Lindo, AN, Thorson, JF, Bedenbaugh, MN, McCosh, RB, Lopez, JA, Young, SA, Meadows, LJ, Bowdridge, EC, Fergani, C, Freking, BA, Lehman, MN, Hileman, SM and Lents, CA

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Running Title: NK3R, NKB and kisspeptin in gilts

Abbreviated Title: KNDy neurons in pigs; distribution and effect of a progestin

Summary Sentence: Components of the KNDy system in the pig are characterized for the first time and pig specific features such as a lack of immunopositive NK3R expression in the ARC and resistance to inhibition of kisspeptin protein expression by a progestin are identified.

Published by Oxford University Press on behalf of Society for the Study of Reproduction 2021.

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Keywords: Gonadotropin-releasing hormone (GnRH), Kisspeptin, Neuropeptides, luteinizing

hormone (LH/LH receptor), Neurokinin B, Pig

Grant support: NIH Grant P20GM103434 to the West Virginia IDeA Network for Biomedical Excellence WVU Microscope Imaging Facility, Agriculture and Food Research Initiative Competitive Grant Numbers USDA-NIFA-2011-67015-30059 (CAL) and 2013-67015-20956 (SMH) from the National Institute of Food and Agriculture, United States Department of Agriculture (USDA).

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Disclosure Statement: The authors have nothing to disclose.

ABSTRACT

Mechanisms in the brain controlling secretion of gonadotropin hormones in pigs, particularly luteinizing hormone (LH), are poorly understood. Kisspeptin is a potent LH stimulant that is essential for fertility in many species, including pigs. Neurokinin B (NKB) acting through neurokinin 3 receptor (NK3R) is involved in kisspeptin-stimulated LH release, but organization of NKB and NK3R within the porcine hypothalamus is unknown. Hypothalamic tissue from ovariectomized (OVX) gilts was used to determine the distribution of immunoreactive kisspeptin, NKB, and NK3R cells in the arcuate nucleus (ARC). Almost all kisspeptin neurons coexpressed NKB in the porcine ARC. Immunostaining for NK3R was distributed throughout the preoptic area (POA) and in several hypothalamic areas including the periventricular and retrochiasmatic areas but was not detected within the ARC. There was no colocalization of

NK3R with gonadotropin-releasing hormone (GnRH), but NK3R-positive fibers in the POA were in close apposition to GnRH neurons. Treating OVX gilts with the progestin altrenogest decreased LH pulse frequency and reduced mean circulating concentrations of LH compared with OVX control gilts ($P < 0.01$), but the number of kisspeptin and NKB cells in the ARC did not differ between treatments. The neuroanatomical arrangement of kisspeptin, NKB, and NK3R within the porcine hypothalamus confirm they are positioned to stimulate GnRH and LH secretion in gilts, though differences with other species exist. Altrenogest suppression of LH secretion in the OVX gilt does not appear to involve decreased peptide expression of kisspeptin or NKB.

Introduction

The pig is a species of significant agricultural and biomedical importance and understanding mechanisms that control gonadotropin secretion and reproductive cycles in gilts is critical for managing their reproduction. Secretion of luteinizing hormone (LH), and to a lesser extent follicle-stimulating hormone (FSH), in gilts is driven by episodic secretory pulses of gonadotropin releasing hormone (GnRH) from the hypothalamus [1-4]. The pulsatile secretion of GnRH and gonadotropin hormones is absolutely essential for maintenance of reproductive cycles in pigs [5-8]. A group of neurons that coexpress kisspeptin, neurokinin B, and dynorphin A (KNDy) have been identified within the hypothalamic arcuate nucleus (ARC) of laboratory animals and small ruminants [9-11]; and these KNDy neurons have been linked to the central regulation of GnRH pulse secretion in these species [12-15]. In ewes, neurokinin B (NKB) acts through neurokinin 3 receptors (NK3R) within the preoptic area (POA) and ARC to stimulate LH secretion [16], an effect that in nonhuman primates requires kisspeptin signaling [17]. Within the ARC of ewes, KNDy neurons express NK3R [18] but not kisspeptin receptors [19]. The current proposed model for KNDy control of the GnRH pulse generator is that a GnRH pulse is initiated when KNDy cells release NKB, triggering a positive feedback loop through NK3R that stimulates release of kisspeptin to induce secretion of GnRH [20]. Subsequently, dynorphin is secreted from KNDy cells and triggers autoregulatory negative feedback through kappa opioid receptors to inhibit release of kisspeptin and terminate the GnRH pulse. It is unknown it this model is fully consistent with LH secretion in pigs.

In pigs, as in other species, kisspeptin is a potent stimulant of LH secretion [21, 22] and loss of function mutation in kisspeptin receptors results in hypogonadotropic hypogonadism and failure of pigs to attain puberty [23]. A survey of recent literature, however, demonstrates that an

understanding of KNDy regulation of reproductive neurobiology and gonadotropin secretion in pigs remains largely unknown [24]. Insufficient secretion of gonadotropins can lead to pubertal failure of gilts, failure of sows to resume cyclicity after lactation, and seasonal infertility of breeding females [7, 25-27]. The lack of understanding about regulatory mechanisms controlling LH secretion is an important problem as it constrains the ability to manage pig reproduction. To date, reports on the localized expression of kisspeptin and NKB within the porcine hypothalamus have focused exclusively on mRNA expression [28-30]. Localized expression of kisspeptin and NKB protein within the POA and ARC of the pig remains unconfirmed. Moreover, expression of NK3R in the pig hypothalamus is completely unknown.

Both pulsatile and surge secretion of LH in pigs is controlled by feedback actions of ovarian steroids at the hypothalamus [31-33]. Though GnRH neurons do not express estrogen receptor α and progesterone receptor, KNDy neurons do [34-36] and they are generally believed to be involved in the mechanism by which gonadal estrogens regulate gonadotropin secretion. Indeed, the *KISS1* gene in the ARC and POA of gilts demonstrates different patterns of expression relative to the onset of an estradiol-induced ovulatory surge of LH [28], suggesting that KNDy neurons in these areas of the pig hypothalamus have different roles in mediating positive and negative estradiol feedback in pigs. Altrenogest (17-α-allyl-estratriene-4,9,11,17β-ol-3-one) is a progestin that is widely used to synchronize estrus and ovulation in breeding gilts because of its ability to suppress LH secretion and follicle growth [37-40]. Progesterone inhibition of LH secretion in ewes has been shown to involve the KNDy neuronal network [14, 36, 41], and it is thus speculated that KNDy neurons are involved in altrenogest-induced inhibition of LH secretion in gilts.

The objective of the present study was to fill critical knowledge gaps about the role KNDy

neurons have in regulating the reproductive biology of gilts. Immunohistochemistry was used to establish the localized expression of kisspeptin and NKB in the porcine hypothalamus, and the hypothesis that kisspeptin and NKB are colocalized in the porcine ARC was tested. Further, the distribution of NK3R in the POA of the gilt is established and the neuroanatomical relationship of NK3R-expressing cells and fibers with the GnRH neurons of the gilt is examined. Lastly, it was hypothesized that altrenogest suppresses LH secretion by inhibiting the expression of kisspeptin and NKB in gilts.

MATERIALS AND METHODS

Animals and treatments

Experiments were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching [42] and approved by the U.S. Meat Animal Research Center (USMARC) Institutional Animal Care and Use Committee. White crossbred (Yorkshire x Landrace) prepubertal gilts from a resource population at USMARC were used in two experiments. Gilts were housed individually in pens (1.5 m^2) in a climate-controlled facility (21.8 ± 0.3^oC) with 12-hour light/dark cycles. Animals were fed a corn-soybean meal diet twice daily (0800 and 1600h) formulated to meet nutrient requirements [43] and had *ad libitum* access to water. The amount of feed was adjusted to maintain a daily gain of 0.33 ± 0.02 kg/d. Gilts were ovariectomized (OVX) by midventral laparotomy and fitted with an indwelling venous catheter to allow collection of serial blood samples. The OVX was performed at 191.4 ± 0.8 d of age and 122.2 ± 0.7 d in experiment 1 and 2, respectively. These ages correspond to the average age (experiment 1) and the youngest age (experiment 2) that puberty is attained in the resource population. Gilts had not previously been exposed to boars and their prepubertal state

was confirmed by examination of ovaries at OVX.

On day 0, animals were blocked by body weight and assigned to one of two treatments; OVX control (OVX; n = 3, experiment 1; n = 6, experiment 2) or OVX treated with altrenogest (OVX+A; n = 4, experiment 1; n = 6, experiment 2). Altrenogest (15 mg, 0.22% solution, Matrix, Intervet Inc., Millsboro, DE, USA) was top-dressed once daily over the morning feed. This dose of altrenogest suppresses gonadotropin secretion and prevents ovulation in pigs [40]. On day 9 after the start of altrenogest treatment, serial blood samples were drawn (every 12 min for 4.6 and 6 h in experiment 1 and 2, respectively). Serum was separated by centrifugation (2,500 x *g*, 30 min, 4^{o}C) and stored at -20^{o}C until analysis.

Gilts were euthanized with barbiturates according to established guidelines for swine [44] on day 10 after the start of altrenogest treatment. Gilts were 219.4 ± 0.8 d of age and 146.3 ± 1.0 d of age at tissue collection in experiment 1 and 2, respectively: corresponding to age at first mating (experiment 1) and average age at start of boar exposure (experiment 2) in the resource population. The head was immediately removed and perfused bilaterally through the carotid arteries with 6 L of fixative (4% paraformaldehyde, PAF; in 0.1 M phosphate buffered saline, PBS; 0.2 L/min). A block of tissue containing the hypothalamus and POA was removed as described [45] and placed in 700 mL of 4% PAF overnight at 4^{o}C followed by 20% sucrose in PBS at 4^{o}C until the tissue sank. A freezing microtome was used to cut tissue into 50 µm sections. Every 5th section was collected in series (250 µm interval between sections) and vials were separated into either the MBH or POA and stored in cryoprotectant (30% sucrose, 1% polyvinylpyrrolidinone) at -20°C until sections were subjected to immunohistochemistry.

Immunohistochemistry

All immunostaining procedures were performed using free-floating sections. Sections were washed with 0.1 M PBS between all incubations and were treated with 1% H_2O_2 for 10 min before incubation with primary antibodies unless otherwise indicated. Blocking solution was 0.1 M PBS, 4% normal goat serum (NGS; Jackson ImmunoResearch, West Grove, PA, USA) and 0.4% Triton X-100 (phosphate buffered saline Triton, PBST; Sigma-Aldrich, St. Louis, MO, USA) unless otherwise specified. Tissue sections were mounted on Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA, USA) before being cover slipped using Eukitt Mounting Reagent (Fisher Scientific). Immunohistochemistry was performed for kisspeptin, NKB, NK3R, dynorphin, and GnRH. Preabsorbing primary antisera with increasing concentrations of the specific peptide antigen (10 to 50 µg/ml) abolished immunostaining. As explained in the discussion below, immunohistochemistry for dynorphin A was unsuccessful.

Kisspeptin

Tissue sections were evaluated from the ARC of OVX (n = 9 animals) and OVX+A (n = 10 animals) gilts from both experiments and were used to determine the influence of treatment on the number of kisspeptin cells. Examination of POA sections revealed the presence of very few detectable kisspeptin neurons in these OVX animals, thus, kisspeptin cell numbers in the POA were not evaluated. Tissue sections were selected from experiment 1 such that regional distribution (rostral, medial, and caudal) of kisspeptin could be assessed. Regions of the ARC were determined by the shape of the infundibular stalk and third ventricle and location of the fornix and mammillothalamic tract as described for the porcine hypothalamus [46-48]. Tissue was selected from the medial region for analysis in experiment 2 based on the outcome of the regional analysis and the fact that puberty-related changes in LH pulse frequency are correlated

with changes in kisspeptin cell numbers within the medial ARC for sheep [49]. Tissue sections were incubated for at least 1 hr in a blocking solution containing 20% NGS before incubation with rabbit anti-kisspeptin serum (Cat# AB9754; 1:2,000; Millipore Sigma, Billerica, MA, USA) at room temperature for 16 hr. After washing, tissue sections were incubated successively with biotinylated goat anti-rabbit IgG (Cat# BA-1000; 1:400; Vector Laboratories, Burlingame, CA, USA) for 1 hr, streptavidin horseradish-peroxidase conjugate (Vectastain Elite ABC; 1:600; Vector Laboratories) for 1 hr, and 3,3-diaminobenzidine (DAB; 10 mg; Cat# D5905 Sigma-Aldrich) as a chromagen with 25 µl hydrogen peroxide (30% stock; Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4) for 10 min.

NKB

Tissue sections from OVX (n = 7 animals) and OVX+A (n = 8 animals) gilts from both experiments were used to assess the presence of NKB cells within the ARC and the effect of altrenogest treatment on the number of NKB expressing cells. The DAB immunostaining protocol described for kisspeptin was used with the exception that blocking solution contained 4% NGS. The primary antibody was rabbit anti-NKB (Cat# NB300-102; 1:2000; Novus Biologicals, Littleton, CO). The degree to which kisspeptin and NKB colocalize in the ARC of the pig was assessed using dual-label immunofluorescence. Three ARC sections from each of three OVX gilts (experiment 2) were incubated with 10% H_20_2 in PBS for 10 minutes. After incubation in PBST with 20% NGS for 1 hr, tissue sections were incubated with rabbit anti-kisspeptin (1:50,000; Lot# 564; gift from Professor Alain Caraty, France) for 16 hr at room temperature before incubation with biotinylated goat anti-rabbit secondary antibody (Cat# BA-1000; 1:400; Vector Laboratories, Burlingame, CA, USA) for 1 hr. Tissue sections were then

placed in Vectastain Elite ABC for 1 hr before incubation with Biotinyl-Tyramide (1:250; Cat# NEL700A; Perkin Elmer; Waltham, MA, USA) and 3% H_20_2 (1µl H_20_2/ml solution) for 10 min. After washing, sections were incubated with Alexa 555 conjugated to streptavidin (1:100, Fisher Scientific) diluted in PBS for 30 min and then incubated with guinea pig anti-NKB (1:1,000; gift from Dr. Philippe Cioffi, INSERM, Bordeaux, FR) for 16 hr at room temperature before being incubated for 1 hr in anti-guinea pig Alexa 488 (1:100; Life Technologies, Carlsbad, CA, USA).

NK3R

To analyze the distribution of NK3R-containing cells within the hypothalamus and POA, tissue was blocked with 4% NGS, then incubated for 1 hr with rabbit anti-NK3R (1:20,000; Cat# 300-102; Novus Biologicals, Littleton, CO, USA) followed by DAB immunostaining. Tissue sections were selected in a series spanning the entire POA and hypothalamus using tissue from three OVX gilts from experiment 2. Dual-label immunofluorescence was used to ascertain whether GnRH neurons expressed NK3R or were apposed by NK3R-containing fibers. After incubation with rabbit anti-NK3R (1:1,000), tissue sections were incubated with mouse anti-GnRH antibody (1:1,000; Cat # MBS2001271, MyBioSource; San Diego, CA, USA) for 16 h at room temperature. Tissue sections were then successively incubated with anti-rabbit Alexa 555 (1:200; 1 h; Cat # A31572, Molecular Probes, Carlsbad, CA, USA) and anti-mouse DyLight Green (1:200; 1 h, Cat # 35503, Fisher Scientific). To determine whether NK3R fibers apposed GnRH neurons, NK3R-positive contacts were counted via confocal microscopy for 10 randomly chosen GnRH neurons per animal in 3 to 4 medial POA sections from OVX and OVX+A gilts (n = 3 animals each from experiment 2).

Single-label immunofluorescence was used to assess the influence of altrenogest treatment on the number of NK3R-positive cell numbers within the POA and the PVN, RCh, and ARC

from OVX (n = 6 animals) or OVX+A (n = 6 animals) gilts. Tissue sections were incubated with rabbit anti-NK3R (1:1,000;1 h), biotinylated goat anti-rabbit IgG (Cat# BA-1000; 1:400; 1 h; Vector Laboratories, Burlingame, CA, USA), and anti-rabbit DyLight green (Cat# 35553; 1:200; 1 h; ThermoFisher Scientific)

Data Analysis

Luteinizing Hormone

Concentrations of LH in serum were determined at USMARC in serial samples using an RIA validated for porcine serum [50]. Reference standard for LH (AFP-10506A) was provided by Dr. A. F. Parlow (Scientific Director for the NIH, NIDDK, National Hormone and Peptide Program, Torrance, CA, USA). Sensitivity (90% of Bo) was 0.08 ng/mL. Pools of porcine serum with LH concentrations of 0.84, 1.74 and 5.64 ng/mL were included in each assay and had an average intra-assay and inter-assay CV of 7.5% and 11.9%, respectively. Mean concentrations of LH were estimated using a mixed-model analysis of variance (ANOVA) with repeated measures. The model included treatment, time, and experiment as fixed effects. Animal within treatment was included as the random effect. A compound symmetric function was used to model the covariance structure for the repeated measure, and degrees of freedom for the pooled error term were calculated using Kenward-Roger approximation. Post hoc comparisons were made with Tukey's honestly significant difference (HSD) test. Pulses of LH in serum were determined as described previously [51]. Briefly, three criteria had to be met; the amplitude of the pulse had to be greater than the sensitivity, the peak had to occur within two samples of the nadir, and the LH concentration at the peak of the pulse had to exceed the 95% confidence limit (based on overall assay variability) of the concentration for the preceding and subsequent nadir. The interval in minutes between LH pulses (inter-pulse interval; IPI) was used to estimate differences in LH

pulse frequency. If an animal did not display a pulse, then the entire period of blood collection was used as a conservative estimate of the IPI. A general least squares ANOVA was used to test the effects of treatment and experiment on IPI followed by a Tukey's HSD test for post hoc comparisons. Difference between treatments in LH pulse amplitude in experiment 1 was estimated with Students t-test. Due to lack of LH pulses in OVX+A gilts in experiment 2, treatment differences in LH pulse amplitude could not be estimated for altrenogest-treated gilts in this experiment.

Immunohistochemistry

An Olympus VS120 Slide Scanning microscope was used to visualize immunopositive cells in tissue sections. The total number of cells for each gilt was counted in a manner that was blind to treatment using Olympus software (OlyVIA). The total number of immunopositive cells in each section (n = 5 per gilt) was determined and then averaged to generate an estimate for total cell counts per section for each animal. Effects of treatment were estimated by Student's t-test with $P < 0.05$ considered significant. Regional distribution of kisspeptin cell numbers and the influence of altrenogest treatment on that distribution was determined by repeated measures ANOVA with region, treatment, and the region x treatment interaction as main effects. Total number of NK3R positive cells was determined in each tissue section of the DBB, POA, PeV, PVN, SON, RCh and ARC and averaged to generate an estimate for each hypothalamic area for each gilt. The effect of treatment on the number of NK3R containing cells in these hypothalamic areas was estimated with Student's t-test.

To determine the percentage of GnRH neurons that coexpressed NK3R, images were captured using an Upright LSM 510 Violet Confocal microscope (Zeiss) with a Plan

Apochromat x 63/1.4 oil objective. For assessment of close contacts, confocal Z-stacks were taken at 1 µm intervals from a total of 9 to 10 GnRH neurons/animal randomly selected from 3 to 4 medial POA sections. Each contact was analyzed using Zeiss Zen software. Orthogonal views were used to confirm that contacts were touching in all planes. The average number of contacts per GnRH neuron was calculated for each animal. Co-localization and number of close contacts between NK3R and GnRH neurons, and estimated effects of altrenogest on numbers of close contacts, was analyzed by Student's t-test. A chi-square analysis was used to compare the percentage of GnRH neurons that expressed these contacts between treatments.

Results

LH secretion

Representative LH profiles for OVX and an OVX+A gilts in each experiment are shown in Fig. 1. Clear differences in LH pulse patterns between treatments can be observed in both experiments. Mean circulating concentrations of LH in each experiment are illustrated in Fig. 2A. There were no three- or two-way interactions between experiment, treatment, or time for mean LH ($P > 0.50$). Altrenogest treatment decreased ($P < 0.0001$) overall mean circulating concentrations of LH by 62% (1.67 ± 0.15 ng/ml and 0.64 ± 0.14 ng/ml for OVX and OVX+A, respectively). In both experiments, altrenogest treatment reduced LH pulse frequency as indicated by a greater IPI for OVX+A gilts compared with OVX control gilts ($P < 0.01$; Fig. 2B). There was an experiment by treatment interaction ($P < 0.01$) for IPI resulting from a greater IPI in OVX+A gilts in experiment 2 compared with OVX+A gilts in experiment 1 ($P < 0.0001$; Fig. 2B), but the IPI for OVX control gilts did not differ between experiments. Only one LH pulse in a single altrenogest-treated gilt was observed in experiment 2, which contributed to the greater IPI of OVX+A gilts in this experiment. In addition to fewer LH pulses in OVX+A gilts,

altrenogest treatment increased LH pulse amplitude in experiment 1 (1.42 ± 0.22 ng/ml vs 0.88 ± 0.07 ng/ml for OVX+A and OVX gilts, respectively; $P < 0.04$).

Expression of kisspeptin, NKB, NK3R, and dynorphin cells in the POA and hypothalamus

As mentioned previously, kisspeptin neurons were not readily observed in the POA or periventricular area in numbers that could be meaningfully analyzed, however, in the ARC kisspeptin positive cell soma were evident (Figure 3A). Analysis of regional distribution (Figure 3B) showed an effect of region ($P < 0.02$), with kisspeptin cell numbers being highest in the rostral and middle portion of the ARC. Although kisspeptin cell numbers were numerically higher in every region for altrenogest-treated gilts, there was not a significant effect of altrenogest treatment ($P > 0.10$) or region x treatment interaction ($P > 0.50$). The number of kisspeptin and NKB-positive cells did not differ between experiments ($P > 0.10$). When data were pooled across experiments, there was a tendency ($P = 0.06$) for kisspeptin cells in the ARC to be greater for OVX+A gilts. The number of NKB-positive cells (Fig. S1) in the ARC (overall mean 114.3 ± 38.6 cells/section) did not differ between treatments ($P > 0.30$; Fig. 4B). Dynorphin immunostaining in the hypothalamus was not detected (data not shown).

Dual immunofluorescence for kisspeptin and NKB (Figure 5A-C) showed that virtually all (99.3 ± 0.4%) kisspeptin neurons coexpressed NKB and that the vast majority of NKB neurons (92.1 ± 1.0 %) coexpressed kisspeptin (Fig. 6). In contrast, there was no evidence for GnRH neurons colocalizing with NK3R (Fig. 5D-E). Nonetheless, NK3R-immunopositive neurons were evident in close proximity to GnRH neurons in the POA. Several GnRH neurons appeared to be contacted by NK3R- positive appositions (Fig. 5F-H). There was no difference between treatment groups in the percentage of GnRH neurons contacted by NK3R cells (48 ± 16% for

OVX and 34 ± 18% for OVX+A gilts; $P > 0.90$). The average number of NK3R-positive close-contacts associated with each GnRH-positive neuron was low (1.0 ± 0.3) and not influenced by altrenogest treatment ($P > 0.90$).

Cells containing NK3R were distributed throughout the POA and hypothalamus. These NK3R-positive cells were readily identified by their robust cytoplasmic staining in all areas examined except for the ARC (Fig. 7A). The average number of NK3R cells (Fig. 7B) ranged from 150 to 900 cells in the DBB, POA, SON, PeV, PVN, and RCh with numbers being greatest in the DBB and PVN. Altrenogest did not affect ($P > 0.10$) the number of NK3R-containing cells in the POA, PVN and RCh (Fig. 8).

Discussion

Neural mechanisms underlying pulsatile secretion of GnRH and LH have been described, at least in part, for species such as laboratory animals and small ruminants, where it is evident that KNDy neurons in the ARC are involved [13, 15, 20]. In the KNDy model, it is proposed that release of NKB from KNDy neurons triggers a positive ultrashort feedback loop through NK3R which stimulates release of kisspeptin, that acting through its cognate receptor on GnRH neurons, initiates a GnRH pulse. Almost simultaneously, KNDy neurons are triggered to release dynorphin that initiates a negative feedback loop through kappa opioid receptors (KOR) to suppress secretion of kisspeptin and terminate the GnRH pulse. Despite the fact that under numerous circumstances inadequate secretion of LH pulses underlies and contributes to infertility and reproductive failure in pigs, surprisingly little is known about KNDy neurons in swine or how they function in regulating the reproductive biology of gilts [24, 52]. This is the first report of kisspeptin and NKB protein expression and localization in the ARC of pigs. Additionally, expression of NK3R in the POA of the pig hypothalamus is characterized for the

first time, and differences in the patterned expression of these important components of the KNDy system in pigs from that in other species is identified.

Previous studies on kisspeptin expression in the gilt hypothalamus have been focused exclusively on *KISS1* gene expression [28-30]. In the current study, numerous neurons expressing kisspeptin peptide were observed within the ARC of the porcine hypothalamus. The distribution of kisspeptin neurons in this area was generally similar to that observed in sheep and cattle. Furthermore, it is our experience that kisspeptin-containing neurons in the ARC of the ewe often extend to the edge of the stalk median eminence [53]. However, kisspeptin neurons in the pig hypothalamus were typically found in the mediodorsal portion of the ARC and the distribution of cell bodies did not usually extend to the medioventral border of the ARC or stalk median eminence. Expression of NKB in the ARC of these gilts followed a similar pattern of localization. These observations confirm that the expression pattern of kisspeptin and NKB protein in the porcine ARC is consistent with the recent report of the pattern of either *KISS1* or *TAC3* (encodes NKB) gene expression in the gilt ARC [30]. When sections dual-labeled for kisspeptin and NKB were examined, virtually all kisspeptin neurons in the ARC were found to coexpress NKB. It has been previously noted in sheep and mice that most kisspeptin neurons in the ARC coexpress NKB [9, 13], and similar observations were reported in heifers [54].

Neurons that contained immunoreactive NK3R, the high affinity receptor for NKB, were identified in several areas of the pig hypothalamus including the POA, RCH, and PVN. Expression of NK3R in these areas has also been observed in other species [18, 55-57]. The roles of NK3R in these various areas are unknown in swine and have only been partly elucidated in other species. Administration of senktide, an NK3R agonist, within either the POA or RCH in sheep elicits surge-like secretion of LH [16]. The patterned expression of NK3R within the PeV

of the pig hypothalamus is of particular interest in that it mirrors the expression pattern of the *KISS1* gene that is upregulated in the PeV of gilts experiencing an estradiol-induced ovulatory surge of LH [28]. We did not observe readily identifiable kisspeptin cells in this area, likely because gilts in our study were not exposed to high levels of estradiol as would be typical for inducing the GnRH surge. The identity of these NK3R expressing cells in the PeV of the pig remains to be determined.

Evidence in sheep suggests that NK3R expressing cells in the ARC are involved in tonic secretion of LH [58]. We have previously determined that expression of *TAC3R* (the gene that encodes NK3R) in the MBH is greater in peripubertal gilts than in prepubertal gilts [45]. It was thus surprising to observe no NK3R-positive immunostaining in the ARC of gilts in the current study. Neither NK3R positive cells nor fibers were evident within the ARC, even though ARC sections were run concurrently with sections from other areas in which NK3R immunostaining was clearly present. In view of this finding, several additional approaches were used in a further effort to uncover the presence of NK3R immunostaining in the porcine ARC. These additional approaches included increasing concentration of the antibody, inclusion of tyramide amplification, and antigen retrieval methods. The failure of these approaches to reveal any NK3R immunostaining in the ARC, and the ample expression of NK3R in other areas of the gilt hypothalamus, seemingly indicates either very low or no expression of NK3R protein in the gilt ARC. The functional consequence of this observation, or whether it may be related to the lack of estrogen in these OVX gilts, remains to be determined. This negative result should be interpreted with caution, but it may indicate that the KNDy model as proposed for sheep and laboratory rodents is not fully applicable to pigs.

Nonetheless, abundant expression of NK3R was observed in regions of the hypothalamus

that contain GnRH neurons [46]. Examination in the current study of whether GnRH neurons coexpressed NK3R demonstrated that receptor expression is absent from GnRH neurons in gilts, which is consistent with previous reports in ewes [18]. At least some GnRH neurons in rodents express NK3R [59, 60], indicating potential for differences between species. Interestingly, NK3R-positive appositions were observed in direct contact with GnRH neurons in these gilts, and similar observations have been reported in ewes [18]. The functional importance of such putative inputs remains unknown but may indicate an ability of NKB to act presynaptically to influence other afferent inputs to GnRH neurons. The identity of the neurons giving rise to these NK3R-positive appositions in gilts or those expressing NK3R within many of these hypothalamic areas in the pig will require further investigation.

As expected, treatment of gilts with altrenogest suppressed LH pulsatility and decreased mean circulating concentrations of LH. Although these gilts lacked ovarian estrogen, reduced LH secretion in response to altrenogest treatment was consistent with that of ovary-intact luteal phase gilts in commercial swine production. Because the vast majority of KNDy neurons in the ARC express progesterone receptor [61], it was hypothesized that altrenogest suppression of LH pulses in gilts would be reflected by a decrease in kisspeptin- and NKB-containing cell numbers in the ARC. Instead, no statistical difference in the number of cells expressing kisspeptin or NKB within the ARC were observed with altrenogest treatment. This is the first study to examine the singular role of a synthetic progestin on regulation of KNDy neuron peptides and although there were no statistical differences, there was a statistical trend towards more kisspeptin neurons in altrenogest-treated gilts. This may reflect a primary inhibition by progesterone of cellular secretory activity rather than protein expression, though that hypothesis remains to be tested. These results in OVX gilts are in line with those in OVX mice, where

progesterone treatment increased kisspeptin staining in the ARC [62]. In primates, progesterone caused a 60% decrease in the number of *KISS1*-expressing neurons [63]. Similarly, progesterone alone can decrease expression of *KISS1* in the ARC of OVX ewes, but the negative regulation of *KISS1* expression in ovary-intact ewes is mostly related to the estrogen rather than changes in progesterone concentrations per se [61, 64].

The hypothalamic location and neural mechanisms responsible for progesterone suppression of LH pulses in the gilt are believed to be the same as those for altrenogest. One possible explanation for the inability of altrenogest to influence kisspeptin cell numbers in OVX gilts may lie in the lack of a supportive role of estrogen. Estrogen stimulates progesterone receptor synthesis and expression [65-67], and the number of kisspeptin neurons in the ARC expressing progesterone receptor varies throughout the estrous cycle of the mouse [68]. Treating gilts with exogenous gonadotropins to increase circulating concentrations of estradiol does not lead to an increase of progesterone receptors in the hypothalamus or anterior pituitary gland [69], suggesting that expression of progesterone receptors in the pig hypothalamus is not entirely dependent upon changing concentrations of estrogen. The suppressive effect of progesterone on LH secretion in OVX ewes is lost after 3 to 4 months without estrogen priming [70, 71]. Such is not the case for gilts, which remain responsive to the negative effects of progesterone 4 months after OVX; although, estrogen priming did increase the sensitivity of these long-term OVX gilts to progesterone negative feedback [33]. Nevertheless, altrenogest suppression of LH pulse secretion in the present study suggests that there were sufficient progesterone receptors in these OVX gilts to elicit the correct biological response.

Administering a general EOP receptor antagonist (naloxone) to progesterone-treated, prepubertal OVX gilts of sufficient age to reach sexual maturity, which were comparable to the

age of gilts used in the current study, increased LH secretion [33]. The exact EOP receptors involved in progesterone inhibition of LH secretion in gilts is not known, but data in ewes suggest KOR on KNDy and/or GnRH neurons may be involved [36, 72, 73]. It was therefore hypothesized that altrenogest-induced suppression of LH secretion in gilts would be accompanied by increased expression of dynorphin, however, we were unable to detect dynorphin-positive cells within the ARC. Two different antibodies were tested (Table S1) in the labs at West Virginia and Mississippi with the same result. These antibodies work in sheep tissue and their dynorphin A 1-17 epitope is conserved in pigs. Nonetheless, their use in pig tissue remained unsuccessful in our hands despite several procedural modifications to increase detection (e.g., increasing antibody concentrations, antigen retrieval, tyramide amplification). Similar observations on absence of dynorphin A staining in the porcine hypothalamus have been made by others as well (personal communication, Dr. Casey Nestor, North Carolina State University). Ovariectomy of adult ewes reduced prodynorphin gene expression in the ARC to minimally detectable levels and progesterone replacement failed to prevent this decrease [41]. The use of OVX gilts may have limited the ability to detect dynorphin in the ARC. Altrenogest may stimulate EOP other than dynorphin in OVX gilts to suppress LH pulses, and there may be other neurological mechanisms at play as well. For example, depleting hypothalamic norepinephrine in progesterone-treated OVX gilts eliminates the naloxone-stimulated increase in LH secretion [74].

These studies describe in part the neuroanatomical arrangement of the kisspeptin and NKB neurons in female pigs. Much like other mammalian species, kisspeptin neurons in the ARC of the pig coexpress NKB. The effects of the progestin altrenogest on LH secretion were as predicted, but kisspeptin and NKB positive cell numbers were unchanged. Dynorphin cells,

which are predicted to mediate altrenogest suppression of LH secretion, were not detected in the ARC. Likewise, receptors for NKB were scattered throughout the porcine hypothalamus and were readily evident, with the surprising exception of the ARC. Clearly further study of the porcine KNDy system is required to gain a better understanding of these unexpected results and to determine whether the proposed model of KNDy regulated LH secretion is fully applicable to the pig.

Acknowledgments

The authors acknowledge Michelle McManus, Rebecca Kern, Troy Gramke, Bruce Larson, and Scott Whitcomb of the U.S. Department of Agriculture (USDA) for technical assistance. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. The USDA prohibits discrimination in all its programs and activities on the basis of race, color, national origin, age, disability, and where applicable, sex, marital status, familial status, parental status, religion, sexual orientation, genetic information, political beliefs, reprisal, or because all or part of an individual's income is derived from any public assistance program (Not all prohibited bases apply to all programs.). Persons with disabilities who require alternative means for communication of program information (Braille, large print, audiotape, etc.) should contact USDA's TARGET Center at (202) 720-2600 (voice and TDD). To file a complaint of discrimination, write to USDA, Director, Office of Civil Rights, 1400 Independence Avenue, S.W., Washington, DC 20250-9410, or call (800) 795-3272 (voice) or (202) 720-6382 (TDD). USDA is an equal opportunity provider and employer.

Data Availability

Data are available upon request.

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FIGURE LEGENDS

Lindo et al., Figure 1

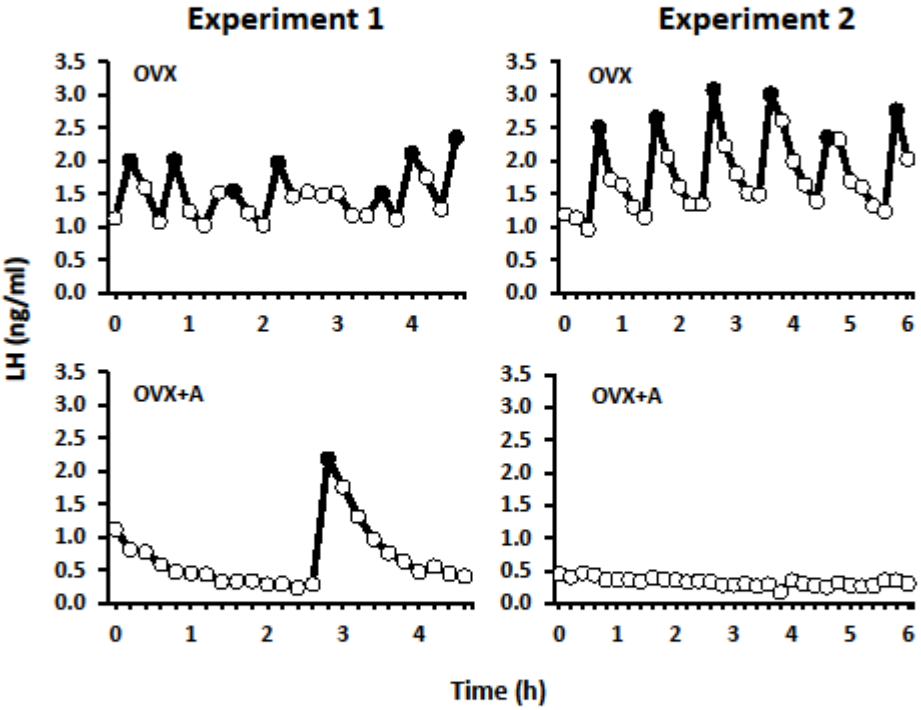

Fig 1. Representative LH profiles for ovariectomized (OVX) control gilts and OVX gilts treated with the progestin altrenogest (OVX+A; top and bottom, respectively) from experiment 1 and experiment 2. Solid circles indicate LH pulses.

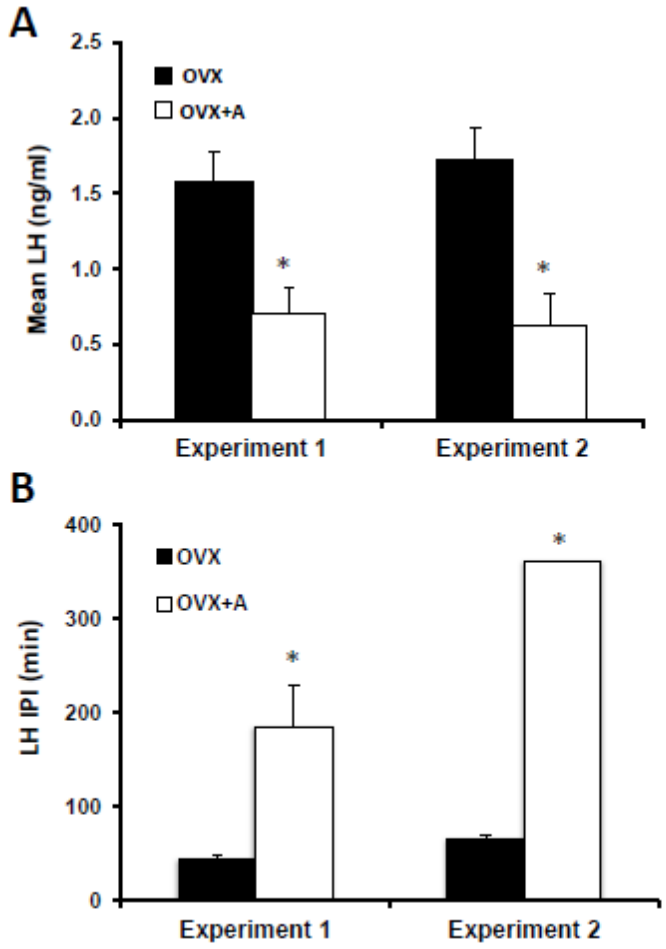

Fig 2. Mean (±SEM) (A) concentrations of LH and (B) LH inter-pulse interval (IPI) for ovariectomized (OVX) control gilts and OVX gilts treated with the progestin altrenogest (OVX+A) from experiments 1 and 2, respectively. Asterisk indicates difference in overall means between treatments ($P < 0.05$).

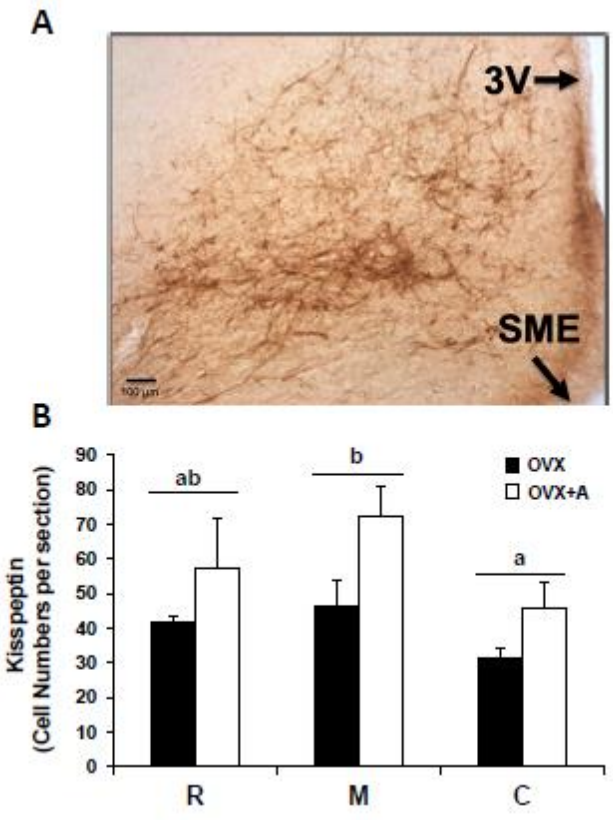

Fig. 3. (A). Photomicrograph (10x) of kisspeptin immunostaining in the arcuate nucleus (ARC), 3V, third ventricle; SME, stalk median eminence. (B). Mean (±SEM) kisspeptin-positive cell numbers per section in the rostral (R), medial (M), and caudal (C) regions of the ARC in ovariectomized (OVX) control gilts and OVX gilts treated with the progestin altrenogest (OVX+A) from experiment 1. Letters indicate significant differences between regions ($P < 0.05$).

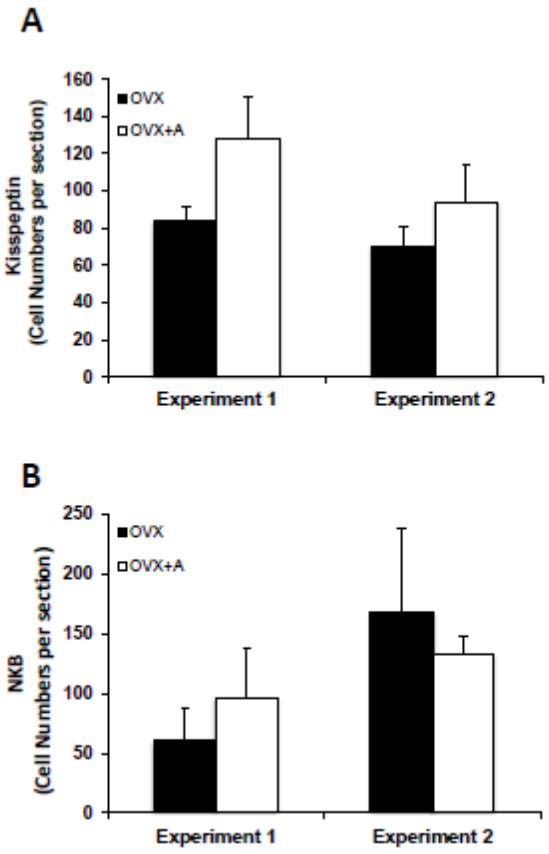

Fig 4. Mean (±SEM) numbers of (A) kisspeptin-positive cells (B) or neurokinin B (NKB)-positive cells per section in the arcuate nucleus (ARC) of the hypothalamus in ovariectomized (OVX) control gilts and OVX gilts treated with the progestin altrenogest (OVX+A) from experiments 1 and 2, respectively.

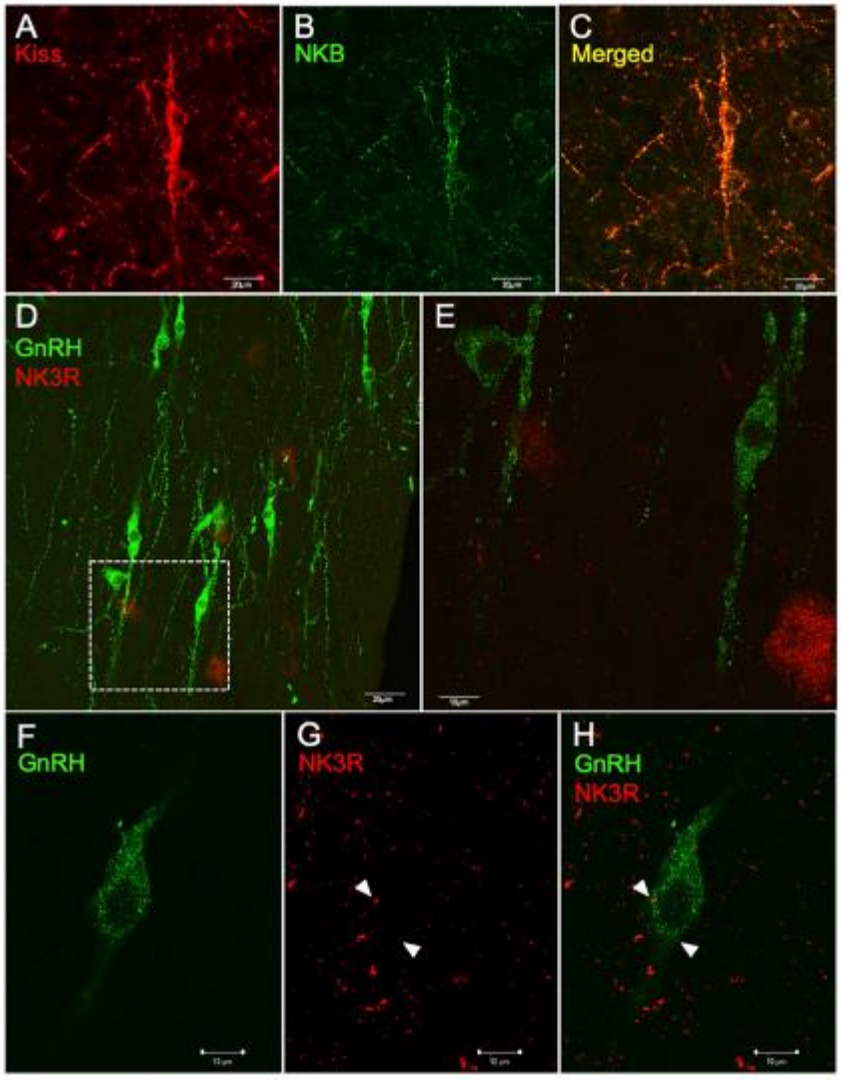

Fig 5. Panels A-C; photomicrograph showing coexpression of kisspeptin and neurokinin B (NKB) in the arcuate nucleus (ARC). Dual immunofluorescence (viewed at 60x) for two neurons in the ARC expressing kisspeptin (red, A) and NKB (green, B). The merged image (C) clearly demonstrates colocalization of the two peptides. Panels D-E; photomicrograph showing the lack of colocalization for GnRH (green) and neurokinin 3 receptor (NK3R; red) at 20X (D) and 63X(E). Panels F-H; photomicrographs showing NK3R-containing close contacts (red) on GnRH neurons (green). White arrowheads denote two NK3R-containing appositions in contact with a single GnRH neuron.

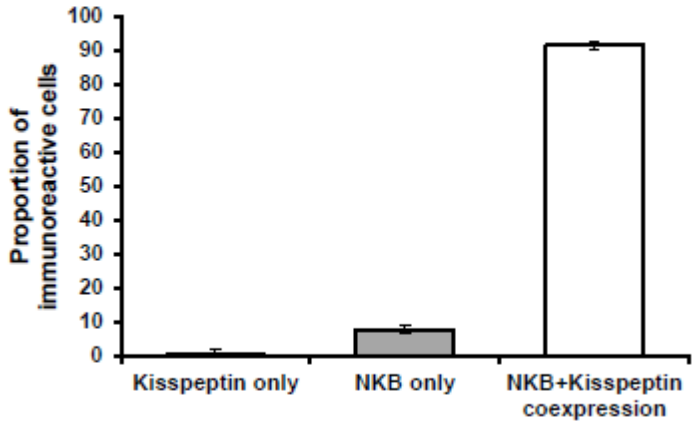

Fig 6. The proportion (± SEM) of immunoreactive neurons per section in the arcuate nucleus of ovariectomized gilts that are only kisspeptin, only neurokinin B (NKB) or in which kisspeptin and NKB are colocalized.

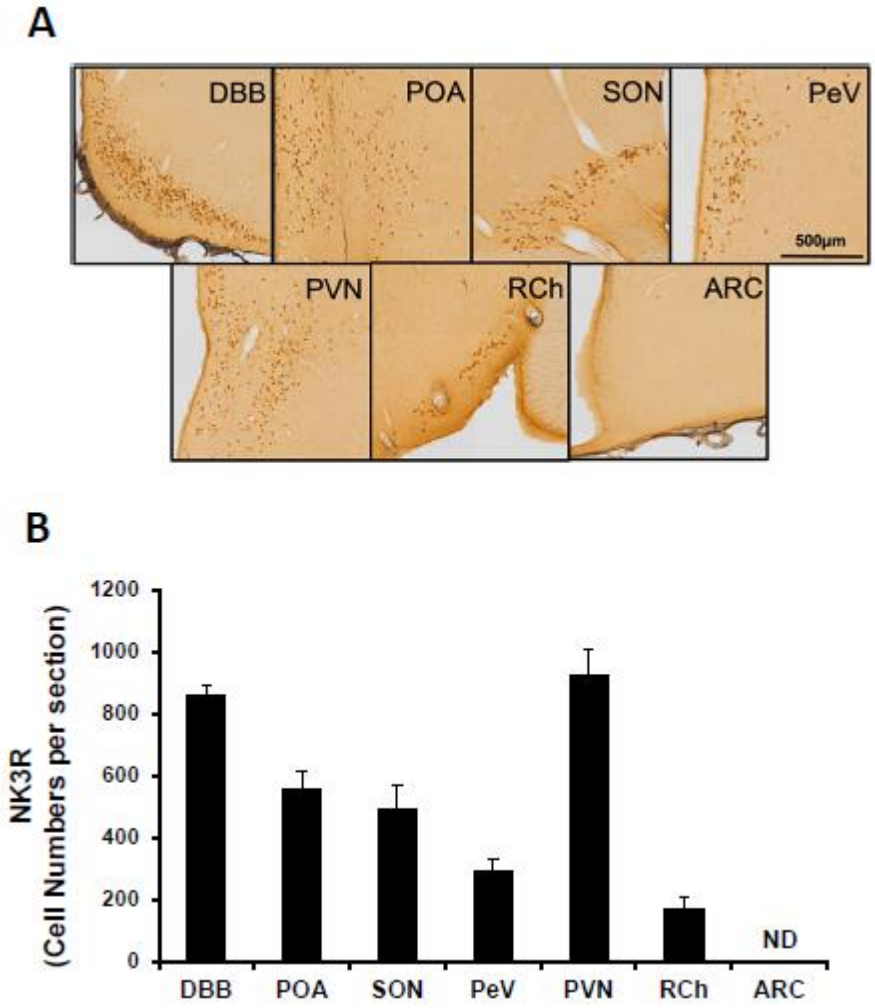

Fig 7. Panel A: Representative photomicrographs (20x) of coronal sections from a gilt through the preoptic area (POA) and hypothalamus illustrating expression of neurokinin 3 receptor (NK3R). Cells were readily apparent within the diagonal band of Broca (DBB), POA, supraoptic nucleus (SON), periventricular area (PeV), the paraventricular nucleus (PVN), and retrochiasmatic area (RCh). Note the lack of detectable staining within the arcuate nucleus (ARC). Panel B: Mean (± SEM) NK3R-immunopositive cell numbers per section throughout the POA and hypothalamus in OVX gilts (n = 3); ND = not detectable.

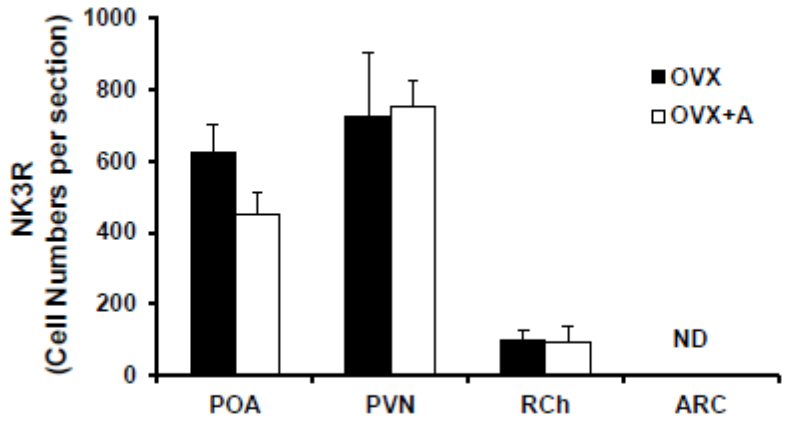

Fig 8. Mean number (± SEM) of neurokinin 3 receptor (NK3R) cells per section in the preoptic area (POA), paraventricular nucleus (PVN), retrochiasmatic area (RCh), and arcuate nucleus (ARC) of ovarectomized (OVX) control gilts and OVX gilts treated with the progestin altrenogest (OVX+A).