ORIGINAL ARTICLE

The impact of undernutrition on KNDy (kisspeptin/neurokinin B/dynorphin) neurons in female lambs

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Funding information
National Institute of Food and Agriculture, Grant/Award Numbers: 2019-67016-29408, Hatch 1012905

Abstract

Undernutrition limits reproduction through inhibition of gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) secretion. Because KNDy neurons coexpress neuropeptides that play stimulatory (kisspeptin and neurokinin B [NKB]) and inhibitory (dynorphin) roles in pulsatile GnRH/LH release, we hypothesized that undernutrition would inhibit kisspeptin and NKB expression at the same time as increasing dynorphin expression. Fifteen ovariectomized lambs were either fed to maintain pre-study body weight (controls) or feed-restricted to lose 20% of pre-study body weight (FR) over 13 weeks. Blood samples were collected and plasma from weeks 0 and 13 were assessed for LH by radioimmunoassay. At week 13, animals were killed, and brain tissue was processed for assessment of KNDy peptide mRNA or protein expression. Mean LH and LH pulse amplitude were lower in FR lambs compared to controls. We observed lower mRNA abundance for kisspeptin within KNDy neurons of FR lambs compared to controls with no significant change in mRNA for NKB or dynorphin. We also observed that FR lambs had fewer numbers of arcuate nucleus kisspeptin and NKB perikarya compared to controls. These findings support the idea that KNDy neurons are important for regulating reproduction during undernutrition in female sheep.

KEYWORDS
dynorphin, kisspeptin, LH, neurokinin B, undernutrition, xxxx, fx1

1 | INTRODUCTION

Reproduction is vital for the preservation of mammalian species and is influenced by numerous environmental cues through the central regulation of gonadotropin-releasing hormone (GnRH) secretion. Sufficient energy intake is one such external factor that can determine reproductive capacity, as illustrated by undernutrition delaying puberty onset,[1–3] prolonging postpartum anestrus,[4–6] and inhibiting ovulatory cycles.[7] Although caloric restriction has been shown to inhibit GnRH release, and in turn reduce luteinizing hormone (LH) secretion,[8] GnRH neurons are devoid of receptors

for metabolic signals such as leptin and insulin.[9–13] Thus, reduced energy availability during undernutrition is likely relayed to GnRH neurons through as of yet to be fully elucidated afferent neuronal inputs consisting of reduced excitatory drive and/or an elevated inhibitory drive.

It is well-established that kisspeptin signaling is essential for reproductive success. Initial evidence of this importance came from genetic mutations in humans and mice that resulted in impaired fertility.[14,15] Given that kisspeptin has elicited either GnRH or LH secretion in all species studied to date[16–23] and that the vast majority of GnRH neurons coexpress the receptor for kisspeptin, Kiss1R,[16,24–26]

kisspeptin is considered to be a key stimulatory element of GnRH/LH secretion. Kisspeptin perikarya primarily reside in the arcuate nucleus (ARC)[27] and the anteroventral periventricular nucleus/rostral periventricular area of the third ventricle of rodent species,[28–30] or in the preoptic area of non-rodent species.[31] The ARC kisspeptin population highly coexpresses two other neuropeptides relevant to reproduction, neurokinin B (NKB) and dynorphin,[31] and were thus termed KNDy neurons.[32] Similar to kisspeptin, NKB is considered to play a stimulatory role in GnRH/LH secretion because deletion of NKB in humans leads to infertility,[33] and multiple groups have shown that activation of the NKB receptor, neurokinin 3 receptor (NK3R), increases LH secretion.[34–37] However, unlike kisspeptin, NKB appears to stimulate GnRH neurons indirectly via local activation of KNDy neurons, which highly coexpress NK3R.[38] Dynorphin, on the other hand, is known for mediating the inhibitory influence of progesterone on GnRH/LH secretion,[39] which could be directly exerted on GnRH neurons and/or on KNDy neurons given that both cell types express receptors for dynorphin, kappa-opioid receptors.[40,41] Together with evidence indicating that at least 45%–60% of GnRH neurons receive input from KNDy neurons,[42] this population of cells has been suggested to regulate the pulsatile nature of GnRH/LH secretion.[43]

There is growing evidence indicating that KNDy neurons may also play a key role in mediating the effects of energy insufficiency on GnRH neurons during chronic undernutrition. Given that ARC kisspeptin neurons, which are presumptively KNDy neurons, express receptors for both leptin and insulin,[13,44–46] they possess the potential to directly detect changes in energy balance and influence GnRH/LH secretion. Work conducted in ovariectomized (OVX) rodents has shown that extended periods of nutritional restriction (2–6 weeks) reduces ARC mRNA abundance of kisspeptin.[47,48] In addition, studies using chronic feed restriction for 6–10 months in OVX adult sheep have also demonstrated that undernutrition inhibits ARC kisspeptin mRNA abundance.[45] In support of this, our recent work in young (less than 1 year of age), castrated male sheep demonstrates that a shorter duration of feed restriction (approximately 13 weeks) is sufficient to reduce mRNA and protein expression of ARC kisspeptin.[49] Similar to kisspeptin, ARC NKB mRNA abundance in rodents[47,48] and protein expression in young male sheep[49] are reduced with chronic undernutrition. However, unlike kisspeptin and NKB, there has been no report of the impact of undernutrition on dynorphin expression in sheep, and the current rodent data for dynorphin are inconsistent because chronic undernutrition reduces ARC mRNA for dynorphin in mice[48] but has no effect on mRNA for dynorphin in rats.[47]

With such a high degree of coexpression, it is likely that the impact of undernutrition on ARC kisspeptin, NKB, and dynorphin resides largely within KNDy neurons. However, there has yet to be a report detailing the impact of chronic undernutrition on these reproductively relevant neuropeptides using methods to detect both mRNA and protein within the same animal. Given their stimulatory (kisspeptin and NKB) and inhibitory (dynorphin) roles in GnRH/LH secretion, we hypothesized that chronic undernutrition, which we have shown suppresses LH secretion in young, castrated male sheep,[49] would inhibit expression of kisspeptin and NKB at the same time as stimulating dynorphin expression in the ARC and within KNDy neurons of OVX sheep. In the present study, we used OVX sheep, a longstanding animal model in reproduction,[50] to directly examine the nutritional effects on LH secretion apart from changes in sensitivity to gonadal steroids. In addition, in the present study, we used maintenance fed females as controls. Because this is a period when lambs are typically growing, maintaining body weights during this period represents a mild form of growth restriction, but provides a relatively constant metabolic background devoid of additional growth-related cues with which to compare our feed restricted animals. Furthermore, because we previously reported that ARC dynorphin is undetectable when using immunohistochemistry in young male and female sheep,[40,49] in the present study we use a relatively new fluorescent in situ hybridization technique, RNAscope, to simultaneously assess mRNA for kisspeptin, NKB, and dynorphin together with classic immunohistochemistry for protein detection of kisspeptin and NKB in the ARC of young, OVX sheep during chronic feed restriction.

2 | MATERIALS AND METHODS

2.1 | Institutional Review Board Statement

All procedures were approved by the North Carolina State University Animal Care and Use Committee (#17-020-B) and followed the National Institutes of Health guidelines for use of animals in research.

2.2 | Animals

Fifteen Suffolk ewe lambs from single, twin, or triplet pregnancies were approximately 4–5 months of age at the start of this study, which was conducted from July through October 2019. Prior to the study, ewes were group-housed in an open barn for a minimum of 14 days, received ad libitum access to water, and were fed hay supplemented with the same diet used during the experiment (Mule City Specialty Feeds; crude protein, 12%; crude fat, 2.5%; crude fiber, 5.0%; 3.28 Mcal kg^{-1}). Once moved indoors for the study, ewe lambs were housed individually (2.32 m^2 per pen) except for when pair housed (1.39 m^2 per pen) 7 days prior to and 14 days after OVX, performed as described previously[37] at the North Carolina State University School of Veterinary Medicine. No corpora lutea were present on any ovaries at the time of OVX. Animals were housed in raised-floor penning made of polyvinyl chloride or stainless steel with a clear view of other sheep, were fed once daily with the experiment diet, were allowed ad libitum access to water, and provided with indoor lighting automated to mimic natural photoperiod.

2.3 | Experimental design

Thirty days after OVX, animals were placed into one of two groups, fed to maintain (FM) pre-study body weight ($n = 7$) or feed-restricted

(FR) to lose 20% of pre-study body weight by week 13 ($n = 8$), with animals from single, twin, and triplet pregnancies present in each group. All animals were weighed weekly, and the amount of feed was adjusted on an individual animal basis to achieve the desired body weight. Biweekly, blood samples (3–4 mL per sample) were taken via jugular venipuncture every 12 min for 4.5 h (276 min), and plasma samples were stored at $-20°C$ until assayed for LH by a previously established radioimmunoassay.[49] At the end of the experiment (week 13), ewes were given heparin (20,000 U, i.v.) and killed with an i.v. overdose of sodium pentobarbital (Euthasol; Patterson Veterinary). Heads were removed and perfused via the carotid arteries with four liters of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) containing 0.1% sodium nitrite. Blocks of hypothalamic tissue were removed and stored in 4% PFA for 24 h at 4°C and transferred to a 20% sucrose solution until sectioning. Frozen coronal sections were cut at 45–50 μm with a freezing microtome into a series of five vials and stored in cryopreservative solution until RNAscope and immunohistochemistry.

2.4 | RNAscope in situ hybridization

For detection of mRNA for kisspeptin, NKB, and dynorphin, RNAscope was conducted similarly to that reported previously.[49] Briefly, RNAscope was performed on four medial ARC hemisections (at least 250 μm apart) per animal using the RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics; catalog. no. 323100), and all incubations at 40°C were completed using an ACD HybEZ II Hybridization System with EZ-Batch Slide System (Advanced Cell Diagnostics; catalog. no. 321710). On day 1, hemisections were incubated in 0.1 M phosphate buffered saline (PBS) (pH 7.4) on a rocking shaker at 4°C overnight. On day 2, hemisections were mounted onto Superfrost/Plus microscope slides (Fisher Scientific), allowed to air dry for 2 h, and then the slides were heated on a slide warmer to 60°C for 90 min. Next, slides were incubated in 4% PFA at 4°C for 1 h, rinsed four times in 0.1 M PBS (5 min per rinse), and then incubated in increasing concentrations of ethanol (50%, 70%, 100%, and 100%) for 5 min at each concentration. Then slides were air-dried at room temperature (RT) for 5 min followed by incubation in hydrogen peroxide solution (Advanced Cell Diagnostics; catalog. no. 322335) for 10 min at RT. Next, slides were briefly rinsed three times with deionized water, incubated with target retrieval solution (Advanced Cell Diagnostics; catalog. no. 322001) for 10 min at 94°C, and rinsed three times in deionized water followed by submersion in 100% ethanol three times and then allowed to air dry. A hydrophobic barrier was created around each hemisection using an ImmEdge Pen (Advanced Cell Diagnostics; catalog. no. 310018), and slides were stored overnight at RT. On day 3, sections were treated with RNAscope Protease III (Advanced Cell Diagnostics; catalog. no. 322337) for 30 min at 40°C. Probes for target genes and positive controls were mixed at a concentration of 50:1:1 for the channel 1 probe, channel 2 probe, and channel 3 probe, respectively, and all probe solutions, including the negative control solution, were heated to 40°C for 10 min in a water bath and cooled to RT before

application. Following Protease III, tissue was incubated with RNAscope target probes from Advanced Cell Diagnostics (NKB, Oa-TAC3-O1, catalog. no. 481411; dynorphin, Oa-PDYN-O1-C2, catalog. no. 481421-C2; kisspeptin, Oa-KISS1-C3, catalog. no. 497471-C3) or control probes from Advanced Cell Diagnostics (positive controls: Oa-POLR2A, catalog. no. 516171; Oa-PPIB, catalog. no. 457031-C2; Oa-UBC-C3, catalog. no.516181-C3; negative control: 3-plex Negative Control Probe, catalog. no. 320871) for 2 h at 40°C. Next, slides were washed twice (2 min each) at RT with 1 × Wash Buffer (Advanced Cell Diagnostics; catalog. no. 310091) followed by sequential tissue application and incubation of the following at 40°C with 2 min washes using 1 × Wash Buffer between applications: RNAscope Multiplex FL v2 AMP 1 (Advanced Cell Diagnostics; catalog. no. 323101) for 30 min, RNAscope Multiplex FL v2 AMP 2 (Advanced Cell Diagnostics; catalog. no. 323102) for 30 min, and RNAscope Multiplex FL v2 AMP 3 (Advanced Cell Diagnostics; catalog. no. 323103) for 15 min. Following the final incubation with AMP 3, slides were rinsed twice (2 min each) with 1 × Wash Buffer at RT followed by tissue application of RNAscope Multiplex FL v2 HRP C1 (Advanced Cell Diagnostics; catalog. no. 323104) for 15 min at 40°C. Next, sections were incubated with Opal 690 (Fisher Scientific; catalog. no. NC1605064) in RNAscope TSA buffer (Advanced Cell Diagnostics; catalog. no. 322809) at a final concentration of 1:1500 for 30 min at 40°C. Following two rinses (2 min each) with 1 × Wash Buffer at RT, RNAscope Multiplex FL v2 HRP Blocker (Advanced Cell Diagnostics; catalog. no. 323107) was applied to tissue for 15 min at 40°C. Slides were then rinsed twice (2 min each) with 1 × Wash Buffer at RT followed by tissue application of RNAscope Multiplex FL v2 HRP-C2 (Advanced Cell Diagnostics; catalog. no. 323106) for 15 min at 40°C. Sections were next incubated with Opal 570 (Fisher Scientific; catalog. no. NC1601878) in RNAscope TSA buffer at a final concentration of 1:1500 for 30 min at 40°C, and followed by two rinses (2 min each) in 1 × Wash Buffer at RT. Then, RNAscope Multiplex FL v2 HRP Blocker was applied to tissue for 15 min at 40°C. Finally, slides were then rinsed twice (2 min each) with 1 × Wash Buffer at RT followed by tissue application of RNAscope Multiplex FL v2 HRP-C3 (Advanced Cell Diagnostics; catalog. no. 323106) for 15 min at 40°C. Sections were next incubated with Opal 520 (Fisher Scientific; catalog. no. NC1601877) in RNAscope TSA buffer at a final concentration of 1:1500 for 30 min at 40°C, and followed by two rinses (2 min each) in 1 × Wash Buffer at RT. Then, RNAscope Multiplex FL v2 HRP Blocker was applied to tissue for 15 min at 40°C. Finally, slides were coverslipped with Invitrogen ProLong Gold Antifade Mountant (Fisher Scientific; catalog. no. P36930) and stored at 4°C until image acquisition.

2.5 | Immunohistochemistry

Dual-label immunohistochemistry was completed for free-floating hypothalamic hemisections for the detection of kisspeptin and NKB using primary antisera validated for use in sheep.[49,51] Three medial-caudal ARC sections (at least 250 μm apart) were selected per animal from a series of every fifth hypothalamic section. On day 1, sections were washed 12 times (5 min each) in 0.1 M PBS at RT. Subsequent

steps were also performed at RT. Sections were incubated in 0.1 M PBS containing 1% hydrogen peroxide (H_2O_2) for 10 min followed by three washes in 0.1 M PBS (5 min each). This was followed by incubation in 0.1 M PBS solution with 0.4% Triton X-100 (Sigma-Aldrich) and 20% normal goat serum (NGS) (Jackson ImmunoResearch Laboratories, Inc.) for 1 h. Then, sections were incubated with primary antibody for detection of kisspeptin peptide (1:50,000; rabbit anti-kisspeptin; Gift from I. Franceschini, #566) diluted in 0.1 M PBS containing 0.4% Triton X-100 and 20% NGS overnight for 16 h. On day 2, sections were washed in 0.1 M PBS three times (5 min each) and subsequently incubated in biotinylated goat anti-rabbit IgG (1:400; Vector Laboratories; catalog. no. BA-1000) in 0.1 M PBS with 0.4% Triton X-100 and 20% NGS for 1 h. After washing three times in 0.1 M PBS (5 min each), tissue was sequentially incubated in Vectastain ABC (1:600 diluted in 0.1 M PBS; Vector Laboratories; catalog. no. PK-6100), Biotinyl-Tyramide (1:250 diluted in 0.1 M PBS to 1 μL mL^{-1} of 3% H_2O_2; Perkin Elmer; catalog. no. NEL700A), and DyLight 488 green conjugated to streptavidin (1:200 diluted in 0.1 M PBS; Thermo Fisher Scientific; catalog. no. 21832), for 1 h, 10 min, and 1 h, respectively, with three rinses in 0.1 M PBS (5 min each) between each step. Tissue was covered to prevent light exposure following the application of fluorescent dyes. After DyLight application, tissue was rinsed four times in PBS (5 min each) and incubated in 0.1 M PBS with 0.4% Triton X-100 and 20% NGS for 1 h followed by incubation in rabbit anti-NKB (1:250; Phoenix Pharmaceuticals, Inc.; catalog. no. H-046-26) diluted in 0.1 M PBS with 0.4% Triton X-100 and 4% NGS overnight for 16 h. On day 3, tissue was washed in 0.1 M PBS three times (5 min each) and incubated in goat anti-rabbit Alexa-555 (1:200; Thermo Fisher Scientific; catalog. no. A-21428) diluted in 0.1 M PBS with 0.4% Triton X-100 and 20% NGS for 1 h. After finally washing four times in 0.1 M PBS (5 min each), tissue sections were mounted on slides, air dried, coverslipped using gelvatol, and stored in the dark at 4°C.

2.6 | Data analysis

2.6.1 | LH assay

Luteinizing hormone concentrations were measured in duplicate for each sample with a radioimmunoassay using 50–200 μL of plasma and reagents purchased from the National Hormone and Peptide Program as described previously.[52] Assay sensitivity was 0.20 ng per tube with the intra- and inter-assay coefficients of variation being 8.26% and 12.60%, respectively. Pulses were identified using three previously described criteria[53]: (1) a peak must exceed assay sensitivity; (2) a peak must occur within two data points of the previous nadir; and (3) a peak must exceed a 95% confidence interval (CI) of the previous and following nadirs.

2.6.2 | RNAscope in situ hybridization

Imaging of mRNA for kisspeptin, NKB, and dynorphin was used to identify ARC cell numbers of kisspeptin neurons, NKB neurons,

dynorphin neurons, and KNDy neurons. Images from two non-overlapping confocal z-stack images, one dorsal-medial and one ventral-medial, were captured at 1-μm optical sections from ARC sections (four hemisections per animal) using a Zeiss 880 confocal laser scanning microscope equipped with a Plan Apochromat 20×/0.8 dry objective with consistent acquisition settings for all images and images were acquired by an observer blinded to treatment within 3 weeks following RNAscope in situ hybridization. Following acquisition, each image was opened using Zen 2.3 SP1 Black (Zeiss), where individual cells were marked using ScreenMarker MFC Application 1.0.0.1 (Uptodown), ensuring that each cell was only counted once. Then, the blinded observer used Fiji/ImageJ[54] to quantify the average number of cells expressing each transcript, as well as the percentage of cells that coexpressed multiple transcripts. In addition, images of individual KNDy neurons (39–40 cells per animal), which expressed mRNA for kisspeptin, NKB, and dynorphin, were used to determine the integrated density of each transcript within KNDy neurons. Confocal z-stack images of randomly selected KNDy cells that encompassed each cell were captured at 1 μm optical sections through the cell with a Zeiss 880 confocal laser scanning microscope
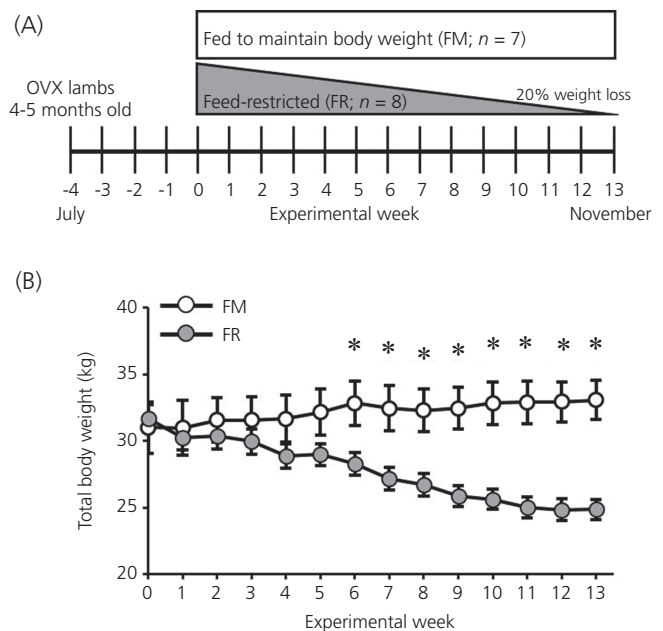
FIGURE 1 (A) Schematic illustration of the experimental design where young (4–5 month old) female sheep were ovariectomized (OVX) in the month of July, which was 30 days prior to week 0, when they were placed into either a fed to maintain pre-study body weight group (FM; $n = 7$) or a feed-restricted to lose 20% of their pre-study body weight (FR; $n = 8$) by week 13 group (November). (B) Mean ± SEM weekly total body weights were recorded throughout the study and feed intake was adjusted weekly to achieve the desired weight change in each group. A repeated-measures two-way ANOVA found a significant interaction between the effects of treatment and time, and fed and feed-restricted groups were compared weekly via F tests for simple effects. From week 6 to week 13, average body weights were lower (*$p < 0.05$) in FR animals compared to FM animals
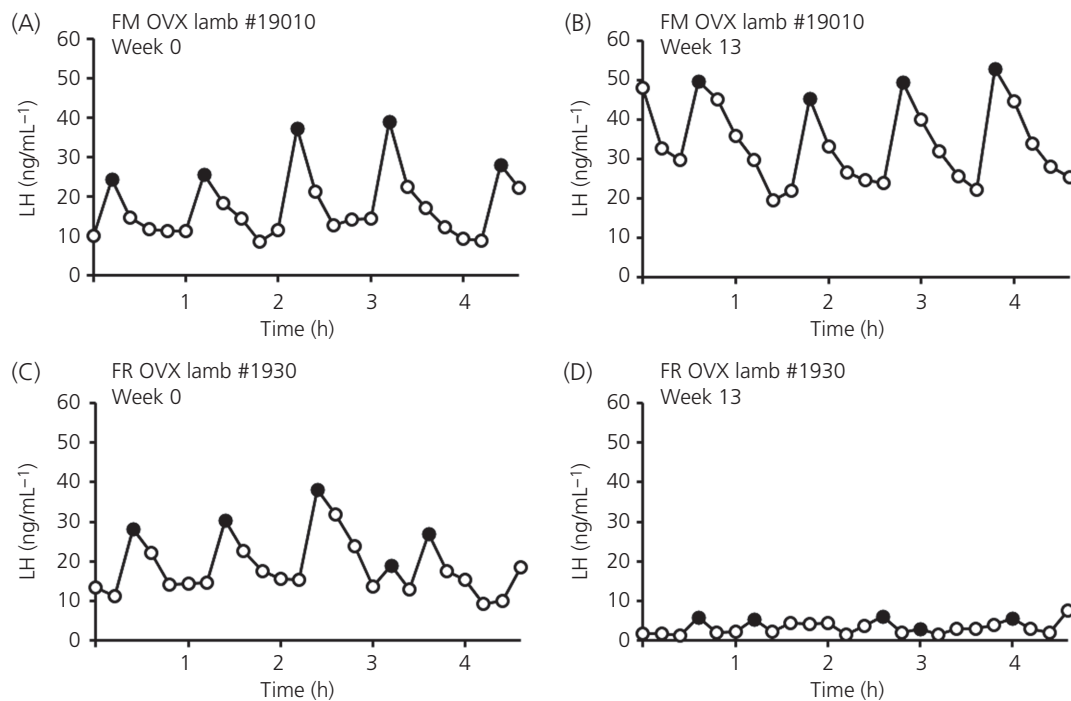
FIGURE 2 Representative luteinizing hormone (LH) pulse profiles at week 0 (A,C) and week 13 (B,D) for a fed to maintain (FM) (A,B) ovariectomized (OVX) lamb and a feed-restricted (FR) (C,D) OVX lamb. LH pulses are denoted by closed circles

equipped with a Plan Apochromat 63×/1.4 oil objective with acquisition settings held constant for all images and images were acquired by an observer blinded to treatment within 3 weeks following RNAscope in situ hybridization. Following image acquisition, an observer blinded to treatment using Fiji/ImageJ converted images to 8-bit and applied a region of interest (312 × 312 pixels) directly over each cell to determine integrated density. An automatic minimum threshold was recorded for each channel corresponding to its specific label in all optical slices to calculate an average threshold for each channel. These respective averages were used as the fixed threshold intensity for integrated density analysis to normalize results across treatment. Three optical slices from the center of each cell, as determined by the extent of detectable signal throughout the cell, were used for analysis with the sum of the integrated density values calculated per cell and then averaged per animal for statistical comparison.

2.6.3 | Immunohistochemistry

To assess the number of kisspeptin-positive, NKB-positive, and neurons coexpressing both peptides following immunohistochemistry for protein detection, ARC tissue sections were imaged using a MIF Olympus VS120 Slide Scanner. Images were taken by an experimenter blinded to treatment using a U PlanS Apochromat 20×/0.75 dry objective with consistent acquisition settings for all images and neurons from each hemisection were counted by an experimenter blinded to treatment using OlyVIA, version 2.9.1 (Olympus), and the number

of cells per hemisection was averaged per animal for statistical comparison.

2.7 | Statistical analysis

Outlier analysis was conducted on mean LH from week 0 using the Igewicz and Hoalglin's robust test for multiple outliers (two sided test) with an outlier criterion modified z score (≥ 3.5) (online outlier test; Contchart Software). Average body weights were analyzed using repeated-measures ANOVA allowing for effects of time and treatment and with a first-order autoregressive covariance structure. The covariance structure was selected using Akaike information criterion. Between group comparisons were run within each week using an F test. The percent change in bodyweight is also reported. Mean LH concentrations, LH pulse amplitude, and the log-transformed average LH interpulse interval were each analyzed using repeated measures two-way ANOVA allowing for effects of time and treatment and a variance components structure on the covariance matrix. Analyses of cell numbers (RNAscope and immunohistochemistry), percent coexpression (RNAscope and immunohistochemistry), and integrated density (RNAscope) were conducted using a one-tailed, unpaired Student's t test with Satterthwaite's approximation to adjust for unequal variances. All values are reported as means and 95% CIs. Model fits were assessed via visual inspection of residual diagnostics. $p < 0.05$ was considered statistically significant. Statistical analyses were conducted using SAS, version 9.4 (SAS Institute Inc.).

3 | RESULTS

3.1 | Body weights

Average weekly body weights from FM OVX lambs and FR OVX lambs throughout the study are illustrated in Figure 1. The repeated-measures model found a strong interaction between week and treatment ($F_{13,169} = 9.54$, $p < 0.001$). This interaction term can be explained by the increasing difference in body weights between treatment groups through the duration of the study. At the beginning of the experiment (week 0), there was no evidence to suggest that total body weights were different ($F_{1,169} = 0.15$, $p = 0.703$) between FM OVX lambs (30.99; 95% CI = 28.52–33.47 kg) and FR OVX lambs (31.65; 95% CI = 29.33–33.97 kg). Beginning at week 6 through to the end of the study, total body weights were lower ($F_{1,169} = 6.94$; $p < 0.05$) in FR OVX lambs compared to FM OVX lambs. At week 13 (day of tissue collection), the average total body weight was higher ($F_{1,169} = 22.82$, $p < 0.001$) in FM OVX lambs (33.07; 95% CI = 30.59–35.55 kg) compared to FR OVX lambs (24.86; 95% CI = 22.545–27.180 kg), and, as designed, the average percent change in pre-study body weight for the FM and FR OVX lambs at week 13 was 7.35% (95% CI = 2.35%–12.36%) and −21.36% (95% CI = −23.43% to 19.28%), respectively.

3.2 | LH data

Representative LH pulse profiles from a FM and a FR OVX lamb taken at the beginning (week 0) and end (week 13) of the study are shown in Figure 2. At week 0, a time when all animals exhibited equivalent body weights, one animal (FM #19008) was identified as an outlier based on mean LH concentrations (#19008, 4.28 ng mL^{-1}; all other animals, 17.61 ± 1.2 ng mL^{-1}). Data from this ewe were excluded from all further analysis. Quantification of mean LH concentration, LH interpulse interval, and LH pulse amplitude for FM and FR OVX lambs at weeks 0 and 13 are illustrated in Figure 3. There was an interaction of time × treatment for mean LH concentration ($F_{1,12} = 5.31$, $p = 0.040$). The interaction term for the LH pulse amplitude was not quite sufficiently large to meet $p < .05$ for statistical significance ($F_{1,12} = 3.78$, $p = 0.076$). The effect is significant at the $p < 0.1$, and post-hoc tests were still run. Although not different at week 0, at week 13 the mean LH concentration (Figure 3A) and LH pulse amplitude (Figure 3B) were lower ($F_{1,12} = 9.79$, $p = .009$ and $F_{1,12} = 7.94$, $p = 0.016$, respectively) in FR OVX lambs compared to FM OVX lambs. No significant differences between groups were detected for LH interpulse interval (Figure 3C) ($F_{1,12} = 0.27$, $p = 0.6144$).

3.3 | Kisspeptin data

We assessed mRNA for kisspeptin in the ARC between FM and FR OVX lambs using RNAscope. Cells expressing mRNA for kisspeptin were readily visible in the ARC of both FM OVX lambs (Figure 4A) and FR OVX lambs (Figure 4B). The average number of kisspeptin cells (Figure 4C) was lower in FR OVX lambs compared to FM OVX lambs (difference of means = 55.42, SE = 28.2, $t_{9.5} = 1.96$, $p = 0.040$), but the percentage of kisspeptin neurons that coexpressed NKB and dynorphin in the FM and FR animals did not differ (difference of means = 0.44, SE = 9.87, $t_{10.4} = .05$, $p = 0.483$) and was 76.0% (SE = 6.45) and 76.4% (SE = 7.72), respectively. The expression of mRNA for kisspeptin within KNDy cells (Figure 4D) was lower (difference of means = 2,807,598, SE = 915,588, $t_{10.7} = 3.07$, $p = 0.006$) in FR OVX lambs compared to FM OVX lambs.
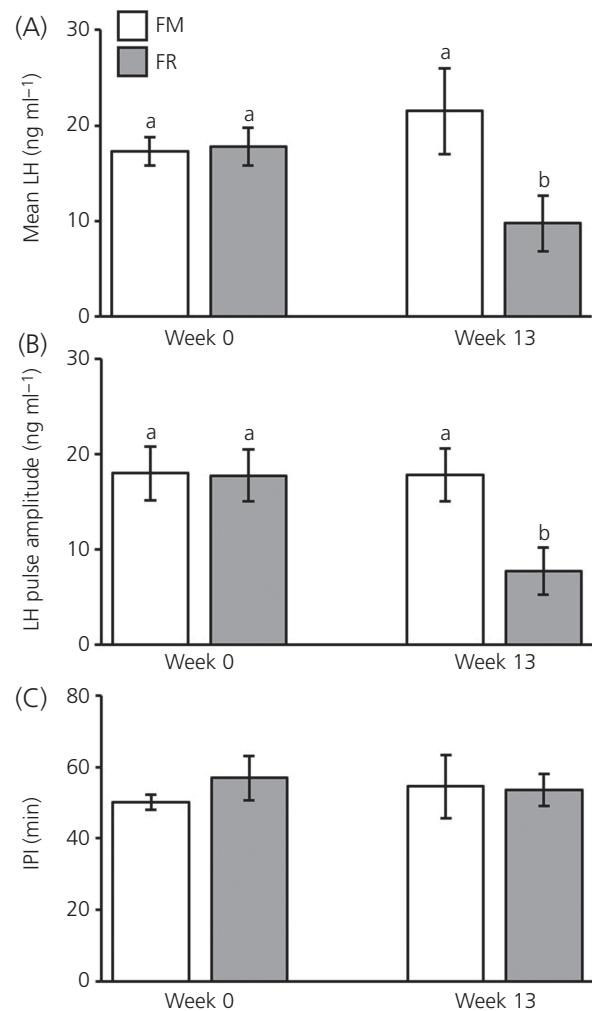
FIGURE 3 Mean ± SEM concentrations of luteinizing hormone (LH) (A), LH pulse amplitude (B), and LH interpulse interval (IPI) (C) from fed to maintain (FM; $n = 6$) and feed-restricted (FR; $n = 8$) ovariectomized (OVX) lambs. Mean LH was significantly lower in FR OVX lambs compared to FM OVX lambs at week 13 ($F_{1,12} = 9.79$, $p = 0.009$), but not at week 0. LH pulse amplitude was significantly lower in FR OVX lambs compared to FM OVX lambs at week 13 ($F_{1,12} = 7.94$, $p = 0.016$, respectively), but not at week 0. IPI was not significantly different between groups at either time point ($F_{1,12} = 0.27$, $p = 0.6144$). Statistical significance ($p < 0.05$) is indicated by differing superscripts and was determined using a repeated measure two-way ANOVA

FIGURE 4 Kisspeptin mRNA (Kiss1) in the arcuate nucleus (ARC) of fed to maintain (FM) and feed-restricted (FR) ovariectomized (OVX) lambs. Confocal images (20×) of Kiss1 cells (green) from a FM (A) and a FR (B) OVX lamb. Insets: 1 μm optical sections; 63× objective. (C) Mean ± SEM number of Kiss1 perikarya in the ARC was significantly different (difference of means = 55.42, SE = 28.2, $t_{9.5}$ = 1.96, p = 0.040) between FM (n = 6) and FR (n = 8) OVX lambs. (D) Mean ± SEM integrated density of mRNA for Kiss1 within KNDy neurons was significantly lower (difference of means = 2,807,598, SE = 915,588, $t_{10.7}$ = 3.07, p = 0.006) in FR OVX lambs compared to FM OVX lambs. Scale bars = 50 μm. Inset scale bars = 10 μm. Statistical significance (*p < 0.05) was determined using a one-tailed, unpaired Students t test
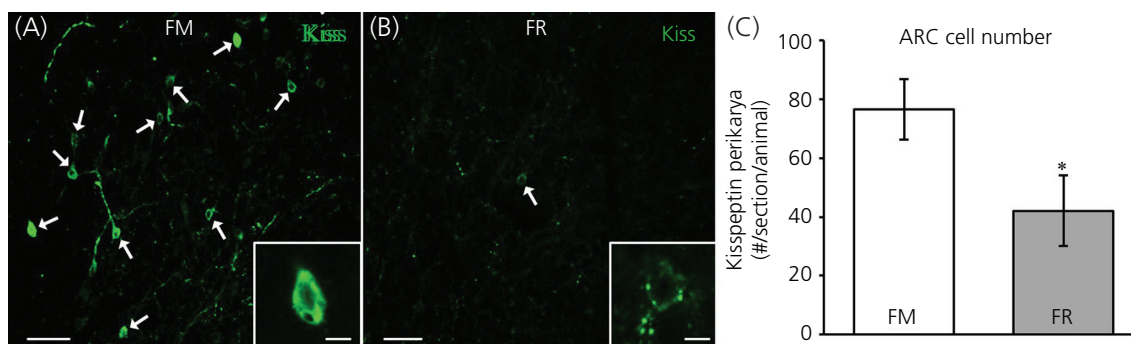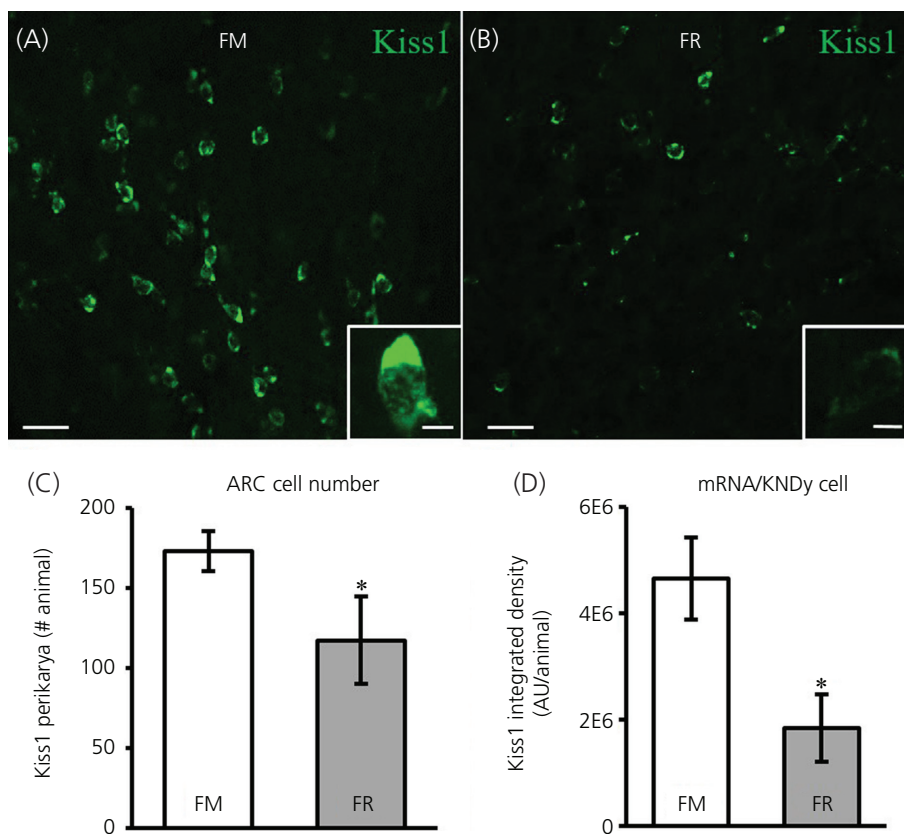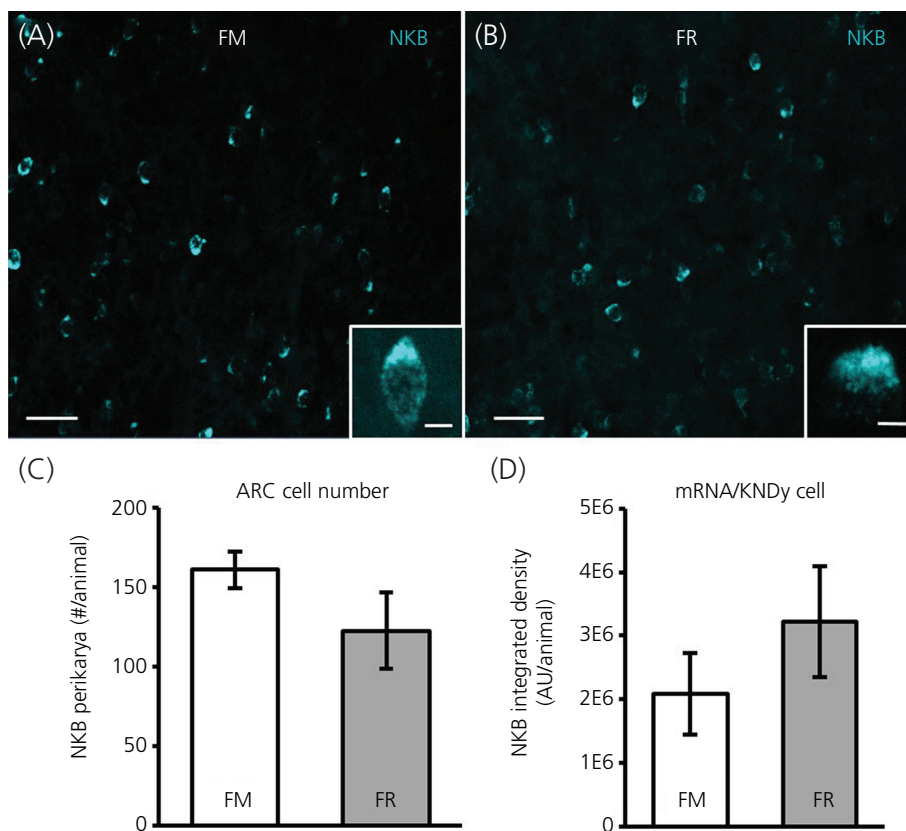

FIGURE 5 Kisspeptin peptide in the arcuate nucleus (ARC) of fed to maintain (FM) and feed-restricted (FR) ovariectomized lambs. (A,B) Representative confocal images (1 μm optical section; 20× objective) from a FM (A) and a FR (B) OVX lamb. Individual kisspeptin perikarya (green) are indicated with white arrows. Insets: 1 μm optical sections; 40× objective. (C) Mean ± SEM number of kisspeptin perikarya in the ARC was significantly lower (difference of means = 34.54, SE = 14.66, t_{12} = 2.36, p = 0.018) in FR OVX lambs (n = 8) compared to FM OVX lambs (n = 6). Scale bars = 50 μm. Inset scale bars = 10 μm. Statistical significance (*p < 0.05) was determined using a one-tailed, unpaired Students t test

In addition, we assessed kisspeptin peptide expression in the ARC between FM and FR OVX lambs using immunohistochemistry. Perikarya and neuronal fibers expressing peptide for kisspeptin were readily visible in the ARC of both FM OVX lambs (Figure 5A) and FR OVX lambs (Figure 5B). Analysis of kisspeptin-immunopositive perikarya revealed that FR OVX lambs had fewer (difference of means = 34.54, SE = 14.66, t_{12} = 2.36, p = 0.018) numbers of kisspeptin cell bodies compared to FM OVX lambs (Figure 5C). The percentage of kisspeptin neurons that coexpressed NKB in the FM and FR animals did not differ (difference of means = 0.48, SE = 5.23, $t_{10.4}$ = .09, p = 0.465) and was 73.9 (SE = 4%) and 73.4 (SE = 4%), respectively.

3.4 | NKB data

We assessed mRNA for NKB in the ARC between FM and FR OVX lambs using RNAscope. Cells expressing mRNA for NKB were readily visible in the ARC of both FM OVX lambs (Figure 6A) and FR OVX lambs (Figure 6B). Although NKB cell numbers were reduced by 23% (Figure 6C), this difference was not statistically significant (difference of means = 38.28, SE = 24.96, $t_{9.7}$ = 1.53, p = 0.079). The percentage of NKB neurons that coexpressed kisspeptin and dynorphin in the FM and FR animals was not different (difference of means = 13.81, SE = 10.88, $t_{11.8}$ = 1.27, p = 0.114) and was 81.4 (SE = 8%) and 67.6

FIGURE 6 NKB mRNA in the arcuate nucleus (ARC) of fed to maintain (FM) and feed-restricted (FR) ovariectomized (OVX) lambs. Confocal images (20×) of NKB cells (blue) from a FM (A) and a FR (B) OVX lamb. Insets: 1 μm optical sections; 63× objective. (C) Mean ± SEM number of NKB perikarya in the ARC was not significantly different (difference of means = 38.28, SE = 24.96, $t_{9.7}$ = 1.53, p = 0.079) between FM (n = 6) and FR (n = 8) OVX lambs. (D) Mean ± SEM integrated density of mRNA for NKB within KNDy neurons was not significantly different (difference of means = −1,112,851, SE = 1,008,531, $t_{11.7}$ = −1.12, p = 0.8581) between FM and FR OVX lambs. Scale bars = 50 μm. Inset scale bars = 10 μm. Statistical significance was determined using a one-tailed, unpaired Students t test
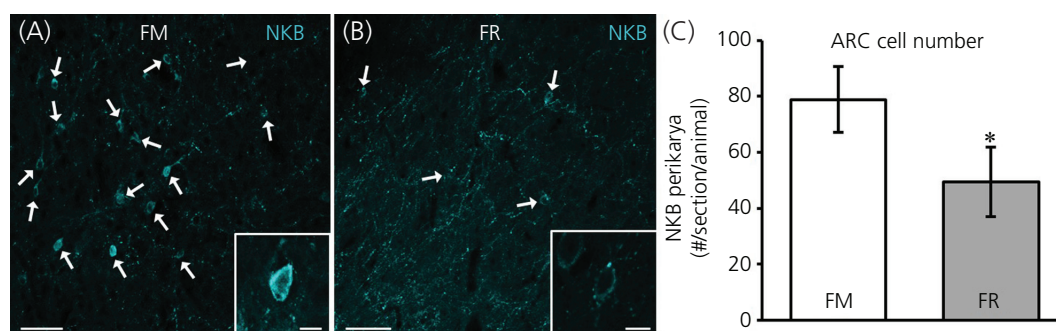
FIGURE 7 NKB protein in the arcuate nucleus (ARC) of fed to maintain (FM) and feed-restricted (FR) ovariectomized lambs. (A,B) Representative confocal images (1 μm optical section; 20× objective) from a FM (A) and a FR (B) OVX lamb. Individual NKB perikarya (blue) are indicated with white arrows. Insets: 1 μm optical sections; 40× objective. (C) Mean ± SEM number of NKB perikarya in the ARC was significantly lower (difference of means = 29.42, SE = 15.77, $t_{11.9}$ = 1.87, p = 0.043) in FR OVX lambs (n = 8) compared to FM OVX lambs (n = 6). Scale bars = 50 μm. Inset scale bars = 10 μm. Statistical significance (*p < 0.05) was determined using a one-tailed, unpaired Students t test

(SE = 8%), respectively. Likewise, the expression of mRNA for NKB within KNDy cells (Figure 6D) was not different (difference of means = −1,112,851, SE = 1,008,531, $t_{11.7}$ = −1.12, p = 0.8581) between groups.

In addition, we assessed protein for NKB in the ARC between FM and FR OVX lambs using immunohistochemistry. Perikarya and neuronal fibers expressing protein for NKB were readily visible in the ARC of both FM OVX lambs (Figure 7A) and FR OVX lambs (Figure 7B). Analysis of NKB-immunopositive perikarya revealed that there was a statistically significant difference (difference of means = 29.42, SE = 15.77, $t_{11.9}$ = 1.87, p = 0.043) with FR OVX lambs having fewer
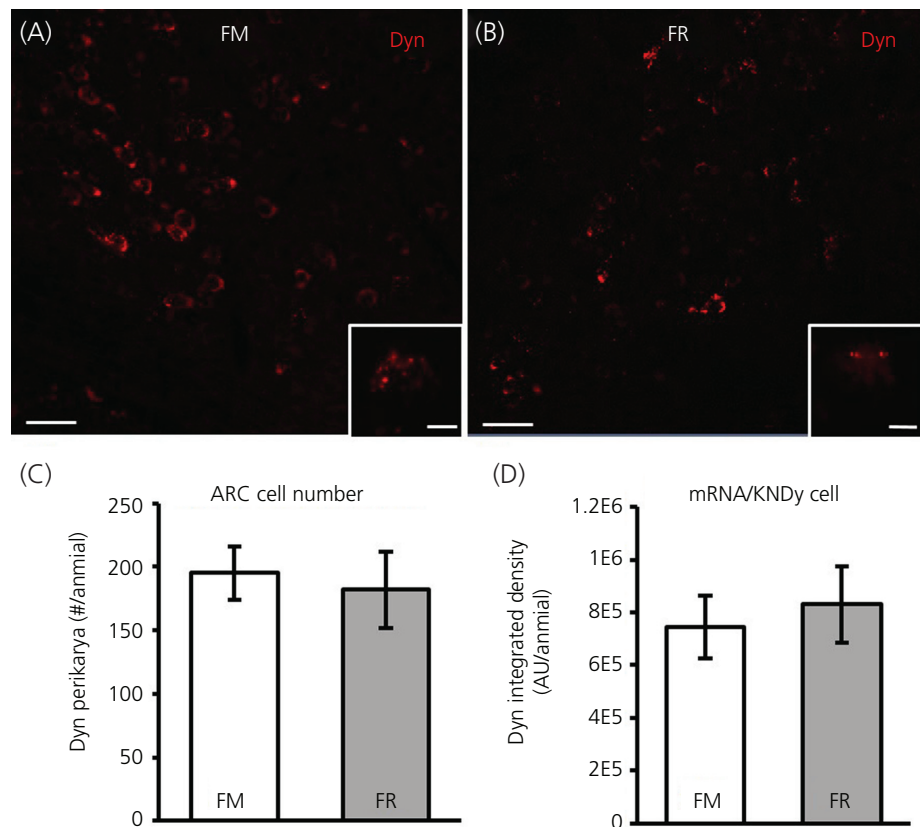
numbers of NKB cell bodies compared to FM OVX lambs (Figure 7C). The percentage of NKB neurons that coexpressed kisspeptin in the FM and FR animals was different (difference of means = 12.34, SE = 4.49, $t_{10.4}$ = 2.75, p = 0.010) and was 72.5 (SE = 4%) and 60.1 (SE = 3%), respectively.

3.5 | Dynorphin data

We assessed mRNA for dynorphin in the ARC between FM and FR OVX lambs using RNAscope. Unlike that previously reported for

FIGURE 8 Dynorphin (Dyn) mRNA in the arcuate nucleus (ARC) of fed to maintain (FM) and feed-restricted (FR) ovariectomized (OVX) lambs. Confocal images (20×) of Dyn cells (red) from a FM (A) and a FR (B) OVX lamb. Insets: 1 μm optical sections; 63× objective. (C) Mean ± SEM number of Dyn perikarya in the ARC was not significantly different (difference of means = 13.35, SE = 34.35, $t_{11.5}$ = 0.39, p = 0.352) between FM (n = 6) and FR (n = 8) OVX lambs. (D) Mean ± SEM integrated density of mRNA for Dyn within KNDy neurons was not significantly different (difference of means = −85,149, SE = 172,570, t_{12} = −0.49, p = 0.685) between FM and FR OVX lambs. Scale bars = 50 μm. Inset scale bars = 10 μm. Statistical significance was determined using a one-tailed, unpaired Students t test

dynorphin protein,[40,49] cells expressing mRNA for dynorphin were readily visible in the ARC of both FM OVX lambs (Figure 8A) and FR OVX lambs (Figure 8B). The average number of dynorphin cells (Figure 8C) were not different (difference of means = 13.35, SE = 34.35, $t_{11.5}$ = 0.39, p = 0.352) between groups, but the percentage of dynorphin neurons that coexpressed kisspeptin and NKB in the FM and FR animals was different (difference of means = 20.91, SE = 7.61, $t_{8.67}$ = 2.75, p = 0.012) and was 67.0 ± 3% and 46.1% ± 8%, respectively. The expression of mRNA for dynorphin in KNDy cells (Figure 8D) was not different (difference of means = −85,149, SE = 172,570, t_{12} = −0.49, p = 0.685) between groups.

4 | DISCUSSION

The data obtained in the present study provide evidence indicating that chronic undernutrition differentially regulates kisspeptin, NKB, and dynorphin within KNDy neurons of female sheep. Chronic feed-restriction in OVX lambs, which produced a significant reduction in LH secretion, resulted in the reduction in the total number of ARC cells expressing mRNA for kisspeptin, less mRNA for kisspeptin within KNDy neurons, and fewer ARC neurons expressing peptide for kisspeptin. Furthermore, although this model of undernutrition in ewe lambs did not alter the number of ARC neurons expressing mRNA for NKB nor the abundance of mRNA for NKB within KNDy neurons, there were fewer ARC neurons expressing protein for NKB with undernutrition. By contrast, we did not observe an impact of feed

restriction on either total number of ARC cells expressing mRNA for dynorphin or on the abundance of mRNA for dynorphin within KNDy neurons.

Chronic undernutrition has been shown to impair reproductive success through the central inhibition of GnRH release from the hypothalamus which, in turn, reduces LH secretion.[8,55] Following previous models as an approach to feed-restriction in young, gonadectomized sheep,[2,56,57] we first established a model of chronic undernutrition in wethers that reduced mean LH concentrations and LH interpulse interval but did not inhibit LH pulse amplitude.[49] In the present study, we observed that feed-restriction in OVX lambs inhibits mean LH concentrations and LH pulse amplitude but does not inhibit LH interpulse interval. Given that the present study used the same model of undernutrition and pulse identification criteria as our previous study,[49] we consider that this may constitute a sex difference in the central mechanism governing the suppression of LH secretion during undernutrition. Furthermore, although the central mechanism by which undernutrition impairs reproduction is shared between young and adult sheep, a much longer duration of feed restriction is required to suppress LH secretion in adult ewes,[58] presumably because of a larger metabolic reserve (i.e., adipose tissue) in adult animals. Moreover, although we have used LH concentrations as an index for GnRH secretion, there is the possibility that undernutrition could act at the level of the pituitary to reduce gonadotrope responsiveness to GnRH. However, studies in rats[59] and sheep[60] have demonstrated normal LH secretion following administration of exogenous GnRH during undernutrition. Taken together with evidence that GnRH neurons are

devoid of receptors for leptin and insulin,[9–13] the reduction in LH secretion with feed restriction very likely reflects a central suppression of GnRH secretion mediated through other afferent neurons.

Although it is generally accepted that KNDy neurons are important for GnRH/LH pulse generation, the role that these neurons play in mediating the negative impact of undernutrition on the reproductive axis is less well developed. Given that kisspeptin has been shown to stimulate GnRH and/or LH secretion in all mammalian species to date[16–23] and that the vast majority of GnRH neurons express Kiss1R,[16,24–26] kisspeptin is considered to provide direct stimulatory drive to GnRH neurons. With up to 60% of GnRH neurons receiving input from KNDy neurons[42] and the majority of ARC kisspeptin neurons (presumptive KNDy neurons) expressing receptors for peripheral metabolic hormones such as leptin and insulin,[13,44–46] kisspeptin within KNDy neurons may serve as a key component of the nutritional regulation of GnRH release. Previous reports in rodents[47,48] and in sheep[45] using a single probe for kisspeptin demonstrated that chronic undernutrition reduces ARC mRNA abundance for kisspeptin. More recently, through the use of RNAscope, we have shown that chronic undernutrition in castrated male sheep reduces mRNA for kisspeptin within kisspeptin/NKB expressing cells.[49] However, although only 52%–65% of kisspeptin cells expressed NKB in castrated males,[49] possibly reflecting the lower degree to which males coexpress kisspeptin and NKB in the ARC,[35,61] we were unable to draw a definitive conclusion about the impact of chronic undernutrition on KNDy neurons because we did not assess dynorphin expression. In the present study using OVX lambs, we observed that undernutrition reduces the number of ARC cells that express mRNA and peptide for kisspeptin, which aligns with our previous report in castrated male sheep.[49] Furthermore, we report novel evidence that feed restriction inhibits mRNA abundance of kisspeptin within KNDy neurons and propose that this reduction led to fewer detectable ARC kisspeptin cells using RNAscope and immunohistochemistry. Although it is assumed that these changes in mRNA and peptide occur within the same cell, further work examining mRNA and peptide within the same sample (i.e., RNAscope combined with immunohistochemistry) is needed to determine whether this change occurs specifically within KNDy neurons and/or within non-KNDy neurons.

Similar to kisspeptin, NKB also plays a stimulatory role in GnRH/LH secretion.[34–37] However, unlike kisspeptin, the actions of NKB are assumed to be mediated, at least in part, via ARC kisspeptin neurons given that they express NK3R, whereas GnRH neurons do not.[38] With the vast majority of NKB perikarya residing in the ARC,[38,62,63] there is growing evidence to suggest that NKB neurons in the ARC play an important role in regulating GnRH/LH secretion during undernutrition. Work in female rodents has shown that a 2–4-week period of chronic undernutrition reduces mRNA for NKB in the ARC.[47,48] Our recent report in male sheep also showed that feed restriction for 13 weeks inhibits the number of ARC cells that express mRNA and protein for NKB.[49] In the present study, using the same undernutrition paradigm that we first established in males,[49] we did not detect a significant reduction in ARC cells that express mRNA for NKB, but did observe a significant reduction in ARC cells expressing NKB protein,

albeit not to the degree that we reported in the males. In addition, in the present study, we demonstrate that the mRNA abundance for NKB within KNDy neurons is unaltered with undernutrition. Because the impact of undernutrition on LH secretion appears to differ between male and female sheep, in that feed restriction reduces LH pulse frequency in males[49] but reduces LH pulse amplitude in females (present study), we postulate that the preserved LH pulsatility in feed restricted female sheep is a result of conserved mRNA for NKB within the ARC, as well as a sufficient number of cells expressing protein for NKB, which highlights the possibility for sex differences in NKB signaling and regulation of NKB cells during undernutrition. Furthermore, work in young female pigs using short-term (10 days) feed restriction designed to hinder weight gain (but not induce weight loss) has shown an increase in mean LH, LH pulse amplitude, and ARC mRNA abundance for NKB.[64] Although the data may appear paradoxical (i.e., feed restriction increases LH secretion), taken together with data obtained in the present study indicating that feed restriction reduces mean LH, LH pulse amplitude, and ARC NKB protein, they support the existing idea that NKB signaling may be the driving stimulus for GnRH pulse initiation[65,66] and leave open the possibility that NKB may also play a role in GnRH pulse amplitude in females. Further work is needed to determine central mechanisms governing the difference in NKB signaling that may exist between sexes, species, and models of undernutrition.

Dynorphin is considered to play a dominant role in mediating progesterone negative feedback[39] and may act directly on GnRH neurons and/or ARC kisspeptin neurons given both express kappa-opioid receptors.[40,41] Although substantial data exist to support the idea that KNDy-derived dynorphin may be part of the "pulse generator" and act to terminate GnRH pulses,[67] much less is known about the role dynorphin may play to regulate GnRH/LH secretion during undernutrition. In mice, chronic undernutrition has been shown to reduce and increase ARC mRNA abundance for dynorphin in OVX and OVX+ oestradiol (E_2) mice, respectively.[48] However, work in OVX and OVX + E_2 rats failed to find a change in ARC mRNA for dynorphin with undernutrition.[47] In the present study, we observed that feed restriction in OVX lambs did not alter the total number of ARC cells expressing mRNA for dynorphin or the abundance of mRNA for dynorphin in KNDy neurons. Because we previously were unable to detect dynorphin protein by immunohistochemistry in young male or female sheep,[40,49] we must rely on mRNA assessment alone to suggest that dynorphin may not play a role in the nutritional regulation of GnRH/LH secretion independent of sex steroids in female sheep. However, given that blockade of dynorphin signaling elicits an increase in LH secretion in prepubertal OVX + E_2 lambs even in the absence of detectable ARC dynorphin immunostaining,[40] it is tempting to speculate that a similar mechanism may exist for dynorphin signaling by which undernutrition may impair GnRH/LH secretion in the presence of E_2, aligning with ovarian hormones playing both activational[68] and organizational[69] roles in the hypothalamus. Moreover, given that dynorphin cells also reside in hypothalamic areas outside of the ARC,[70] further examination of dynorphin signaling during undernutrition in non-ARC regions in both the presence and absence of E_2 is warranted.

Although the data obtained in the present study provide clear evidence that undernutrition reduces kisspeptin within KNDy neurons, it is still unknown whether the effect of chronic undernutrition is exerted directly on KNDy neurons or whether afferent neuronal networks are responsible for relaying energy status to these key reproductive neurons. There is evidence that ARC kisspeptin neurons (presumptive KNDy neurons) express receptors for leptin,[44–46] although leptin administration in 48 h fasted adult sheep[12] and normally fed rats[47] does not induce expression of p-STAT3, a cellular marker of functional leptin receptors, in ARC kisspeptin neurons. Thus, for longer periods of feed restriction in young animals, it is still unknown whether leptin is capable of directly activating ARC kisspeptin neurons. In addition, more recent work in sheep has revealed that ARC kisspeptin neurons also express insulin receptors,[13] although it has yet to be shown that insulin administration during undernutrition can activate kisspeptin neurons. Although direct metabolic regulation should not be ruled out, recent findings in sheep[71] and mice[72] showing that the majority of ARC kisspeptin neurons express melanocortin 3 receptors support the idea that KNDy neurons may be governed by neurons within the ARC that have classically been associated with regulating energy homeostasis (i.e., pro-opiomelanocortin and agouti-related peptide neurons). Furthermore, although larger litter sizes during pregnancy result in lower birth weights in sheep,[73] the data obtained in the present study are not sufficient to determine whether litter size has a long-term impact on hypothalamic neurons during undernutrition and this warrants further investigation.

In conclusion, the data obtained in the present study support the hypothesis that undernutrition acts through KNDy neurons to reduce GnRH/LH secretion in young, OVX lambs. Furthermore, the data raise the possibility that differences exist in the central mechanism by which undernutrition and metabolic status regulates pulsatile LH secretion in male and female sheep. Although we did not find evidence to support a role for dynorphin during chronic feed restriction, we cannot rule out the possibility of a role for dynorphin in the presence of E_2. Additionally, the role of circulating concentrations of markers for metabolic status such as leptin and insulin and the expression of their respective receptors in interneurons that are important for controlling GnRH/LH secretion may be of importance in the future investigation of chronic undernutrition. Thus, future studies will be needed to fully elucidate the central mechanisms underlying the nutrition-induced suppression of GnRH release.

AUTHOR CONTRIBUTIONS

KaLynn Harlow: Data curation; formal analysis; investigation; methodology; project administration; writing – original draft; writing – review and editing. **Max Griesgraber:** Data curation; investigation; methodology; project administration. **Andrew Seman:** Investigation; methodology. **Sydney Shuping:** Investigation; methodology; project administration. **Jeffrey Sommer:** Methodology; project administration; supervision. **Emily H. Griffith:** Formal analysis. **Stanley M. Hileman:** Conceptualization; data curation; formal analysis; funding acquisition; project administration; resources; software; supervision; writing – review and editing. **Casey Nestor:** Conceptualization; data curation; formal analysis; funding

acquisition; project administration; resources; software; supervision; writing – original draft; writing – review and editing.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are available from corresponding author upon reasonable request.

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