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Research Article

Energy balance affects pulsatile secretion of luteinizing hormone from the adenohypophysis and expression of neurokinin B in the hypothalamus of ovariectomized gilts[†]

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Abstract

The pubertal transition of gonadotropin secretion in pigs is metabolically gated. Kisspeptin (*KISS1*) and neurokinin B (NKB) are coexpressed in neurons within the arcuate nucleus of the hypothalamus (ARC) and are thought to play an important role in the integration of nutrition and metabolic state with the reproductive neuroendocrine axis. The hypothesis that circulating concentrations of luteinizing hormone (LH) and expression of *KISS1* and tachykinin 3 (*TAC3*, encodes NKB) in the ARC of female pigs are reduced with negative energy balance was tested using ovariectomized, prepubertal gilts fed to either gain or lose body weight. Restricted feeding of ovariectomized gilts caused a rapid and sustained metabolic response characterized by reduced concentrations of plasma urea nitrogen, insulin, leptin, and insulin-like growth factor-1 and elevated concentrations of free fatty acids. The secretory pattern of LH shifted from one of low amplitude to one of high amplitude, which caused overall circulating concentrations of LH to be greater in restricted gilts. Nutrient-restricted gilts had greater expression of follicle-stimulating hormone and gonadotropin-releasing hormone receptor, but not *LH* in the anterior pituitary gland. Expression of *KISS1* in the ARC was not affected by dietary treatment, but expression of *TAC3* was greater in restricted gilts. These data are consistent with the idea that hypothalamic expression of *KISS1* is correlated with the number of LH pulse in pig, and further indicate that amplitude of LH pulses may be regulated by NKB in the gilt.

Summary Sentence

Nutritional restriction affects pulsatile secretion of LH with no effect on expression of kisspeptin but increased neurokinin B in the arcuate nucleus of the hypothalamus of ovariectomized gilts.

Key words: pig, nutrition, kisspeptin, neurokinin B, luteinizing hormone, metabolism.

Introduction

Puberty in the pig is a complex developmental process that culminates in follicular maturation and expression of estrus followed shortly by ovulation and establishment of normal luteal function. This process is dependent on the pattern of luteinizing hormone (LH) pulses [1, 2]. Mean circulating concentrations of LH in young gilts are initially high because of high amplitude, high frequency LH pulses. At about 100 days of age, mean concentrations of LH are reduced because of decreased LH pulse frequency, which remain suppressed until just before puberty [3]. Approximately 10 to 15 days before puberty occurs, secretion of LH in gilts shifts from low frequency and high amplitude pulses to a higher frequency and lower amplitude pulse pattern [4–7]. Secretion of LH in pigs depends upon the pattern of gonadotropin-releasing hormone (GnRH) release [8], but there is little information about the mechanisms within the central nervous system that bring about the timing of this relationship for proper activation of the reproductive neurosecretory axis and initiation of puberty in gilts.

Puberty occurs around 200 days of age in pigs but substantial variation exists [9] with a significant proportion of gilts failing to reach puberty by 250 days of age [10]. Genome-wide association studies for age at puberty in gilts, which is analogous to age at menarche in women, reveal genomic association with growth and metabolism [10–12]. It is well understood that gonadotropin secretion in pigs is nutritionally gated [13], but identifying a critical level of body weight or minimal percentage of body fat for initiation of puberty in gilts has proven elusive [14]. It has been suggested that metabolic state is an important determinant of physiological maturity and timing of puberty in pigs [15, 16]. Changes in circulating concentrations of metabolic fuels (glucose; free fatty acids, FFA) and hormones such as insulin, leptin, and insulin-like growth factor-I (IGF-1) may provide important cues to the hypothalamus that link metabolic status with the reproductive neuroendocrine axis. Leptin, for example, stimulates secretion of GnRH from the porcine hypothalamus and can stimulate release of LH from the anterior pituitary gland of the pig [17]. Additionally, circulating concentrations of leptin are genetically correlated to age at puberty in pigs [9]. However, GnRH neurons in many species do not express receptors for leptin [18–20], and so it is not sufficiently clear how metabolic cues such as leptin may act to affect the pulsatile secretion of GnRH and LH in gilts [21].

A group of cells within the arcuate nucleus of the hypothalamus (ARC) called kisspeptin-neurokinin B-dynorphin (KNDy) neurons co-express kisspeptin, neurokinin B (NKB), and dynorphin [22–24] and have been proposed to be central to the regulation of the GnRH pulse generator [25–28]. The importance of KNDy neurons for maintenance of gonadotropin secretion is evident in that loss of function mutations in kisspeptin and NKB or their respective receptors results in hypogonadotropic hypogonadism and failure to attain puberty in rodents and humans [29–32], as well as pigs [33]. The purported role of NKB in promoting secretion of LH in prepubertal animals is dependent upon autaptic inputs onto KNDy neurons. Unlike GnRH neurons, KNDy neurons express the NKB receptor (tachykinin 3 receptor; TAC3R) necessary to induce the secretion of

kisspeptin [23, 26, 34]. Kisspeptin stimulates gonadotropin secretion in prepubertal animals [35–38] through direct receptor action on GnRH neurons [39, 40]. Finally, electrophysiology studies in goats [41, 42] and studies of kisspeptin and neurokinin receptor antagonism in sheep [28] suggest that modulations in activity of KNDy neurons are correlated with LH pulses.

The prevailing view is that NKB acts through an autoregulatory mechanism involving TAC3R to induce the release of kisspeptin, which acts directly on the GnRH neuronal network to stimulate a pulsatile release of GnRH and subsequently LH. The KNDy neurons are therefore positioned to relay information about interoceptive signals, such as gonadal steroids and metabolic hormones, to modulate frequency and amplitude of GnRH pulses. Indeed, expression of kisspeptin and NKB in several species, including pigs, is altered under different conditions of gonadal feedback [43–47]. Moreover, KNDy neurons in the ARC of rodents and sheep appear to be targets for metabolic hormones such as leptin [48–50] and IGF-1 [51], suggesting they play a key role to integrate metabolism with reproduction. Key differences in metabolism (e.g. glucose metabolism and lipogenesis) exist between pigs and ruminants or laboratory species [52]. These factors make it important to elucidate how nutrition and metabolism impinge upon the reproductive axis of gilts and the specific role KNDy neurons play in nutritional regulation of reproduction in pigs. Therefore, the objective of the study was to better understand how nutrition and metabolic state affects gonadotropin secretion in gilts. We hypothesized that negative energy balance would decrease expression of *KISS1* and *TAC3* (encodes NKB) mRNA in the ARC resulting in reduced circulating concentrations of LH in ovariectomized gilts.

Materials and methods

Animals and dietary treatments

Experiments were conducted with procedures that were in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching [53], and approved by the U.S. Meat Animal Research Center. Prepubertal, white cross-bred gilts ($n = 12$; maternal-line Yorkshire $\times$ Landrace) were individually housed in pens ($\geq 1.5 \text{ m}^2$) in a climate controlled facility with 12 h light-dark cycles. Animals had ad libitum access to water and were offered twice daily (0800 and 1600 h) a fortified corn-soybean meal diet (Table 1) formulated to meet nutrient requirements [54]. Animals were weighed weekly and the amount of feed offered was adjusted to maintain an average daily gain of 0.68 kg/day from 102 to 153 days of age when animals were ovariectomized. Reproductive status of gilts was confirmed by examining their ovaries at removal for absence of luteal structures. Gilts were ovariectomized by mid-ventral laparotomy and fitted with an indwelling venous catheter to allow collection of serial blood samples. Animals were blocked by body weight and assigned to treatment. From experiment day -7 to 0 all animals were allocated an amount of feed to supply three times their maintenance energy requirements (full-fed). On day 0, half the gilts ($n = 6$) remained on the full-fed treatment and the other half were assigned an allocation of feed that supplied 0.7 times their

Table 1. Diet composition, % as fed.

Ingredient	Composition
Ground corn	79.08
Soybean meal, 44% CP	17.55
Soybean oil	1.00
Dicalcium phosphate	0.61
Ground limestone	0.82
Sodium chloride	0.30
Vitamin mix ^a	0.20
Trace mineral mix ^b	0.20
L-Lys	0.12
Thr	0.01
BMD ^c	0.03
Total	100.00
Calculated nutrient composition	
ME, kcal/kg	3388
CP, %	15.00
Calcium, %	0.55
Phosphorus, %	0.46
Digestible Lys, %	0.73

^aVitamin A (retinyl acetate), 2200,000 IU/kg; vitamin D₃ (cholecalciferol), 440,000 IU/kg; vitamin E (DL- α -tocopheryl acetate), 17,600 IU/kg; vitamin K (menadione sodium bisulfate complex), 2200 mg/kg; niacin, 22,000 mg/kg; D-pantothenic acid (D-calcium-pantothenate), 12,100 mg/kg; riboflavin, 4400 mg/kg; and vitamin B₁₂, 22 mg/kg.

^bFerrous sulfate heptahydrate, 35.05%; copper sulfate pentahydrate, 1.77%; manganese oxide, 9.62%; calcium iodate, 0.016%; sodium selenite, 0.033%; and calcium carbonate, 50.91%.

^cBacitracin methylene disalicylate.

maintenance energy requirement (restricted; $n = 6$) for 11 days. Body weights of gilts were determined on days -7, -4, 0, 5, and 10 and the feed allocation adjusted to maintain dietary treatment. Fasted blood samples were collected into tubes with and without additive (3 and 2 mg NaF and EDTA, respectively, per mL of blood) on days -7, -4, and 0 to 11. Serial blood samples were collected in S-Monovette syringes containing silicate-coated beads (Sarstedt Inc., Newton, NC, USA) at 12-min intervals for 6 h on day 10. Serum and plasma were collected by centrifugation ($2500 \times g$, 20 min, 4°C) and stored at -20°C until analysis. On treatment day 11, gilts were euthanized with barbiturates according to established guidelines for swine [55]. The head was removed and the hypothalamus was

collected as previously described [56]. Hypothalami were frozen over liquid nitrogen vapor and stored at -80°C until sectioning. Coronal sections (20 μ m) through the hypothalamus were made on a cryostat, then thaw-mounted onto SuperFrost Plus slides. A series of every 10th section (200 μ m apart) was used for detection of *KISS1* and *TAC3* mRNA by isotopic in situ hybridization. The anterior pituitary gland was snap-frozen and stored at -80°C until isolation of RNA for quantitative real-time PCR.

In situ hybridization

Using primers (Table 2) designed for the porcine kisspeptin (*KISS1*; AB 466320.1) and neurokinin B (encoded by tachykinin 3; *TAC3*; AY 769936.1) genes, cDNA was produced using total RNA isolated from porcine hypothalamus. Resulting amplicons were cloned into pCR II-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced to confirm the identity of the insert.

Templates containing either a 301- or 455-bp sequence of the porcine *KISS1* and *TAC3* cDNA, respectively, linked to RNA polymerase promoters were produced using procedures for ³⁵S-labeled riboprobe synthesis. Transcription vectors were linearized with EcoRV or BamHI for transcription from the SP6 or T7 promoter, respectively. Linearized plasmids were transcribed in separate reactions with ³⁵S-labeled UTP (Perkin Elmer, Waltham, MA, USA). Probes were purified with phenol-chloroform extraction followed by ethanol precipitation before being resuspended in Tris-EDTA buffer. Hypothalamic sections were fixed in 4% formalin in phosphate-buffered saline, followed by treatment with 0.25% acetic anhydride in 1 M triethanolamine HCL, washed briefly in 2 $\times$ saline sodium citrate buffer (SSC), and dehydrated in ascending concentrations of ethanol. Slides were delipidated in chloroform, washed in ethanol, and air-dried. The ³⁵S cRNA probes were applied at a concentration of 1×10^6 cpm/25 μ L in hybridization buffer (50% formamide, 10% dextran sulfate, 1x Denhardt solution, 2 $\times$ SSC, 500 μ g/mL heparin sodium salt, 0.5 mg/mL yeast tRNA, 0.1% sodium pyrophosphate) at 55°C for 16 h. Sections were washed twice in 1 $\times$ SSC (22°C, 15 min) followed by two washes of 50% formamide in 2 $\times$ SSC (52°C, 20 min) and one wash in 2 $\times$ SSC (22°C, 10 min). Sections were incubated in RNase buffer (0.5 M NaCl, 10 mM Tris-1 mM EDTA, pH 8.0) for 30 min at 37°C. Sections were washed with 2 $\times$ SSC (22°C, 10 min) and incubated with 50% formamide in 2 $\times$ SSC (52°C, 10 min) before washing once in 2 $\times$ SSC (22°C,

Table 2. Sequences and GenBank accession number for oligos used in the study.

Gene ^a	GenBank accession No.	Oligo sequence (5' to 3')	Amplicon size, bp	Primer efficiency
<i>KISS1</i>	AB466320.1	Forward: GGAGACGTCATCTGGCTTTT Reverse: CGGGCCTGTAGATCTAGGATT	301	–
<i>TAC3</i>	AY769936.1	Forward: TGTGAGGAGTCTCAGGAGCA Reverse: GAGGGATGTGGAAGGAGTCA	455	–
<i>LHβ</i>	NM 214080	Forward: CAGCCGGTGTGCACCTA Reverse: CCAGGGAGGCGGATGGA	53	105
<i>FSHβ</i>	NM 213875.1	Forward: CACGTGGTGTGCTGGCTATT Reverse: TCTCGTACACCAGCTCCTTG	104	99
<i>CGA</i>	NM 214446.1	Forward: TCCTGGCCATATTGTCTGTGT Reverse: TTTAGCTTGCATTCTGGGCA	91	98
<i>GNRHR</i>	NM 214273.1	Forward: CCTGGCTCCTCAGTAGCATC Reverse: AGAGAAACCTTCTGTCTGTCCA	95	95
<i>TUBA1B</i>	NM 001.144544.1	Forward: GGTGTCTACTCCTGTGTGCCTG Reverse: GCATTGCCGATCTGGACACC	108	95

^aLuteinizing hormone β polypeptide (*LH β*), follicle stimulating hormone β polypeptide (*FSH β*), common glycoprotein hormone alpha subunit (*CGA*), gonadotropin-releasing hormone 1 receptor (*GnRHR*), tubulin alpha 1b (*TUBA1B*).

10 min) followed by rinsing in deionized water. Slides were dipped in NTB emulsion (Carestream Health, Inc., Rochester, NY, USA) heated to 42°C and air-dried overnight before incubation at 4°C for 10 days. Slides were developed according to the manufacturer's recommendations before counterstaining with toluidine blue. Location of *KISS1* and *TAC3* mRNA-containing neurons was identified under dark- and bright-field microscopy based on density of silver grains 5 × above background. Cell counts were performed in comparable hemisections within the medial arcuate nucleus (mARC) of the hypothalamus (12 hemisections) of each gilt using the Leica Application Suite X imaging software (Leica Microsystems Inc., Buffalo Grove, IL, USA).

Quantitative real-time PCR

Total RNA was isolated from pituitary glands by extraction with Trizol (Thermo Fisher Scientific Inc.) followed by repurification on RNEasy Mini Columns (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol for on-column digestion with DNase. The quantity and quality of RNA were determined by spectrophotometry (NanoDrop 8000; NanoDrop Technologies Inc., Wilmington, DE, USA) and by microfluidic analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA, USA). The average RNA integrity number was 8.9.

Total RNA (1 µg) was reverse transcribed (20 µl reaction) with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA) using both random hexamers and oligo-dT primers. Duplicate reverse transcriptase reactions were conducted and pooled for each animal. Resulting cDNA was diluted 1:6 and used in triplicate amplification reactions (10 µl), which consisted of 1 µl cDNA, 5 µl LightCycler 480 Syber Green I Master mix (Roche Applied Science), 0.4 µl of forward and reverse primer (12 µM), and 3.2 µl of nuclease free water. The quantitative real-time (QPCR) reactions were performed on a Bio-Rad CFX384 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the threshold cycle values were determined with CFX Manager Software using the automatic thresholding settings. Thermal cycling parameters were 1 cycle of 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. Tubulin- α 1B (*TUBA1B*; Table 2) was chosen from eight common reference genes as the housekeeping gene using NormFinder [57] and BestKeep [58]. The 2-log threshold values of the triplicate reactions for each sample were compared and the triplicate was thrown out or repeated if the CV was $\geq 20\%$. The cycle threshold values of within sample replicates were averaged and relative differences in gene expression were calculated [59].

Hormone analyses

Plasma concentrations of glucose and urea nitrogen (PUN) in daily samples were determined on the Technicon AutoAnalyzer II [60]. Plasma concentrations of nonesterified FFAs were determined with an enzymatic colorimetric method (SFA-5; Zen-Bio, Inc., Research Triangle Park, NC, USA). Concentrations of insulin in plasma were determined by RIA (porcine; PI-12K; Millipore Corp., St. Charles, MO, USA). Dilutions of pooled plasma from gilts were parallel to the insulin standard curve. When 12.5 µU and 50 µU of insulin were added to a pool of plasma from restricted gilts, recovery was 95% and 90%, respectively. Pools of porcine plasma with insulin concentrations 5.5 and 8.3 µU/mL had an average intra-assay CV of 3.8%. Sensitivity was 2.7 µU/mL. Concentrations of leptin were determined by RIA (multi-species; XL-85K; Millipore Corp.) according

to the manufacturer's recommendations and validated in pigs [61]. Sensitivity was 1.4 ng/mL and intra-assay CV was 5.8%. Concentrations of cortisol were determined in samples at minute 0, 60, 120, 180, 240, 300, and 360 during the serial blood collection using a coated tube RIA (06B-256440; MP Biomedicals, LLC, Santa Ana, CA, USA) with a sensitivity of 0.24 nmol/l. A pool of porcine plasma with cortisol concentration of 63.6 nmol/l had an intra-assay CV of 6.3%.

Serum concentrations of IGF-1 were determined in duplicate by radioimmunoassay [62, 63] for daily blood samples. Insulin-like growth factor binding proteins (IGFBP) were extracted from serum using a 1:17 ratio of sample to acidified ethanol (12.5% 2 N HCl: 87.5% absolute ethanol) [64]. Extracted samples were centrifuged (12,000 × g at 4°C) to separate IGFBP. A portion of the resulting supernatant was removed and neutralized with 0.855 M Tris base, incubated for an additional 4 h at 4°C, and then centrifuged at 12,000 × g at 4°C to remove any additional IGFBP. When samples of this extract, equivalent to the original serum sample, were subjected to western ligand blot analysis and subsequent phosphorimager, no detectable binding of ^{125}I -IGF-I to IGFBP was observed. Inhibition curves of the neutralized extracted serum ranging from 12.5 to 50 mL were parallel to the standard curve. Recombinant human IGF-1 (GF-050; Austral Biological, San Ramon, CA, USA) was used as the standard and radioiodinated antigen. Antisera AFP 4892898 (National Hormone and Peptide Program, National Institutes of Diabetes, Digestive and Kidney Diseases, Bethesda, MD, USA) was used at a dilution of 1:62,500. Sensitivity of the assay was 19.27 pg/tube. Intra- and interassay CV were 7.9% and 8.8%, respectively.

Concentrations of LH in serum were determined in serial samples by porcine-specific RIA [65]. The reference standards for LH (AFP-10506A) were provided by Dr A. F. Parlow (Scientific Director of the NIH, NIDDK, National Hormone and Peptide Program). Sensitivity of the LH assay was 0.12 ng/mL. Pools of porcine serum with LH concentrations of 0.51 and 5.59 ng/mL had an average intra-assay CV of 10%.

Pulses of LH were quantified based on parameters described by Goodman et al. [66]. An LH pulse was defined as (1) the peak occurred within two samples of the previous nadir, (2) pulse amplitude exceeded assay sensitivity, and (3) the peak was 2 standard deviations above the preceding nadir. Characteristics of LH pulses were quantified within the 6-h period of serial blood collection. Pulse frequency was quantified as the number of pulses per 6 h for each animal. Interpulse interval was quantified as the interval of time between pulse peaks, and means were calculated within animal for statistical analysis. Pulse amplitude was calculated as the difference between the peak concentration of the pulse and the preceding nadir. The exception was when the pulse occurred within two samples of initiation of sampling, then the following nadir was used. Means for pulse amplitude were calculated within animal.

Statistical analyses

Treatment differences in body weight and concentrations of hormones were estimated with a mixed-model ANOVA for repeated measures. The MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary NC, USA) was used and the covariance structure was modeled with either compound symmetry or first-order autoregressive based on the Akaike information criteria. The model included treatment with time and treatment × time interaction. Animal within treatment was the error term to test effects of treatment. The Kenward–Roger estimate was used for denominator degrees of

Figure 1. Least squares mean ($\pm$ SEM) for body weight of ovariectomized gilts that were feed restricted ($0.7 \times$ maintenance energy requirement beginning on day 0) or full-fed ($3.0 \times$ maintenance energy requirement). *Treatment $\times$ day interaction, $P < 0.0001$.

freedom. Treatment differences in the number of cells containing *KISS1* and *TAC3* mRNA were estimated with ANOVA for a split-plot design using the MIXED procedure. A heterogeneous first-order autoregressive function with lag equal to 1 was used to model the covariance structure for hemisection. The model included treatment, hemisection, and their interaction. Because animal was the experimental unit for treatment, animal nested within treatment was used as the error term for the main plot (treatment) and the pooled residual error was used to test effects of the split-unit factor (hemisection) and the interaction. Degrees of freedom for the pooled error term were calculated using the Satterthwaite approximation. Effects of treatment on the number of LH pulses were estimated with the Kruskal–Wallis test for nonparametric, noncontinuous data. Relative differences in gene expression in the anterior pituitary gland were subjected to a one-way ANOVA using the GLM procedure of SAS with treatment as the fixed effect. Differences were considered significant at $P \leq 0.05$ and a tendency toward significance at $P \leq 0.10$.

Results

There was a treatment $\times$ day interaction ($P < 0.0001$) for body weight. Body weight of all gilts increased ($P < 0.0001$) between experiment day -7 and 0 during full feeding, and there were no differences in body weight between treatments during this time (Figure 1). After experiment day 0 , full-fed gilts continued to gain body weight whereas restricted gilts lost body weight such that body weight of restricted gilts was less ($P < 0.0001$) than full-fed gilts at day 5 , and this difference was maintained for the duration of the study.

Metabolites and metabolic hormones

There was a treatment $\times$ day interaction ($P \leq 0.003$) for PUN, glucose, FFA, and IGF-1 (Figure 2). Plasma concentrations of PUN increased ($P = 0.005$) in all gilts between day -7 and 0 during adaptation to full feeding (Figure 2). Thereafter, concentrations of PUN were less ($P < 0.05$) and FFAs were greater ($P < 0.05$) in restricted gilts compared with full-fed gilts beginning 1 day after initiating treatments. These differences continued for the duration of the study (Figure 2). Concentrations of glucose in plasma of restricted gilts were less on day 2 ($P = 0.09$) and day 10 ($P < 0.05$) than in full-fed gilts. There was no treatment $\times$ day interaction ($P = 0.15$) for insulin (Figure 2). Overall circulating concentrations of insulin were

less ($P < 0.01$) in restricted gilts compared with full-fed gilts (3.9 and $7.0 \mu\text{U/mL}$, respectively). Serum concentrations of IGF-1 increased ($P < 0.001$) in all gilts between day -7 and 0 during adaptation to full feeding. From day 2 through 11 of treatment, concentrations of IGF-1 in serum of restricted gilts were reduced ($P < 0.05$) when compared with full-fed gilts (Figure 2). There was a tendency ($P = 0.08$) for a treatment $\times$ day interaction for leptin. Concentrations of leptin in plasma of restricted gilts were less ($P < 0.04$) after dietary treatment when compared with full-fed gilts (Figure 2). Circulating concentrations of cortisol averaged 46.9 nmol/l during the serial blood collection period and did not differ between dietary treatments or over time.

Pulsatile secretion of luteinizing hormone and gene expression

Pulses are clearly evident in secretory patterns of LH in individual gilts from restricted and full-fed treatments (Figure 3). The number of LH pulses and interpulse interval did not differ ($P \geq 0.12$) between treatments; however, the amplitude of LH pulses in restricted gilts was greater ($P < 0.001$) than in full-fed gilts (Table 3). Basal concentrations of LH did not differ ($P = 0.59$), but mean concentrations of LH were greater ($P < 0.01$) in restricted than in full-fed gilts (Table 3).

Expression of *LH β* in the anterior pituitary gland was not affected by dietary treatment; however, restricted gilts had greater ($P < 0.05$) expression of *FSH β* in the adenohypophysis than did full-fed gilts (Figure 4). Pituitary expression of the common gonadotropin alpha subunit (*CGA*) did not differ with treatment, but expression of GnRH receptor (*GnRHR*) tended ($P = 0.07$) to be greater in restricted gilts when compared with full-fed gilts (Figure 4).

Cells expressing *KISS1* and *TAC3* were readily observed in the ARC of these gilts (Figure 5). Expression of *KISS1* was greater ($P < 0.02$) in the more caudal hemisections of the mARC, but the number of cells that expressed *KISS1* in restricted gilts did not differ ($P = 0.93$) from that of full-fed gilts. There was a treatment $\times$ hemisection interaction ($P < 0.05$) for expression of *TAC3*. Expression of *TAC3* was greater ($P < 0.05$) in the more caudal hemisections of the mARC, and this expression was greater ($P < 0.05$) in restricted gilts than in full-fed gilts (Figure 5).

Discussion

Reduced energy intake can alter LH pulsatility [67, 68] and delay puberty in pigs [69, 70]. As an initial step toward gaining greater understanding of what factors may be involved in mediating the effects of energy balance on the hypothalamic–pituitary axis of pigs, we restricted dietary nutrients to ovariectomized, prepubertal gilts. Ovariectomized gilts were used to remove the confounding effects of gonadal steroids and isolate the effects of nutrition. The dietary restriction imposed was designed to generate a modest negative energy balance and produced a slight but steady decline in body weight in comparison to the continued weight gain of full-fed gilts. Restricted gilts demonstrated an adaptive metabolic response to negative energy balance as demonstrated by the profile of metabolites and metabolic hormones. Decreased availability of dietary amino acids coupled with increased utilization of consumed amino acids for tissue homeostasis resulted in reduced circulating concentrations of PUN in restricted gilts. Although plasma concentrations of insulin were reduced during feed restriction, gilts were able to maintain normal concentrations of glucose due to rapid mobilization of FFA to

Figure 2. Circulating concentrations of (A) plasma urea nitrogen (PUN; treatment $\times$ day, $P < 0.0001$), (B) glucose (treatment $\times$ day, $P < 0.003$), (C) free fatty acids (FFA; treatment $\times$ day, $P < 0.0001$), (D) insulin (treatment $\times$ day, $P = 0.15$; treatment, $P < 0.01$), (E) insulin-like growth factor-I (IGF-1; treatment $\times$ day, $P < 0.0001$), and (F) leptin (treatment $\times$ day, $P = 0.08$; treatment, $P < 0.04$) in ovariectomized gilts that were feed restricted ($0.7 \times$ maintenance energy requirement beginning on day 0) or full-fed ($3.0 \times$ maintenance energy requirement).

support gluconeogenesis. Nonetheless, a treatment $\times$ day interaction illustrates that restricted gilts struggled to maintain similar day-to-day concentrations of glucose in circulation.

Mean circulating concentrations of LH were greater for gilts in which feed was restricted. This observation is in agreement with studies in gonadectomized sheep [71] and ovariectomized rats [72, 73], but differs from most studies in feed-restricted gilts. Incongruence regarding the effects of negative energy balance on circulating concentrations of LH in gilts from reported effects in the literature can be attributed to the use of ovariectomized gilts in the current study. Although numerous differences exist between studies, most studies in which changes in concentrations of LH in pigs were measured after feed restriction have used ovary-intact gilts [61, 67, 68]. The reduction in LH pulse frequency and mean concentrations of LH in response to nutritional restriction in the presence of ovarian steroids is likely due to a nutritionally induced increase in sensitivity of gilts to the suppressive effects of estrogen negative feedback [74], as is the case in sheep [71], cattle [75], and rodents [76–78].

Whisnant and Harrell [79] reported that short-term (7 days) feed restriction reduced LH pulse frequency and mean concentrations of LH in serum of ovariectomized gilts. Key differences between that study and the current study are that the degree of nutritional restriction imposed by Whisnant and Harrell [79] was greater (on a body weight basis) and that gilts were ovariectomized after they reached sexual maturity. Sexually mature ovariectomized gilts thus appear to have a different LH secretory response to acute nutritional restriction than do prepubertal ovariectomized gilts. The use of modern maternal genotypes, which are leaner and more energetically efficient than pigs of previous decades, or growth rates before dietary treatments were imposed may have further contributed to differences between experiments.

The increased circulating concentrations of LH in restricted gilts can be ascribed to the significant increase in amplitude of LH pulses. Others have also found that feed restriction of ovariectomized gilts increased LH pulse amplitude [74, 80]. Additionally, the release of LH induced by different doses of GnRH was greater in ovariectomized gilts during restricted feeding than in control-fed gilts [74].

Figure 3. Serum concentrations of luteinizing hormone (LH) in individual ovariectomized gilts that were (A) feed restricted ($0.7 \times$ maintenance energy requirement) or (B) full-fed ($3.0 \times$ maintenance energy requirement). Solid black fill represents peak of LH pulse.

Table 3. Mean and basal concentrations of luteinizing hormone (LH) and characteristics of LH pulses in ovariectomized gilts that were feed restricted or full fed.

Variable ^b	Dietary treatment ^a				<i>P</i> -value
	Restricted		Full-fed		
	Mean	SE	Mean	SE	
Mean LH concentration, ng/mL	1.76	0.09	1.41	0.08	0.01
Basal concentration, ng/mL	1.03	0.08	1.11	0.13	0.59
Pulse amplitude, ng/mL	2.32	0.15	1.02	0.14	0.001
LH pulses, no./6 h	5.5	1.9	7.5	1.0	0.12
Interpulse interval, min	70.3	10.7	52.9	9.8	0.27

^aRestricted, $0.7 \times$ maintenance energy requirement; full-fed, $3.0 \times$ maintenance energy requirement.

^bPulse frequency reported as arithmetic mean with all others reported as least squares means.

Thus, it is likely that increased LH pulse amplitude of feed restricted gilts is a result of increased sensitivity of the anterior pituitary gland to endogenous pulses of GnRH. It is reported here for the first time that feed-restricted ovariectomized gilts have greater expression of *GnRHR* in the anterior pituitary gland, which might account for increased sensitivity to GnRH during nutrient restriction. It is assumed that this change in expression of *GnRHR* in the adenohypophysis of restricted gilts relates to a change in the number of GnRH receptors on the plasma membrane of gonadotrope cells. Gonadectomized sheep subjected to moderate feed restriction, for example, demonstrated increased amplitude of LH pulses along with greater mRNA expression for *GnRHR* and a greater number of GnRH receptors in the anterior pituitary gland than did sheep fed to gain weight [81].

It is possible that increased amplitude of LH pulses in restricted gilts might also reflect increased stores of LH in the hypophysis. Stores of LH in the pituitary were not measured, but no differences between nutritional treatments in expression of *LHβ* in the pituitary gland were observed in the current study. Beckett et al. [81] demonstrated that expression of *LHβ* mRNA in the pituitary of gonadectomized sheep was not dependent upon nutrition, but that sheep fed to lose weight had greater stores of LH in the pituitary than did sheep fed to gain weight. Of course, other factors such as increased input of NPY to the GnRH pulse generator during nutrient restriction [82] may also be at play to alter amplitude of GnRH pulses.

Kisspeptin stimulates secretion of gonadotropins in the pig [38] through its direct action on GnRH release [39, 83]. Kisspeptin, along with NKB and dynorphin, has been proposed to regulate GnRH pulses [25, 47], and their activity in the ARC is critical to this function [28]. The level of kisspeptin expressed in the ARC is related to gonadotropin secretion in rodents [84–86] and sheep [87, 88], and expression of kisspeptin is most commonly reduced during negative energy balance [89–91]. Expression of *KISS1* in hypothalamic blocks of tissue containing the ARC, as determined by QPCR, was reduced 40% in sexually mature gilts after long-term feed restriction that resulted in excessive loss of body weight and cessation of estrus cycles [92]. Nutritional-induced anestrus in pigs, as seen in Zhou et al. [92], is caused by the complete absence of LH pulses [74]. In contrast, short-term feed restriction and moderate loss of body weight in the current study did not affect expression of *KISS1* in the mARC of gilts as determined by *in situ* hybridization, an approach that allows for a more precise estimate of where differences in gene expression might occur. We indeed observed greater expression of *KISS1* in the most caudal sections as compared with those most rostral, which agrees with the previous report in pigs [46]. The lack of a treatment effect on expression of *KISS1* is consistent with the lack of diet-induced change in LH pulse frequency. The current data are consistent with the previous report in which feeding high-energy diets to gilts reduced age at puberty, but did not affect expression of *KISS1* in the ARC as determined by QPCR [93]. Moreover, there was no change in *KISS1* expression in the dissected ARC of gilts during the peripubertal period [94], which differs from rats [84] and sheep [44]. It appears that expression of *KISS1* in the mARC of the hypothalamus of ovariectomized gilts is not sensitive to modest changes in energy balance, and may be suppressed only under severe conditions of nutritional restriction.

A member of the tachykinin family of neuropeptides, NKB regulates secretion of LH in prepubertal animals [90, 95] as a result of its expression and action within the ARC [24, 27, 28]. Similar to the female rat [90], expression of NKB and its receptor in the hypothalamus of the pig increases with sexual maturation [56]. The inhibition of pubertal onset in rats caused by nutrient restriction was overridden when they were treated with a neurokinin 3 receptor-specific agonist [90], indicating that NKB may play a role in metabolic regulation of gonadotropin secretion. We report for the first time the spatial expression of NKB (*TAC3*) in the mARC of the pig. Similar to kisspeptin, expression of NKB was greater in the more caudal sections of the mARC than in the more rostral sections. This is consistent with the high degree of coexpression of these two neuropeptides observed in mice and sheep. This is the first report, that we are aware of, demonstrating the effects of nutritional restriction on gene expression for NKB in a species other than rodents. Here it was observed that gene expression for NKB (*TAC3*) in the mARC was greater in feed-restricted gilts than in full-fed gilts. True et al. [96] reported that although severe caloric restriction

Figure 4. Relative expression of mRNA (LS mean $\pm$ SEM) for luteinizing hormone beta polypeptide (A; *LHβ*), follicle-stimulating hormone beta polypeptide (B; *FSHβ*), glycoprotein hormones alpha subunit (C; *CGA*), and gonadotropin-releasing hormone 1 receptor (D; *GnRHR*) in the anterior pituitary gland of gilts that were feed restricted (0.7 $\times$ maintenance energy requirement) or full-fed (3.0 $\times$ maintenance energy requirement). Means without a common superscript are different (^{a,b} $P < 0.05$; ^{x,y} $P = 0.07$).

suppressed abundance of mRNA for NKB in the ARC of ovariectomized estradiol-treated rats, such as was not the case during a more moderate food restriction. This moderate food restriction is comparable to the current study in which ovariectomized gilts were subjected to a 30% reduction from nutritional requirements in metabolizable energy. In mice, gene expression for NKB in the ARC was dependent upon the degree and duration of energy restriction as well as gonadal steroids and even genetic strain [78]. As has been proposed for the rat [96], there may be a critical threshold of nutrient restriction required to negatively impact NKB in the ARC of the gilt and the degree of nutritional restriction required to meet this threshold is likely very high in ovariectomized gilts. The increased expression of mRNA for NKB and increased circulating concentra-

tions of LH in feed-restricted gilts are consistent with the overall idea that NKB is important for promoting gonadotropin secretion in the pig. Although NKB is thought to be associated with LH pulse frequency, these data indicate that NKB may play a role in modulating amplitude of LH pulses in the gilt.

Leptin is thought to be a key metabolic signal for the integration of energy balance with the reproductive axis of the pig [97]. There is an age dependent increase in the expression of leptin receptor in the hypothalamus [98] and serum concentrations of leptin increase [99] concomitant with changes in LH secretion of gilts during escape from estrogen negative feedback [3, 100]. Serum concentrations of leptin are indeed correlated with the onset of puberty in gilts [9], and leptin stimulates the release of GnRH from the porcine hypothalamus [17]. Reduced leptin in restricted gilts may have affected GnRH release and altered LH pulsatility. The KNDy neurons in the ARC appear to be direct targets for leptin [50, 101], and conditions of leptin deficiency brought about by nutritional restriction are often associated with reduced expression of kisspeptin and NKB in the hypothalamus in rodents [89, 90] and sheep [101]. Previous [61, 79] and current results indicate that negative energy balance produces a rapid and robust suppression of circulating concentrations of leptin in gilts, but present results further demonstrate that this reduction in leptin fails to negatively affect hypothalamic expression of kisspeptin. It may, however, cause changes within the hypothalamus that induce the upregulation of NKB. It has been reported that very few KNDy neurons express leptin receptor [19] so effects of leptin on KNDy neurons are thought to be indirect through second-order neurons. On the other hand, others have shown that effects of leptin on gonadotropins can be mediated through hypothalamic pathways that are independent of KNDy neurons in the ARC [102]. This suggests that KNDy neurons are not the sole neurological pathway through which leptin can act to affect gonadotropin secretion. It was previously proposed that effects of leptin on the reproductive neuroendocrine axis of the gilt may be indirect through hypothalamic pathways involving NPY, which can suppress LH secretion in gilts [103]. Approximately 30% of KNDy neurons in the ARC of the ewe are in close apposition to neural fibers expressing NPY and pro-opiomelanocortin [101], which further supports the idea that leptin might act on KNDy neurons indirectly through NPY neuronal circuits. This remains to be conclusively proven in the pig.

Hypothalamic and peripheral IGF-1 are increased with puberty in rodents [104, 105] and pigs [106]. The role of IGF-1 in directly regulating the hypothalamic-pituitary axis of the gilt is primarily in relation to secretion of growth hormone [107], but it has been reported that IGF-1 can stimulate increased basal secretion of LH from porcine gonadotropes in vitro [108, 109]. This would suggest that IGF-1 may contribute to LH secretion in pigs, and would be consistent with the current observation of altered LH pulses concomitant with reduced concentrations of IGF-1 in feed-restricted gilts. Peripheral and central infusion of IGF-1 stimulated secretion of GnRH in female rats [105, 110] through increased expression of kisspeptin in the hypothalamus [51]. In contrast, intracerebroventricular injection of IGF-1 failed to stimulate secretion of LH in gilts [108]. This would agree with the current observation that decreased IGF-1 in feed-restricted gilts was not accompanied by reduced expression of *KISS1*. Moreover, different circulating concentrations of IGF-1 did not affect GnRH-induced LH secretion in gilts [111]. It is unlikely, therefore, that diet-induced differences in LH pulsatility were due to reduced secretion of IGF-1.

Increases in amplitude and decreases in frequency of LH pulses can occur in stressed animals. Glucocorticoids can negatively affect

Figure 5. Expression of kisspeptin (*KISS1*) and neurokinin B (tachykinin 3; *TAC3*) mRNA in neurons in the arcuate nucleus of the hypothalamus (ARC) of ovariectomized gilts that were (A, C) feed restricted ($0.7 \times$ maintenance energy requirement) or (B, D) full-fed ($3.0 \times$ maintenance energy requirement). Photomicrographs captured at $10\times$ magnification and tiled to depict the distribution of *KISS1* and *TAC3* within the ARC. Inset illustrates the outlined region at $20\times$ magnification. Scale bar = $200\ \mu\text{m}$; 3V, third ventricle. Number of cells (E) in the ARC expressing *KISS1* and (F) *TAC3* (hemisection, $P < 0.0001$; treatment $\times$ hemisection, $P < 0.05$). *Denotes differences between treatment groups ($P < 0.05$).

LH secretion in gilts [112, 113] due to their action at the hypothalamus [114]. Persistent activation of the adrenal gland due to nutritive stress can lead to elevated concentrations of cortisol in food-restricted animals. Circulating concentrations of cortisol, however, were unaffected by nutritional treatment in the current study. These observations agree with those in feed-restricted pigs [68] and sheep [81]. Although longer term feed restriction (≥ 3 weeks) can increase circulating concentrations of cortisol in the pig [115] and acute increases in cortisol can reduce sensitivity of the pituitary gland of gilts to exogenous GnRH [116], it is unlikely that cortisol had a role in nutritional-induced differences in the pulsatile secretion of LH in the current experiment.

Although inhibition of glycolysis can negatively affect LH secretion in prepubertal gilts [61], elevated levels of glucose do not have a net positive effect on secretion of LH in pigs [107, 117]. Pigs are able to maintain euglycemia during acute fasting (72 h) and

short-term feed restriction (< 14 days) by increasing hydrolysis of triglycerides from large peripheral depots of adipose tissue to spare glucose through beta oxidation of FFA in the liver. An alteration in circulating concentrations of FFA in and of itself likely has little influence on activity of the reproductive neuroendocrine axis of the pig [107, 117]. Likewise, concentrations of insulin do not appear to have strong effects on the reproductive hypothalamic-pituitary axis of the gilts. Similar to the current experiment, short-term fasting of ovary intact or ovariectomized prepubertal gilts reduced insulin levels, but LH was unaffected [61, 80]. Moreover, central infusion of insulin failed to alter LH secretion in ovariectomized prepubertal gilts [118]. The lack of a central effect of insulin on LH secretion in gilts agrees with the observation here that expression of *KISS1* in the ARC was not affected by reduced levels of insulin due to feed restriction. It is speculated that pigs may be like mice in that few kisspeptin neurons ($\leq 5\%$) express the insulin receptor and signaling

of the insulin receptor in hypothalamic neurons appears to have minimal to no effect on kisspeptin function in mice [119, 120].

Peripheral signals relay information about nutritional status and body energy stores to integrated neurocircuitries in the hypothalamus that control the proper temporal shift in gonadotropin secretion for the initiation and maintenance of puberty. Short-term feed restriction of gilts causes rapid and pronounced changes in many of the metabolites and metabolic hormones thought to be key signals in this hypothalamic regulation. The result is a shift in LH secretion to a low frequency, high amplitude pulse pattern. These data demonstrate that expression of kisspeptin mRNA in the ARC of the hypothalamus of ovariectomized prepubertal gilts is resistant to changes in metabolic cues brought about by short-term feed restriction, whereas expression of mRNA for NKB was increased in response to nutritional restriction. How these changes in gene expression relate to protein synthesis and secretion in the gilt are not presently known. Whether these observations are connected to changes in other neurocircuits that directly respond to alterations of circulating metabolic signals require further study.

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