Do Substance P and Neurokinin A play important roles in the control of LH secretion in ewes?

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Abstract

There is now general agreement that neurokinin B (NKB) acts via NK3R to stimulate secretion of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) in a number of species, including rats, mice, sheep, and humans. However the roles of two other tachykinins, substance P (SP) and neurokinin A (NKA), which act primarily via NK1R and NK2R respectively, are less clear. In rodents, these signaling pathways can stimulate LH release and substitute for NKB signaling; in humans, SP is colocalized with kisspeptin and NKB in the mediobasal hypothalamus. In this study, we examined the possible role of these tachykinins in control of the reproductive axis in sheep. Immunocytochemistry was used to describe the expression of SP and NK1R in the ovine diencephalon and determine if these proteins are colocalized in kisspeptin or GnRH neurons. SP-containing neurons were largely confined to the arcuate nucleus (ARC), but NK1R-immunoreactivity was more widespread, with relatively high expression in the lateral preoptic area, the ventromedial nucleus, and the ARC. However, there was very low coexpression of SP or NK1R in kisspeptin cells and none in GnRH neurons. We next determined the minimal effective dose of these three tachykinins that would stimulate LH secretion when administered into the third cerebral ventricle of ovary-intact anestrous sheep. A much lower dose of NKB (0.2 nmoles) than of NKA (2 nmoles) or SP (10 nmoles) consistently stimulated LH secretion. Moreover, the relative potency of these three neuropeptides parallels the relative selectively of NK3R. Based on these anatomical and pharmacological data, we conclude that NKB-NK3R signaling is the primary pathway for the control of GnRH secretion by tachykinins in ewes.

Introduction

Recent studies (1-3) on the reproductive effects of mutations in the *TAC3* gene that encodes neurokinin B (NKB) or in the gene (*TACR3*) that encodes its receptor (NK3R) have focused the attention of reproductive neuroendocrinologists on the possible roles of tachykinins in control of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle stimulating hormone (FSH) secretion. In addition to NKB, two other tachykinins have been implicated in the control of reproduction: substance P (SP) and neurokinin A (NKA). These peptides are both encoded by the same gene (*TAC1)* and act via NK1R and NK2R, respectively (4,5). At this time there are no reports that mutations in *TAC1* or the genes for NK1R or NK2R produce reproductive deficits in humans, but other approaches have implicated these two tachykinins in the control of GnRH secretion (4).

A stimulatory role for NKB in control of GnRH in humans was first proposed in 1991 based on the increase in NKB expression in post-menopausal women within the infundibular nucleus (6), which is analogous to the arcuate nucleus (ARC) in other species. These cells also contain kisspeptin and dynorphin and thus are now known as KNDy neurons (7,8). More recently, work across a number of species, spurred on by the human genetic studies, has produced considerable evidence in support of this hypothesis. In most studies NKB, or more often senktide (an NK3R agonist), stimulated LH secretion via GnRH in gonadally-intact animals or when LH secretion was suppressed with exogenous steroids (9-13). Conversely, antagonists to NK3R inhibited LH secretion in ovariectomized (OVX) sheep (14-16), castrated monkeys (16), and normal men and women (17). Interestingly most, but not all (18), studies indicate that these stimulatory effects are mediated by kisspeptin released from KNDy neurons. Thus, KNDy neurons contain NK3R (19-21) and NKB, or senktide, increased their activity, based on Fos expression, in vivo (22,23) or electrical activity in hypothalamic slices (13,24,25). Moreover, these agonists did not stimulate LH secretion in the absence of Kiss1r in mice (26), in the presence of a Kiss1r antagonist in rats (27), or when Kiss1r was down-regulated in primates (28). In contrast to these stimulatory effects of senktide when endogenous GnRH secretion is low, this NK3R agonist inhibits LH

secretion in OVX rats (29,30) and mice (21), probably by stimulating dynorphin release from KNDy neurons (31).

There are fewer studies on the roles of SP and NKA, but recent work in rodents indicates that these tachykinins can have effects similar to NKB on LH and FSH secretion. Thus NKA and SP increased the firing rate of murine KNDy neurons in vitro (25) and specific NK1R or NK2R agonists increased LH and FSH secretion in rats (32) and mice (12), with the latter effects being dependent on Kiss1r signaling (12). Similarly knockout of *Tac1* delayed puberty and decreased ovulation rate and the number of pups/litter in mice (33). Finally, there may well be some redundancy among the three tachykinin signaling pathways in rodents because blockade of all three receptors is required to inhibit LH secretion in OVX rats (34). Similarly, stimulatory effects of NKB on the electrical activity of murine KNDy neurons in vitro is not blocked by each selective NKR antagonist alone, but is blocked by a cocktail of all three receptor antagonists (25).

The limited work on SP and NKA in other species has largely focused on the former. In humans, infusion of SP stimulated LH, but not FSH, secretion (35) and SP expression in the infundibular nucleus is higher in post-menopausal women than in pre-menopausal women (6) and higher than expression in both young and aged men (36). Immunocytochemical (ICC) studies using tissue from post-menopausal women indicated that 25% of NKB-containing and 31% of kisspeptin-containing cell bodies also expressed SP (36) and similar colocalization was observed in close contacts with GnRH axonal fibers in the median eminence (37). On the other hand, in male rhesus monkeys, SP was not found in kisspeptin-containing neurons and iv injection of SP failed to stimulate LH secretion (38). Moreover, an NK1R antagonist increased both LH and FSH concentrations during the descending phase of an estrogen-induced surge in cynomologus monkeys, suggesting a possible inhibitory effect of SP (39).

Studies in sheep have provided important information on the expression and actions of NKB (8,9,14-16,19,40), but there is no information in this species on the role of other tachykinins in control of GnRH secretion. This work addressed this gap in our knowledge by: 1) describing the expression of SP and NK1R in the ovine preoptic area (POA) and hypothalamus, 2) determining if either was colocalized

with kisspeptin- or GnRH-containing neurons and the effect of estradiol (E_2) on their expression, and 3) comparing the minimal dose of NKB, SP, and NKA required to stimulate LH secretion in ovary-intact anestrous ewes. We chose to monitor LH concentrations because they provide a reliable index of episodic GnRH secretion. In contrast, patterns of FSH do not reflect endogenous GnRH pulses (41) and FSH elevations are not seen in response to exogenous GnRH injections that produce LH pulses (41,42).

Materials & Methods

Animals

Adult (4-8 years of age) multiparous blackface ewes of predominantly Suffolk breeding were maintained in an open barn and moved indoors 3–7 days prior to surgeries. Ewes were fed a pelleted alfalfa diet to maintain weight (65-85 kg) and provided free access to water and supplemental minerals. Lighting was adjusted bimonthly to mimic the duration of natural day light. All experiments used anestrous ewes and were performed between the middle of April and the end of July.

Surgical and blood collection procedures

All surgeries were performed under aseptic conditions using 2-4 % isofluorane in oxygen for anesthesia. For OVX, ovaries were exposed via mid-ventral laporatomy, the blood supply ligated, and the ovaries removed. Any blood clots adhering to the uterus or oviducts were then removed with sterile saline, these organs returned to the abdomen, and the peritoneum and skin were sutured closed. For intracerebroventricular (icv) administration of tachykinins, an 18-gauge stainless steel cannula was stereotaxically placed into the third ventricle, cemented in place with dental acrylic, protected with a plastic cap, and the hub plugged to prevent CSF backflow (43). All ewes were treated with dexamethasone and penicillin from 1 day before to 5 days after surgery, and with analgesic (125 mg; Banamine, Phoenix Pharmaceutical, St. Joseph, MO) at the time of anesthesia induction and for 5 days after surgery. Animals were allowed to recover from surgical procedures for at least 7 days before any experimental treatments. Jugular blood samples (3-4 mL) were taken by venipuncture, placed in heparinized tubes, and plasma collected and stored at -20 C until assayed for LH. All procedures were

approved by the West Virginia University Animal Care and Use Committee and conducted in accordance with NIH guidelines on the care and use of animals in research.

Tissue collection

Paraformaldhyde-fixed tissue was collected for immunocytochemistry and histological determination of treatment sites. Ewes were injected with two doses of 20,000 units of heparin (10 mins apart) and then euthanized with an overdose (8–12 mL, iv) of sodium pentobarbital (Euthasol; Patterson Veterinary, Devens, MA). The head was removed when the animal had stopped breathing and perfused via the internal carotids with 6 L of 4 % paraformaldehyde in 0.1 M phosphate buffer containing 0.1 % $NaNO_3$. Tissue blocks containing hypothalamus and preoptic area (POA) were removed and stored in fixative at 4 °C overnight and then in 20% sucrose. After sucrose infiltration, 45-μm-thick frozen coronal sections were cut using a freezing microtome. For ICC, 6 parallel series of sections (270 μm apart) were stored at -20 °C in cryoprotectant.

Experiments 1 and 2. Distribution, colocalization with kisspeptin or GnRH and effect of E_2 on SP and NK1R expression.

All immunohistochemical studies were conducted on tissue collected from a cohort of anestrous ewes that did not undergo intracranial surgeries. Anestrous animals were OVX, as described above, and a 3 cm long E_2-containing Silastic implant was inserted subcutaneously (s.c.) (OVX+ E_2; n=5) or sham inserted (OVX; n=5) at the end of the surgical procedure. Animals were perfused, as described above, and tissue was collected 10 days later.

Experiment 3. LH dose-response to NKB in ovary-intact anestrous ewes

Chronic cannulae were placed in the third ventricle of ovary-intact anestrous ewes (n=4) in the middle of April. This experiment was done in ovary-intact ewes to avoid multiple survival surgeries. A stock solution of NKB (Tocris Bioscience, Ellisville MO) was prepared the day before the first treatments by dissolving 0.6 mg NKB in 0.375 mL of 0.1 N NaOH (1 nmole/μL) (44). Aliquots of this stock solution were stored at -20 C, and then thawed and diluted with sterile artificial CSF (aCSF) (45) on the day of

146 treatments to produce concentrations ranging from 0.05 to 0.5 nmoles/100 μL. Because the stock was
147 diluted 1:200 to prepare the highest dose of NKB, we used 0.1 N NaOH diluted 1:200 with aCSF as
148 vehicle for the 0 nmole treatment. This study was originally designed to determine a dose of NKB that
149 would reliably induce a physiological LH pulse for analysis of receptor turnover (46), so LH was only
150 monitored for 2 hr after injection. Starting 9-10 days after surgery, blood samples were collected every
151 12 min from 24 min before to 2 hrs after icv injection (100 μL) of 0 (100 μL of vehicle), 0.05 nmoles
152 NKB, 0.1 nmoles NKB, 0.2 nmoles NKB, or 0.5 nmoles NKB. This protocol was then repeated four
153 more times, with 3-5 days between treatments so that all animals received all five treatments in a random
154 order. Tissue was collected after the last treatment and location of cannulae in the third ventricle
155 confirmed. In a follow-up experiment the response to 0.2 nmoles of NKB was assessed in a separate set of
156 five ovary-intact anestrous ewes using this protocol.
157 *Experiment 4. What doses of NKA and SP are needed to stimulate LH secretion in ovary-intact anestrous*
158 *ewes?*
159 Chronic third ventricle cannulae were implanted in 5 ovary-intact ewes in early June. This experiment
160 was designed to test two doses of NKA and SP. Based on the results of Exp. 3, and the relative potency
161 of NKB, SP, and NKA in stimulating electrical activity of KNDy neurons in mice (25), we first compared
162 the effects of 2 nmoles of NKA and SP with vehicle controls. We then planned to either increase or
163 decrease the dose of these tachykinins based on the effects at this dose, which resulted in administration
164 of 0.5 nmoles NKA and 10 nmoles of SP to these ewes in the second part of this experiment. Stock
165 solutions of 0.5 nmoles tachykinin/μL of aCSF were prepared before the first treatment, aliquots frozen,
166 and diluted with aCSF to the appropriate concentration on the morning of all treatments. Starting two
167 weeks after neurosurgery, ewes received icv injections of either aCSF, 2 nmoles NKA, or 2 nmoles SP in
168 random order with 3-4 days between treatments. LH concentrations were measured in plasma samples
169 collected from 36 min before to 4 hr after injections. One week after the last set of injections, samples
170 were again collected for 36 min before to 4 hr after injection of either 0.5 nmoles NKA or 10 nmoles SP,

and this treatment repeated with a cross-over design so that each ewe received both treatments. At the end of the experiment, tissue was collected to confirm that cannulae were in the third ventricle.

Immunohistochemistry

Free floating, double-label, immunofluorescence histochemistry was performed in order to determine the distribution of SP and NK1R, and investigate their potential colocalization with kisspeptin and/or GnRH in the sheep POA and hypothalamus. Furthermore, we processed tissue sections from OVX and OVX+ E_2 ewes to determine whether E_2 regulates protein expression of SP or NK1R. Hence, a series of every sixth section, extending from the level of the optic chiasma to the mammillary bodies, was processed for each of the following four combinations: a) SP and kisspeptin, b) NK1R and kisspeptin, c) SP and GnRH, or d) NK1R and GnRH using a modified protocol previously described and routinely used in our laboratory (8). Briefly, all steps were performed at room temperature and with gentle agitation, and tissue sections were washed with 0.1M phosphate buffered (pH 7.35) saline (PBS) between each step. Antibodies were diluted in PBS^+, a solution consisting of 0.1M PBS, 0.4% Triton X-100 (Sigma-Aldrich, St. Louis MO) and 4% normal goat serum (Jackson ImmunoResearch Labortories, West Grove, PA). Before the application of the first primary antibody, sections were treated with 1% hydrogen peroxide (H_2O_2) for 10 min followed by PBS^+ for 1 hr to prevent nonspecific background labeling. Tissue sections were then incubated sequentially with: 1) guinea pig anti-SP (1:4,000, Abcam, Cambridge, MA; Table 1) or rabbit anti-NK1R (1:10,000, Millipore, Billerica, MA; Table 1) for 17 hr, 2) biotinylated goat anti-guinea pig IgG (1:500, Vector Laboratories, Burlingame, CA) or anti-rabbit IgG (1:500, Vector Laboratories), respectively, for 1 hr, 3) avidin and biotinylated horseradish peroxidase complex (Avidin-Biotin Complex, 1:500, Vector Laboratories) for 1 hr, 4) biotinylated tyramine (1:250, PerkinElmer, Waltham, MS), containing 0.003% H_2O_2 for 10 min, and 5) Alexa Fluor 555 conjugated streptavidin (1:100, Invitrogen, Carlsbad, CA) for 30 min. Tissue was protected from light from this step forward. Next, sections were incubated with rabbit anti-kisspeptin (1:1,000, Millipore; Table 1) or rabbit anti-GnRH (1:400, Immunostar, Hudson, WI; Table 1) for 17 hr. After overnight incubation, sections were washed and incubated with goat anti-rabbit DyLight green (1:100, Vector Laboratories) for 1 hr. Tissue

sections were mounted onto slides, air dried, coverslipped with Gelvatol mounting medium, and stored at 4°C until analysis. Negative controls were routinely performed by omission of primary antibody, which eliminated all labeling corresponding to that antigen. In addition, specificity of the SP antibody was tested using peptide blocking controls (47). In short, preabsorption (overnight at 4^0C) of the guinea pig-anti-SP antibody with 15 g/ml of the corresponding SP peptide (Abcam, catalog item ab38217) abolished all staining in the ovine POA and hypothalamus. No blocking peptide was available for the NK1R antibody, but the complete mismatch with the distribution of NK3R (see Results) and reported absence of NK2R in the hypothalamus (48) support the specificity of this antibody.

Image capture and analysis.

The distribution of SP and NK1R was examined in sections through the POA and hypothalamus of each ewe. Colocalization of SP or NK1R with kisspeptin or GnRH as well as the comparison of total cell number between OVX and OVX+ E_2 ewes, was evaluated in sections containing rostral, middle, or caudal levels of the ARC (four sections each), or POA (six to eight sections each) at 20X magnification. We used a digital camera (Microfire A/R, Optronics, Goleta, CA) attached to a microscope (DM500B, Leica Microsystems, Wetzlar, Germany), with the appropriate excitation for DyLight 488 (green fluorescent protein) and Alexa 555 (red fluorescent protein) and Neurolucida software (MicroBrightfield Bioscience, Williston, VT) to superimpose the two images and determine colocalization. For each section, the total number of single and double labeled (Kiss+SP, Kiss+NK1R, GnRH+SP, GnRH+NK1R) cells were counted by flipping through the green and red channels. Counts were averaged per ewe, per brain area. Percentages of the total number of kisspeptin- or GnRH-ir cells containing SP or NK1R as well as the percentages of SP- or NK1R-ir cells containing kisspeptin or GnRH were calculated for each section, and averaged per ewe, per brain area.

In addition to cell counts, the effects of E_2 on SP fiber density in the ARC was examined because SP cell bodies where observed infrequently. A midlevel section of the ARC was selected for imaging and quantification of the density of SP-containing fibers. Fibers were visualized and photographed with a 20X objective lens (Microfire A/R). Data from these images were then analyzed using Image J software (NIH,

Bethesda, MD). A standardized threshold was applied to all images and the proportion of immunolabeling above threshold was quantified for each section. Therefore the data represent the proportional area occupied by labeled fibers, not the specific number of fibers. Two images per animal were analyzed and these data averaged to obtain values for that animal. Group data are expressed as the mean ± SEM.

LH Assay

Ovine LH concentrations in plasma (200 μL) were measured in duplicate with a RIA using reagents provided by the National Hormone and Peptide Program as previously described (49). The sensitivity of the assay averaged 0.04 ng/tube (NIH S24) and the intra- and interassay coefficients of variation were 5.9% and 9.8%, respectively.

Statistical analyses

For Exp. 2, the number of single-labeled kisspeptin, GnRH, or NKR1 perikarya were compared between OVX and OVX+ E_2 groups using a two-way ANOVA (region and hormone treatment as main factors) and Holm-Sidak posthoc test, whenever appropriate. Densities of SP fibers in the ARC were compared between OVX and OVX+ E_2 groups by t-test. For analysis of the effects of tachykinins on LH secretion (Exp. 3 and 4), an increase in LH concentrations following injection of a tachykinin was considered to be a response if it occurred within 2 samples of the injection and peak LH concentrations were two SD above the preinjection value, based on assay variability. For Exp. 3, mean LH concentrations before and for 0-2 hrs after injection were compared by two-way ANOVA with repeated measures (time and dose of NKB were the main factors), and the Holm-Sidak test was used for comparisons within treatments. LH pulse amplitudes and frequencies after injection were determined using established criteria for LH pulses (50). Amplitudes were analyzed by one-way ANOVA with repeated measures and pulse frequencies by the non-parametric Kruskal-Wallis one-way ANOVA on Ranks. For Exp. 4, mean LH concentrations before, for 0-2 hrs after, and for 2-4 hrs after injection were analyzed for NKA and SP separately using a similar two-way ANOVA with repeated measures. Initial analysis of LH pulse frequencies and amplitudes in the

two time bins post-injections indicated no effect of time, so these were analyzed using a simple repeated-measures for the 4 hrs post-injection period. Specifically, amplitudes were analyzed by one-way ANOVA with repeated measures and pulse frequencies by the non-parametric Friedman's repeated measures ANOVA. $P<0.05$ was considered statistically significant.

Results

Experiment 1. Distribution of SP and NK1R and colocalization with kisspeptin or GnRH in the POA and hypothalamus.

SP-immunopositive perikarya were observed very infrequently in the mediobasal hypothalamus of OVX and OVX+ E_2 ewes, with more cell bodies identified in the caudal region of the ARC (range 0-10 cell bodies) compared to middle (range 0-4 cell bodies) and rostral (range 0-1 cell bodies) regions (Fig. 1 and 2). No SP perikarya were observed in the POA (Fig. 1 and 2B). On the other hand, fibers immunopositive for SP were observed throughout the entire length of the POA and hypothalamus. In light of the paucity of SP-containing perikarya, it is not surprising that little colocalization of SP and kisspeptin was observed. Specifically, colocalization of these two peptides in ARC cell bodies (see Supplemental Fig. 1) was only observed on three occasions (over 2300 kisspeptin cell bodies analyzed from 10 ewes). However, both populations of kisspeptin cells (ARC and POA) and their fibers were surrounded by a plethora of SP immunopositive fibers (Fig. 2A and 2B).

NK1R-containing cells were observed in the diagonal band of Broca, inner outline of the globus pallidus, anterior hypothalamic area, lateral hypothalamic area, dorsomedial hypothalamus, ventromedial nucleus, ARC and premammillary nucleus (Fig. 1). Of note, even though NK1R immunopositive cells were numerous throughout the hypothalamus, they were observed more sparsely in the POA (Fig. 2C-2D and Fig. 3C-3D). Within the ARC, expression of cell bodies was concentrated mainly in the rostral and caudal regions (average 34 cell bodies for each) and less in the middle portion of this nucleus (average 18 cell bodies). Analysis of dual-labeled kisspeptin and NK1R staining revealed infrequent colocalization of these

two proteins in the ARC (Fig 2C). Specifically, only 6.2 ± 3.3% of kisspeptin neurons were seen to contain NK1R, and conversely, only 4.6 ± 1.7% of NK1R containing neurons detected in the ARC also expressed kisspeptin. Similarly to SP, NK1R containing fibers in the ARC where observed in close proximity to kisspeptin cells (Fig. 2C).

Quantitative analysis of sections throughout the POA and MBH, revealed no colocalization of GnRH in SP or NK1R-containing neurons (Fig. 3; over 100 GnRH cell bodies analyzed from 10 ewes, for each set of double-label staining). Similarly, double-labeled fibers were not observed in the ARC or POA. However, GnRH cells and fibers were intimately surrounded by SP and NK1R immunopositive fibers in the mediobasal hypothalamus (Fig. 3 and supplemental Fig. 2).

Experiment 2. Effect of E_2 *on SP fiber density and NK1R expression in the ARC.*

As expected, the presence of E_2 downregulated kisspeptin expression in the rostral, middle and caudal aspects of the ARC (Fig. 4) but had no effect on total GnRH cell numbers (3.9 ± 0.6 and 4.4 ± 1.3 cells in OVX and OVX+ E_2 ewes, respectively). Quantification of SP fiber density did not reveal differences between OVX and OVX+ E_2 ewes (4.23 ± 1.96 and 4.92 ± 1.26 % of analyzed area above the threshold for OVX and OVX+ E_2 ewes, respectively). Similarly, E_2 had no effect on NK1R immunoreactivity in the rostral, middle or caudal aspects of the ARC (Fig. 4). In the POA, kisspeptin expression was upregulated by E_2 (12.3 ± 4.2 and 38.7 ± 4.2 cells for OVX and OVX+ E_2 ewes, respectively; $P<0.02$) whereas there was no effect on GnRH expression (18.4 ± 2.7 and 21.4 ± 1.8 cells for OVX and OVX+ E_2 ewes, respectively). No cell bodies immunopositive for SP or NK1R were observed in the vicinity of GnRH neurons in the POA and therefore this area was not included in the analysis.

Experiment 3. LH dose-response to NKB in ovary-intact anestrous ewes

None of the ewes treated icv with 0 nmoles NKB had an increase in LH concentrations that met the criteria for a response and there was only one animal that responded to 0.05 nmoles NKB, whereas doses of 0.1, 0.2, and 0.5 nmoles NKB produced a response 100% of the time. Based on two-way ANOVA there was a significant effect of time, dose, and dose by treatment interaction; there were no significant differences in pre-injection LH concentrations, but mean LH concentrations during the 2 hrs post-

injection of either 0.2 or 0.5 nmoles NKB were significantly greater than those following control injections (Fig. 5). LH pulse frequency was increased (compared to control injections) when animals were injected with 0.1, 0.2, or 0.5 nmoles, but not when they received the lowest does of NKB (Table 2). There were no significant differences among groups in LH pulse amplitude, but only data from the three highest doses were analyzed because there were too few pulses in ewes receiving 0 and 0.05 nmoles NKB. In the follow-up study, 0.2 nmoles NKB again induced a response in all five ewes and increased LH from 1.9 ± 0.3 ng/ml before injection to 3.1 ± 0.4 ng/ml in the 2 hrs post-injection (P=0.015, paired t-test). Pulse frequencies averaged 1.4 ± 0.2 pulses/2hr and amplitudes were 2.5 ± 0.5 ng/ml after the injection in these animals.

Experiment 4. What doses of NKA and SP are needed to stimulate LH secretion in ovary-intact anestrous ewes?

No ewes responded to icv injection of aCSF with an increase in LH concentrations, although occasional LH pulses occurred either before or 1-3 hrs after injection (Fig 6). In contrast 80% of the animals responded to icv injections of 2 nmoles of NKA with a robust and prolonged increment in LH, but such a response was only observed in 1 of the 5 animals given 2 nmoles SP icv (Fig. 6, left panels). Consequently, we next tested 0.5 nmoles NKA and 10 nmoles SP (Fig 6, right panels). With this lower dose of NKA, one ewe had a robust response immediately following injection of the tachykinin, while three ewes responded to the higher dose of SP, and the other two appeared to have a somewhat delayed increase in episodic LH secretion (Fig 6, right panels).

Statistical analysis of mean LH concentrations indicated there was a dose-response to NKA, with 2 nmoles, but not 0.5 nmoles, producing a significant increase in LH concentrations (Fig. 7A). However, this response was fairly brief as LH returned to pre-injection values during the 2-4 hr period. A similar dose-response in the effects of NKA on LH pulse frequency after injection was seen, but NKA had no significant effects on LH pulse amplitude (Fig. 7B).

SP also produced a significant increase in mean LH concentrations in the first 2 hr period post-injection, but this was only seen with the 10 nmole dose of this tachykinin (Fig. 8A). Although LH values were also higher during the 2-4 hrs after injection of 10 nmoles SP, this was not significant because of increased variability. However, these effects of SP on mean LH concentrations were not reflected in either LH pulse frequency or pulse amplitude as neither parameter was significantly increased at either dose (Fig. 8B)

Discussion

This is the first detailed description of neurons containing SP and its cognate receptor, NK1R, in the ovine hypothalamus. Although SP was found in hypothalamic areas critical to the control of GnRH and LH secretion, the absence of NK1R in either GnRH- or kisspeptin-containing neurons argues against an important role for this tachykinin in control of reproductive function. The pharmacological data demonstrating that much higher doses of SP and NKA, than of NKB, are needed to stimulate LH secretion also supports this conclusion.

Although there has been considerable work describing the expression of SP in the ovine peripheral nervous system and one report of SP-immunoreactive cells in the pars tuberalis of sheep (51), there has been no description of SP-containing neurons in the hypothalamus of this species. We observed that these neurons were largely limited to the ARC, a distribution similar to that reported in humans (52) and monkeys (38,53). In contrast, SP-containing neural cell bodies are found in several other hypothalamic regions in rodents, including the POA, anterior hypothalamic area, and premammillary region (PMR) (12,54,55). It is important to point out, however, that an ICC analysis may not detect all SP-producing neurons. For example, in situ hybridization identified cells containing mRNA for SP in the POA and PMR of humans (56) and there is a similar mismatch between mRNA and protein expression for dynorphin in some regions of the ovine hypothalamus (57). In contrast to the limited distribution of SP-immunoreactive cells, NK1R-expressing cells were observed in several different regions of the ovine diencephalon, with relatively high expression in the lateral POA, the ventromedial nucleus, the caudal

ARC, and the PMR. A similar distribution of NK1R has been observed in rats (58) and guinea pigs (59). Although NK1R has been detected in the human hypothalamus (60), there is no detailed description of its expression in specific hypothalamic nuclei.

In contrast to the high level of colocalization of NKB and kisspeptin previously reported in the ovine ARC (8), we found that very few kisspeptin-containing neurons in this nucleus also expressed SP and no colocalization of SP and kisspeptin in the POA was observed. These observations conflict with the recent report that SP-immunoreactivity was found in 30% of kisspeptin neurons in the human infundibular region (36), but are consistent with the lack of *Tac1* mRNA in *Kiss1* neurons in either the AVPV/PeN or ARC of mice (12). These data raise the possibility of differences among species in expression of SP in kisspeptin neurons. In this regard it is interesting to note that essentially no colocalization of these two peptides was recently observed in tissue from gonadally-intact and castrated male monkeys (38). It is also unlikely that endocrine status can account for these differences as tissue from post-menopausal women, OVX mice, and castrated monkeys were used in these three studies.

In light of the accumulating evidence that activation of NK1R signaling stimulates LH secretion in rodents (see Introduction), we hypothesized that NK1R would be found in either GnRH or kisspeptin neurons in the sheep. However, our data do not support this hypothesis: no GnRH neurons or POA kisspeptin neurons contained NK1R, and only 6% of KNDy neurons contained this receptor. In mice, 49 % of KNDy neurons, 27% of AVPV/PeN *Kiss1* neurons, and 23% of GnRH neurons also contain *Tacr1* mRNA, based on single cell RT-PCR analysis (12); data on the expression of NK1R in these neurons is not currently available in any other species. It is possible that immunocytochemistry failed to detect NK1R in our study, but the similar anatomy of NK1R-containg neurons in rodents and sheep, and the robust expression of NK1R in ARC neurons not containing kisspeptin argue against this. Moreover, using a similar approach we have observed that the majority of KNDy neurons contain NK3R in sheep (19). Thus these anatomical differences in NK1R expression most likely reflect functional differences in the role of NK1R signaling between these species. Finally, although we did not examine expression of

NK2R in this study, previous work found no expression of NK2R in the hypothalamus of rats (48) and no coexpression of this receptor in murine kisspeptin or GnRH neurons (12).

In the second part of this study we determined the minimal dose of NKB, NKA, and SP needed to stimulate LH secretion when given into the third ventricle of ovary-intact anestrous ewes. Ewes were much more sensitive to NKB, with 0.2 nmole producing a consistent increase in LH concentrations, while 2.0 nmoles of NKA and 10 nmoles of SP were needed to produce the same effect. A lower dose of NKB (0.1 nmole) consistently produced an initial increase, but mean LH concentrations during the 2 hr post-injection was not significantly higher than controls because of its relatively short duration. Thus, the minimal dose of NKB needed to increase LH secretion probably falls between 0.2 and 0.1 nmoles. The minimal effective dose of NKB in this study is lower than that needed to increase bursts of multi-unit electrical activity (MUA) in goats (44), but this may in part be due to differences in the site of administration, since NKB was injected into the lateral ventricle in that study. It is interesting to note that the relative potency of these three tachykinins (NKB>NKA>SP) parallels the relative selectivity of NK3R, not that of NK1R (SP>NKA>NKB) or NK2R (NKA>NKB>SP) (5). This correlation raises the possibility that each of these three tachykinins produces its stimulatory effects on LH secretion in the ewe via NK3R. This possibility is supported by the recent report that a selective NK3R agonist is much more potent at increasing bursts of MUA and LH pulses in OVX goats than selective NK1R or NK2R agonists (61) and by reports that three different selective NK3R antagonists each inhibits episodic LH secretion in OVX ewes (14-16).

. The conclusion that NK3R-signaling is the predominant pathway by which tachykinins control LH secretion in sheep and goats contrasts with recent data in rodents supporting a role for all three tachykinin receptors. This evidence includes reports that: 1) equivalent doses of selective agonists to NK1R, NK2R, and NK3R increase LH secretion in mice (12) and rats (32); 2) NKA and SP can stimulate electrical activity of KNDy neurons in vitro (25); and 3) antagonists to all three tachykinin receptors are required to completely block the stimulatory effects of NKB on the electrical activity of KNDy neurons

401 (25) and episodic LH secretion in OVX rats (34). Thus there appears to be species differences between
402 rodents and ruminants in the ability of signaling via NK1R or NK2R to stimulate LH secretion.
403 In light of this apparent species difference in redundancy within tachykinin signaling critical for
404 LH secretion, it is of interest to assess the situation in humans and non-human primates. At this time there
405 is no data on colocalization of any tachykinin receptor in GnRH or kisspeptin neurons in these species,
406 but there are a few functional and more extensive genetic studies. In monkeys, the number of SP-
407 containing neurons increases following castration (38), but iv administration of SP had no effect on LH
408 secretion (38) and a selective antagonist to NK3R inhibited LH secretion in castrated males and ovary-
409 intact females during the follicular phase of the menstrual cycle (16). Thus, most data are consistent with
410 a lack of redundancy in tachykinin signaling in non-human primates. The situation in humans appears to
411 be more complex. Two observations support redundancy: 1) in women, expression of mRNA for SP
412 increases after menopause (6) and 2) in men, iv infusion of SP can stimulate LH secretion (35). On the
413 other hand, the infertility observed in patients with mutations that disrupt NKB-NK3R signaling argues
414 against redundancy (1-3,62,63), although the reversibility of this condition in some individuals (2,64)
415 could be due to signaling via other tachykinin receptors. Finally, the recent report that the selective NK3R
416 antagonist, ESN364, inhibits LH secretion in men and women (17), as it does in sheep and monkeys (16)
417 provides strong evidence that signaling through NK1R or NK2R plays a minor role in the control of LH
418 secretion in humans. This conclusion, if correct, would provide a simple explanation for the differences
419 in the severity of infertility produced by genetic disruption of NKB-NK3R signaling in humans (1-
420 3,62,63) and mice (65,66).
421 In summary, this study provides the first detailed description of the expression of SP- and NK1R-
422 immunoreactivity within the ovine POA and hypothalamus. In contrast to data in humans and mice, but
423 consistent with data in male monkeys, we found little colocalization of SP with kisspeptin. Moreover, the
424 lack of expression in NK1R within GnRH- and kisspeptin-containing neurons and the relatively high
425 doses of NKA and SP needed to stimulate LH secretion in ewes, support the hypothesis that NKB-NK3R
426 signaling is the predominant pathway by which tachykinins control LH secretion in this species.

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Figure legends

Fig. 1. Camera lucida drawings illustrating the representative distribution of SP (circles) and NK1R (triangles) cell bodies in the preoptic area and hypothalamus of the ewe. Each marking represents approximately 10 cell bodies. Note that SP cell bodies were found only in the middle and caudal ARC (F-H). The distribution of SP and NK1R is shown unilaterally to allow visualization of labels. A15, A15 dopaminergic neurons; ac, anterior commissure; AHA, anterior hypothalamic area; r-m-c-ARC, rostral, middle and caudal ARC; BNST, bed nucleus of the stria terminalis; CP, cerebral peduncle; DMH, dorsomedial nucleus of the hypothalamus; fx, fornix; GP, globus pallidus; LHA, lateral hypothalamic area; me, median eminence; MM, mammillary body; mPOA, medial preoptic area; mr, mammillary recess; mt, mammillary tract; OC, optic chiasm; ot, optic tract; OVLT, organum vasculosum of lamina terminalis; PH, posterior hypothalamus; PMv, ventral premammillary nucleus; pt, pars tuberalis of the adenohypophysis; PVN, paraventricular nucleus; SON, supraoptic nucleus; TMv, ventral tuberomammillary nucleus; VMH, ventromedial nucleus of the hypothalamus; 3v, third ventricle.

Fig. 2. Representative fluorescence photomicrographs showing lack of colocalization of kisspeptin (green) and SP (red) (Panels A, B) and kisspeptin (green) and NK1R (red) (Panels C, D) in the ARC (A, C) and POA (B, D). All photomicrographs are the computerized merger of two separate images captured simultaneously with the appropriate excitation for either DyLight 488 (green: kisspeptin) or Alexa 555 (red: SP or NK1R). Scale bar: (A), 100 μm; (B-D), 50 μm. 3v: third ventricle.

Fig. 3. Representative fluorescence photomicrographs showing lack of colocalization of GnRH (green) and SP (red) (Panels A, B) and GnRH (green) and NK1R (red) (Panels C, D) in the ARC (A, C) and POA (B, D). All photomicrographs are the computerized merger of two separate images captured simultaneously with the appropriate excitation for either DyLight 488 (green) or Alexa 555 (red).Scale bar: (A), 100 μm; (B-D), 50 μm. GP: globus pallidus; 3v: third ventricle.

Fig. 4. Mean ± SEM total number of kisspeptin (A) and NK1R (B) cells in the rostral, middle and caudal aspects of the ARC, in OVX (white bars) and OVX+ E_2 (black bars) ewes. Representative fluorescence photomicrographs from tissue stained for kisspeptin (green) and NK1R (red) in the caudal ARC of OVX (C) and OVX+ E_2 (D) ewes. Scale bar: 100 μm. 3v: third ventricle. * $P<0.05$ during comparison of OVX and OVX+ E_2 in each aspect of the ARC.

Fig 5. Effects of different doses of NKB administered as a single injection into the third ventricle. Mean (±SEM) LH concentrations before (open bars) and 2 hrs after (solid bars) injection are shown. *$P<0.05$ vs corresponding value for control (0 dose) injection.

Fig 6. Representative LH patterns before and after third ventricular injection (arrows) of artificial CSF (aCSF), NKA, and SP. Left panels depict LH data from the same ewe for the first set of treatments, while data from the second set of treatments in a different ewe are depicted in the right panel. Doses (in nmoles) of NKA or SP are presented in parentheses and peaks of LH pulses are indicated by solid circles.

Fig 7. Top panel (A): Effect of two doses of NKA on mean LH concentrations before (open bars), 0-2 hrs (grey bars) and 2-4 hrs (black bars) after injection. *$P<0.05$ vs corresponding value for control (0 dose) treatment. Bottom panel (B): Dose-response of NKA on LH pulse frequency (bars on left) and pulse amplitude (bars on right) during the 4 hrs after injection of this tachykinin. . *$P<0.05$ vs control (0 dose).

Fig 8. Top panel (A): Effect of two doses of SP on mean LH concentrations before (open bars), 0-2 hrs (grey bars) and 2-4 hrs (black bars) after injection. *$P<0.05$ vs corresponding value for control (0 dose) treatment. Bottom panel (B): Effect of two doses of SP on LH pulse frequency (bars on left) and pulse amplitude (bars on right) during the 4 hrs after injection of this tachykinin. There were no statistically significant effects. Note differences in doses between NKA (Fig. 7) and SP.

706 Table 1. Primary antibodies used

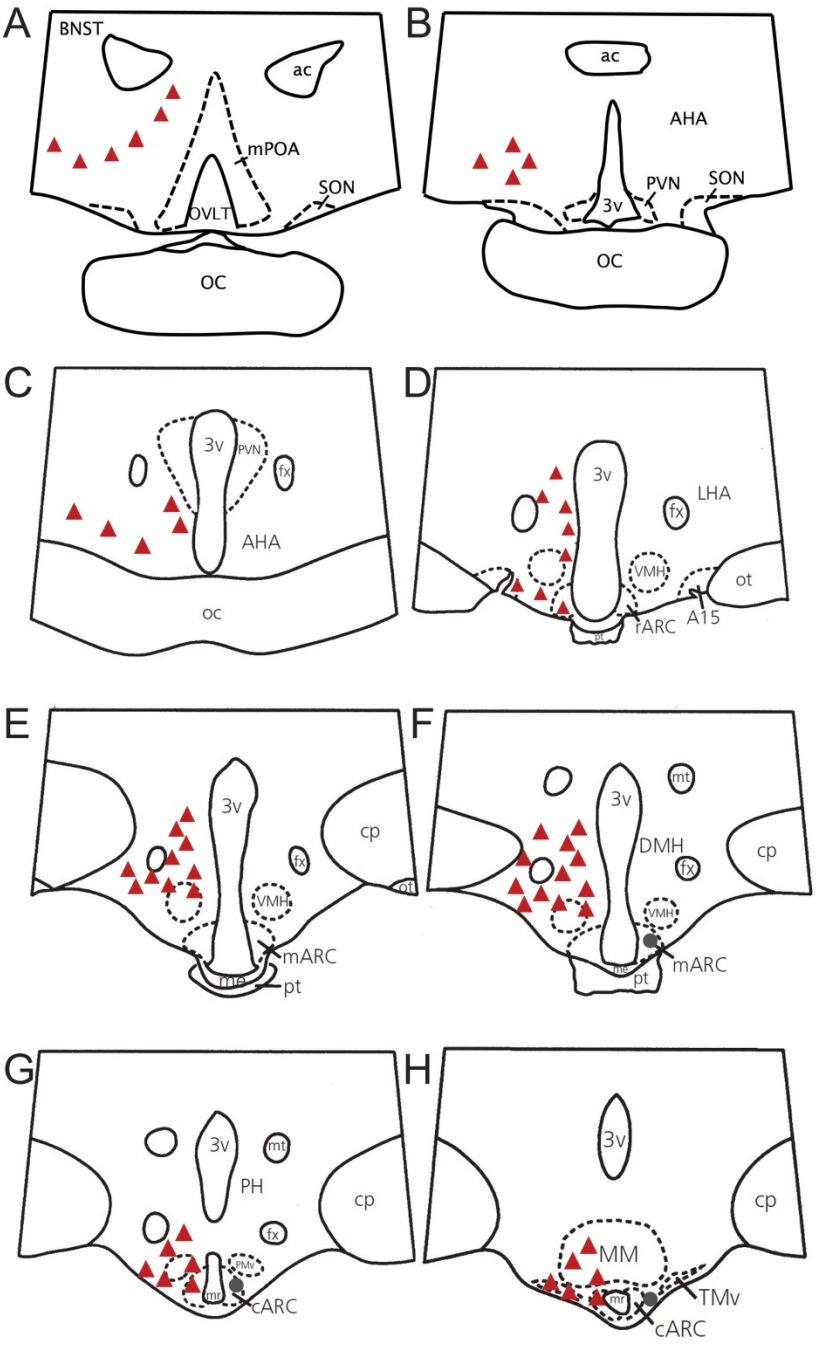
Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
Substance P	CRPKPQQFFGLM	Anti-Substance P antibody	Abcam, ab10353; Lot GR29977-16	Guinea Pig, polyclonal	1:4,000
NK1R	23 aa sequende (385-407) of COOH end of rat SP receptor	Anti-Substance P Receptor antibody	Millipore, AB 5060, Lot 2135068	Rabbit, polyclonal	1:10,000
Kisspeptin	Peptide from mouse kisspeptin 10	Anti-kisspeptin antibody	Millipore, AB9754, and Lot 2397065	Rabbit, polyclonal	1:1,000
GnRH	Synthetic GnRH coupled to keyhole limpet hemocyanin with carbodiimide linker	LHRH antibody	Immunostar, 20075, lot 1037001	Rabbit, polyclonal	1:400

707

Table 2. Effect of NKB on LH pulse frequency and amplitude

Dose of NKB (nmoles)	Frequency (#/2hrs)	Amplitude (ng/ml)
0	0 ± 0	ND
0.05	0.25 ± 0.22	ND
0.1	1.0 ± 0*	2.4 ± 0.5
0.2	1.5 ± 0.3*	4.7 ± 0.9
0.5	1.5 ± 0.3*	3.1 ± 0.9

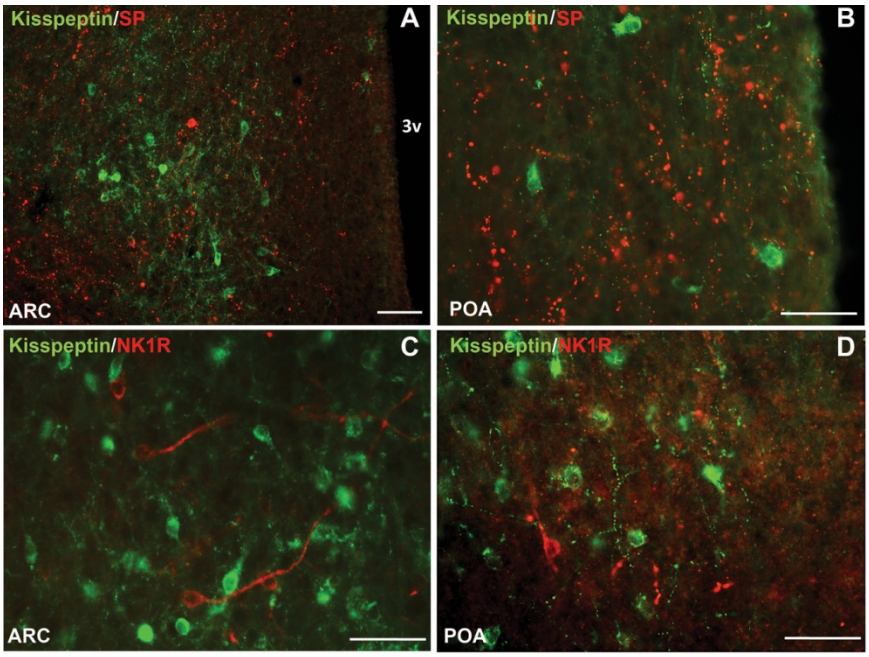
*P<0.05 compared to 0 nmoles NKB; ND: not determined due to low number of pulses



716

717 Fig. 1

718



719

720 Fig. 2

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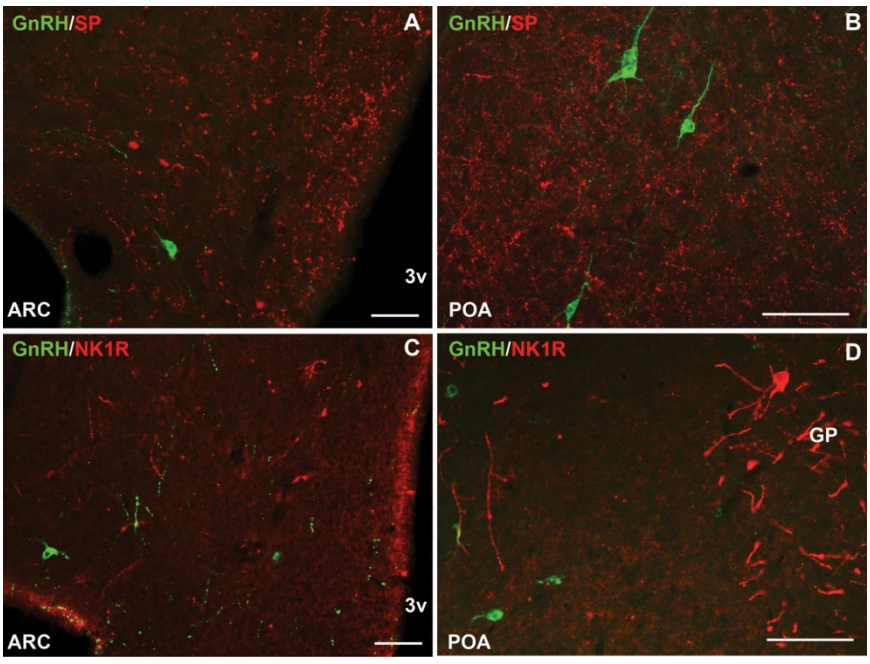
722



723

724 Fig. 3

725

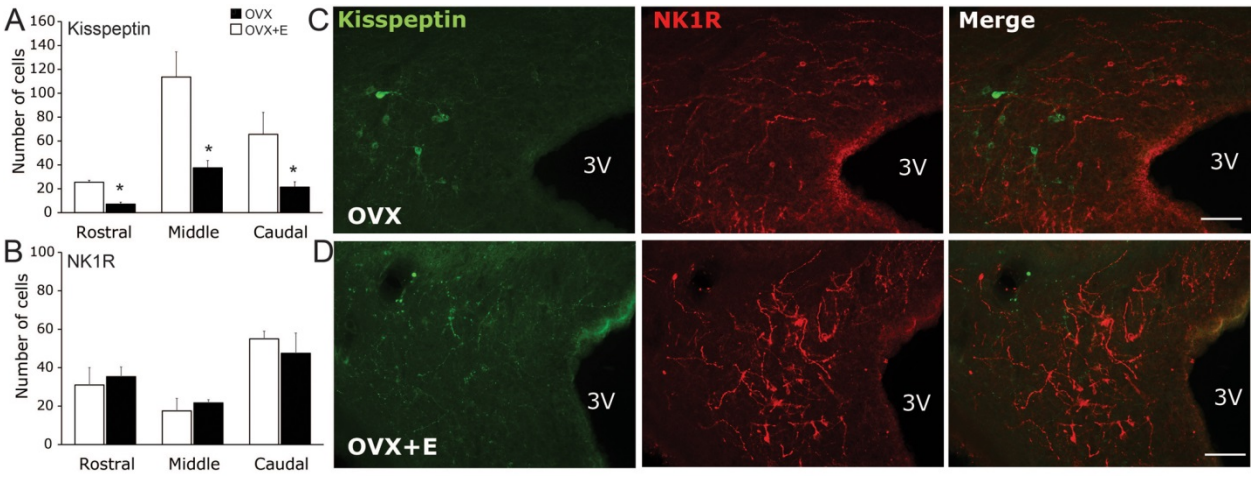

A
Kisspeptin
OVX
OVX+E
Number of cells
Rostral
Middle
Caudal
B
NK1R
C
Kisspeptin
NK1R
Merge
D
3V
OVX
OVX+E

Fig. 4

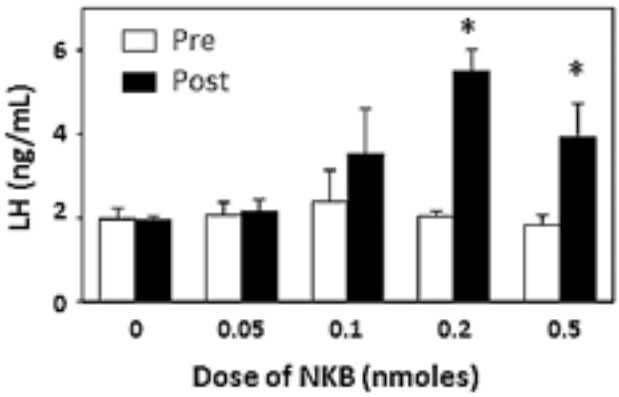
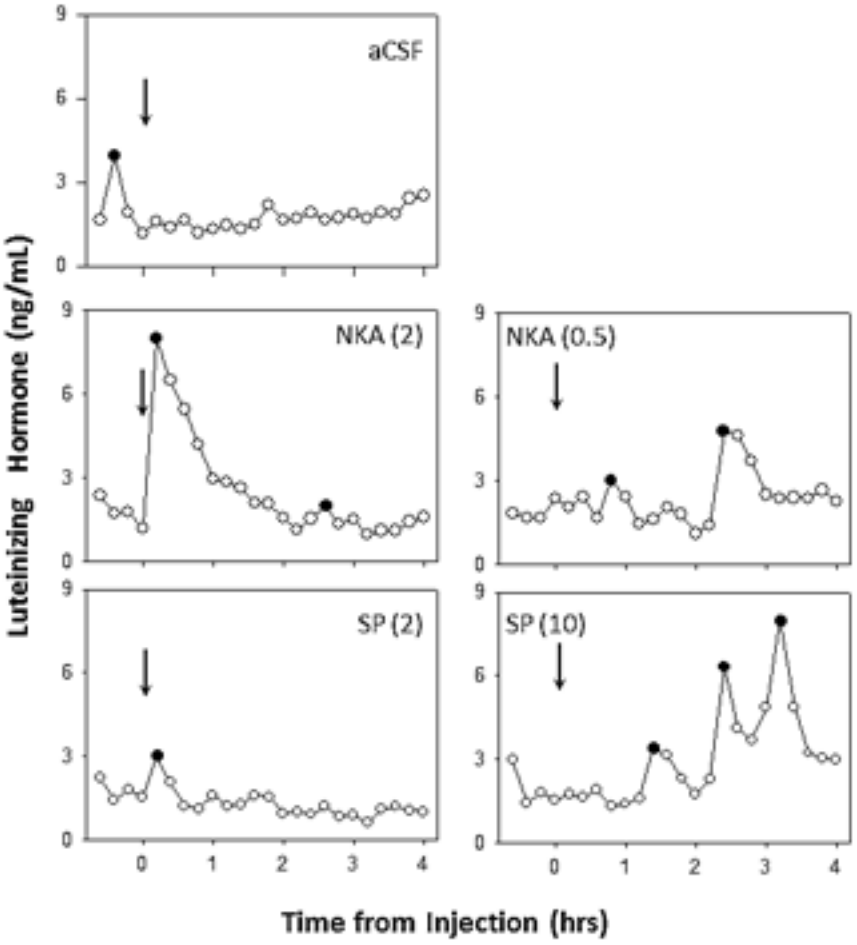

Pre
Post
LH (ng/mL)
Dose of NKB (nmoles)
0
0.05
0.1
0.2
0.5

Fig. 5

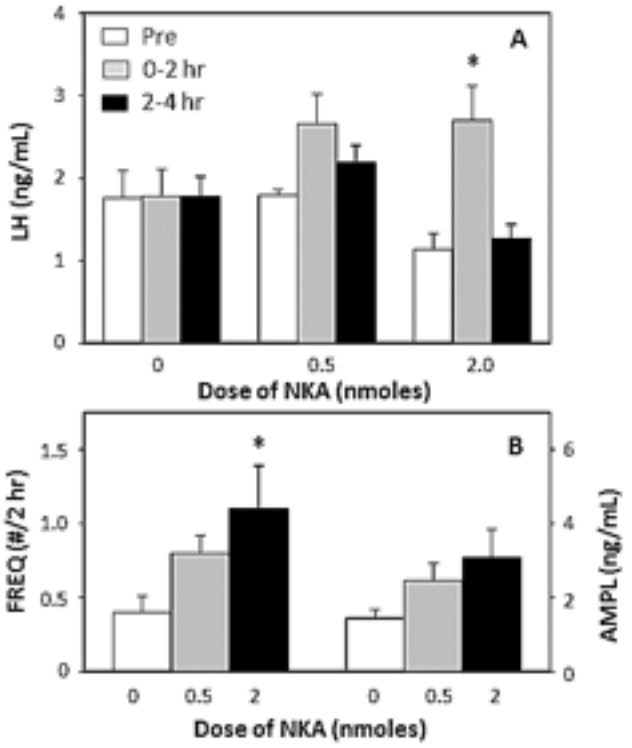


745

746

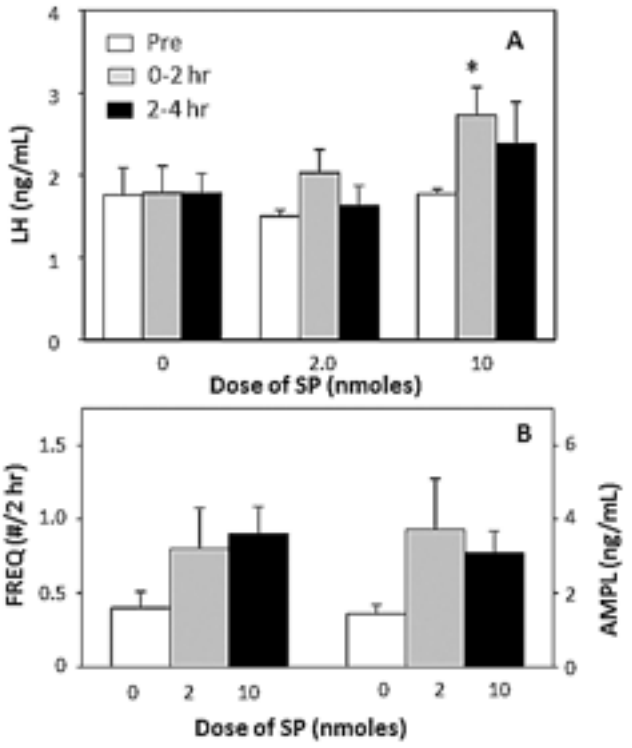
747 Fig. 6

748



749

750 Fig. 7



751

752 Fig. 8