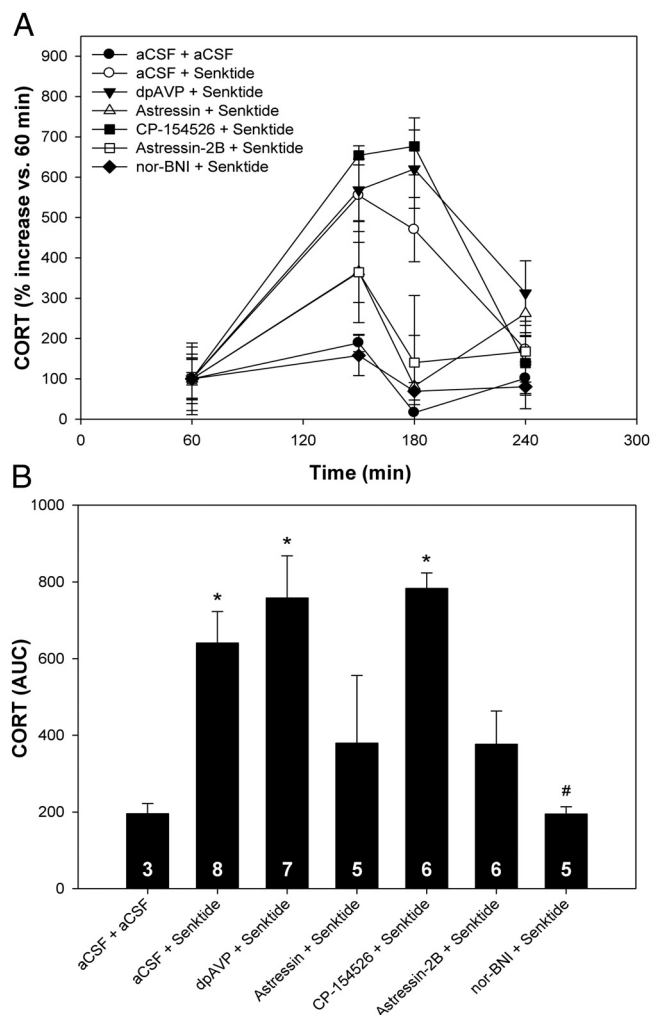
**Figure 5.** Effect of CRH receptor antagonists on the senktide-induced suppression of pulsatile LH secretion. Representative LH profiles demonstrating the effect of icv administration of a selective NK3R agonist, senktide (panels A, B, D, F, and H), or vehicle (panels C, E, and G), following pretreatment with 1) the nonselective CRH receptor antagonist, astresin (panels C and D); 2) the selective CRHR1 antagonist, CP-154526 (panels E and F), or 3) the selective CRHR2 antagonist, astresin-2B (panels G and H), on pulsatile LH secretion in OVX rats. The prolonged suppression of the LH pulse by senktide was unaffected by the intermittent administration of each CRH receptor antagonist, as summarized in panel I. White numbers within the black bars (I) denote the size of the treatment groups. \*,  $P < .05$  vs 2-hour baseline control period within the same treatment group, as well as vs the same 2-hour period within the vehicle-treated group.

with aCSF. These data indicate that under basal conditions (in the absence of acute stress) endogenous tones of AVP and CRH are not involved in the regulation of the GnRH pulse generator. Indeed, coadministration of CRHR1 or CRHR2 antagonists with saline has been previously shown to be without effect on the pulsatile release of LH in the female rat (9). Selective antagonism of AVPR1A, CRHR1, or CRHR2 receptors did not alter the robust suppression of pulsatile release of LH following icv administration of senktide. Simultaneous blockade of CRHR1 and CRHR2 receptors using a nonselective CRH receptor antagonist also had no effect on the senktide-induced suppression of the GnRH pulse generator. These

findings rule out the involvement of central AVP- and CRH receptor-mediated mechanisms in relaying the signal generated by NKB in the ARC to GnRH neurons, which was hypothesized to indirectly inhibit GnRH neurosecretion in female rats under hypoestrogenic conditions.

The role of Dyn as an intermediary effector of NKB inhibition of the LH pulse (32, 33, 66) prompted the hypothesis that any events that involve NK3R signaling and lead to the suppression of the GnRH pulse generator should also be Dyn/KOR dependent. For this reason, we investigated whether nor-BNI (administered icv, at a dose that has previously been shown to effectively block senktide-induced suppression of the LH pulse (32)) would





**Figure 6.** Effect of AVPR1A or CRH receptor antagonism on senktide-induced CORT secretion. Increase in plasma CORT levels (% vs baseline; panel A) in response to icv administration of senktide following pretreatment with vehicle (aCSF), a selective AVPR1A antagonist (dpAVP), a nonselective CRH receptor antagonist (astresin), a selective CRHR1 antagonist (CP-154526), a selective CRHR1 antagonist (astresin-2B), or a selective KOR antagonist (nor-BNI) in OVX rats. As depicted in the histogram showing AUC values for the experiment (panel B), the senktide-induced increase in CORT levels was blocked by nor-BNI, attenuated ( $P > .05$ ) by astresin and astresin-2B, and unaffected by dpAVP or CP-154526 pretreatment. Number of replicates in each treatment group is indicated in white on the black bars in panel B. \*,  $P < .05$  vs negative control (aCSF + aCSF) group; #,  $P < .05$  vs positive control (aCSF + senktide) group.

block the inhibition of LH secretion following LPS challenge and LPS- or senktide-induced CORT release. Although the KOR antagonist, nor-BNI, did not rescue the LH pulse suppressed by LPS challenge and did not affect LPS-induced CORT secretion, it was effective at blocking CORT secretion induced by senktide, which is concomitant with its blockade of senktide-induced suppression of the LH pulse (32, 33). However, these data disagree with the notion of Dyn/KOR-dependent suppression of the LH pulse by increased ARC NKB signaling (9, 32, 33) because

SB222200, but not nor-BNI, was able to block the inhibitory effect of LPS on the LH pulse. This discrepancy can be explained by the fact the central nervous system responses to systemic LPS challenge, even if NKB signaling is involved, are far more widespread than the comparatively limited effects of central NK3R activation. The suppressive effects of iv LPS administration on the GnRH pulse generator have previously been shown to involve the proinflammatory cytokines, TNF- $\alpha$  (67, 68) and IL-1 (68), nitric oxide (69), GABA (65, 69, 70), and the CRH-signaling system (reviewed in Reference 71). The mechanism by which these signaling molecules might trigger the secretion of NKB is currently unknown, but the complexity of this mechanism suggests that a higher dose of nor-BNI is necessary to rule out the involvement of a dynorphinergic component in the mechanism of LPS-induced suppression of the GnRH pulse generator. Indeed, LPS induced CORT secretion considerably more potently than senktide, resulting in a greater peak increase and a more persistent elevation. Moreover, although the mean pulse interval following LPS administration in animals pretreated with nor-BNI was not significantly different from that in positive (aCSF + LPS) controls, there was no detectable prolongation of the LH pulse in 2 of the 6 animals in this treatment group. Finally, CORT levels in the group treated with nor-BNI and LPS declined after peaking 1 hour postinjection, but plateaued in LPS-treated animals pretreated with aCSF or SB222200. For these reasons, based solely on the results of the present study, it might be premature to conclude that Dyn/KOR signaling is required for the elevation in CORT and suppression of pulsatile LH secretion in response to central senktide administration, but not LPS challenge.

Inflammation induced by LPS is a powerful activator of the HPA axis. Senktide also induced a robust increase in circulating CORT levels, indicative of potent HPA axis activation. This is in agreement with a previous report of raised plasma ACTH and CORT levels following sc administration of NKB (72). Furthermore, because SB222200 blocked the suppressive effect of LPS on the LH pulse, NKB is likely released in response to LPS and might contribute to the activation of pituitary corticotropes that underlies adrenal glucocorticoid secretion. However, SB222200 did not affect LPS-induced CORT release, suggesting that adrenocorticotrophic activation by senktide and LPS occurs via parallel, but distinct, mechanisms. Indeed, CORT levels in senktide-treated rats pretreated with nor-BNI were indistinguishable from vehicle-treated controls, whereas the same pretreatment failed to alter the rise in CORT levels after LPS challenge. Taken together these data demonstrate that LPS challenge simultaneously activates the HPA axis and suppresses the hypothalamic-

pituitary-gonadal axis by means of independent signaling pathways.

The observation that the senktide-induced increase in CORT levels was attenuated by CRHR2 antagonism suggests that activation of the HPA axis by the NK3R agonist, at least in part, involves downstream CRHR2 signaling. This notion is supported by the fact that senktide-induced increase in CORT levels was initially attenuated, but later (2 hours postsenktide) allowed to resume in rats pretreated with the nonselective CRH antagonist, astressin, whereas in those pretreated with the selective CRHR2 antagonist, astressin-2B, the CORT levels remained at baseline after the transient senktide-induced increase. Central administration of the selective CRHR1 antagonist, CP154526, did not significantly affect the extent of senktide-induced CORT release, contrary to the widely accepted notion that CRH (acting primarily through CRHR1) is the major hypothalamic ACTH secretagogue (56, 57). The preferential involvement of CRHR2 signaling is also a hallmark of the mechanism by which systemic stress (LPS) suppresses the LH pulse (65). Indeed, there are other striking similarities between the effects of acute exposure to LPS stress and central senktide administration in OVX rats: suppression of LH pulse (9, 32, 33) and multiunit activity volleys recorded from the mediobasal hypothalamus (67), induction of CORT secretion (9), and the aforementioned down-regulation of *Kiss1r* mRNA expression in the mPOA (4, 33). The present report, however, also provides the first evidence to indicate that a common mechanism might underlie these effects.

Data presented herewith suggest that NKB/NK3R signaling is involved in the suppression of the gonadotropic system following LPS challenge. Because CRH receptor antagonists did not affect the suppression of the LH pulse following central senktide administration, recruitment of the NKB-ergic mechanism appears to be consequential rather than causative of LPS-induced CRH neuron activation. Several lines of evidence substantiate this hypothesis: 1) intra-ARC injections of senktide suppressed LH pulses in the OVX + E<sub>2</sub> rat (33) in a dose-dependent manner; 2) an increase in c-Fos expression has been detected in the ARC of follicular phase ewes subjected to LPS challenge and coincided with augmented CRHR2 immunoreactivity in these regions (73); and 3) the ARC receives direct input from the CRH-rich BNST (74–77) that appears to induce activation of GABA-ergic neurons in response to CRH stimulation (15). The BNST is, in turn, innervated by glutamatergic CRH neurons of the central nuclei of the amygdala (78), the neurotoxic lesioning of which abolishes the LPS-induced suppression of the LH pulse in OVX + E<sub>2</sub> rats (14). Based on this evidence we propose that limbic activation of ARC NKB neurons un-

derlies the immunologic stress response, and that ARC NKB/NK3R signaling is responsible for adrenocorticotrophic stimulation and suppression of the hypothalamic-pituitary-gonadal axis following acute stress exposure. An alternative view is that LPS-induced, NK3R-dependent suppression of the LH pulse is secondary to characteristic changes in body temperature. Although a mechanism by which LPS-induced hypothermia might be able to suppress pulsatile LH secretion is unknown, a robust transient decrease in body temperature can be observed in rats administered systemically with LPS (at a dose equal to that used here) (79) or centrally (direct intra-median preoptic nucleus microinjection) with senktide (80). Additionally, icv injections of cannabinoid-1 receptor antagonists block LPS-induced hypothermia (79). It is, therefore, worth exploring whether NK3R antagonists prevent LPS-induced hypothermia and whether cannabinoid-1 receptor antagonists can block senktide-induced hypothermia and suppression of the LH pulse.

Although the data presented in this report support the conclusion that NKB is important for LPS inhibition of pulsatile LH release in OVX rats, NKB is generally stimulatory to LH secretion in ovary-intact rats (32, 34, 81), sheep (82, 83), and humans (84). Changes in gonadal steroid feedback triggers remodeling of ARC neurons (85), which probably accounts for the reversal of the effects of NKB after ovariectomy. In light of the present findings, it is important to assess the involvement of NKB in stress-induced suppression of pulsatile LH secretion in ovary-intact animals. Although little is currently known about the effects of stress on the reproductive physiology of ovary-intact rats, perhaps due to the difficulty of profiling LH pulses in the presence of endogenous ovarian steroids, it is well recognized that exposure to stress has detrimental effects on reproductive function in ovary-intact sheep (73), monkeys (86, 87), and women (88). In the follicular-phase ewe, LPS disrupted sexual behavior and prevented the LH surge, which correlated with the lack of ARC kisspeptin neurons in LPS-treated animals (73). The accompanying doubling of the number of c-Fos-immunoreactive nuclei in the ARC (73), taken in the context of the present report, raises the possibility that nonkisspeptin NKB/Dyn neurons in this region signal in an autocrine/paracrine fashion to restrict NKDy neuron stimulation of GnRH secretion. KNDy neurons are indeed NK3R- (89) and KOR-positive (90), and senktide and the KOR agonist, U50488, have been shown to differentially affect the rate of KNDy neuron spontaneous firing in murine brain slices (91). Future studies should therefore address the involvement of NKB/NK3R and Dyn/KOR signaling in suppressing the activity and stimulatory secretions of kisspeptin

neurons in ovary-intact rats and sheep acutely exposed to experimental stressors.

## Appendix

Artificial cerebrospinal fluid (aCSF) was made up as follows: 100 ml deionized water, 0.724g NaCl, 0.246g KCl, 0.0163g KH<sub>2</sub>PO<sub>4</sub>, 0.214g NaHCO<sub>3</sub>, 0.18g D-Glucose, 0.0368g CaCl<sub>2</sub>, 0.025g MgSO<sub>4</sub>. The salts were mixed with deionized water in the order listed above. The next salt was only added after the previous one had fully dissolved. The solution was then filtered through a sterile filter (Millipore, Watford, UK), aliquoted and stored at -20 C.

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