Reciprocal changes in leptin and NPY during nutritional acceleration of puberty in heifers

Rodolfo C Cardoso^{1,2}, Bruna R C Alves², Ligia D Prezotto^{1,2}, Jennifer F Thorson^{1,2}, Luis O Tedeschi², Duane H Keisler³, Marcel Amstalden² and Gary L Williams^{1,2}

¹Animal Reproduction Laboratory, Texas A&M AgriLife Research Station, 3507 Highway 59E, Beeville, Texas 78102, USA

²Department of Animal Science, Kleberg Center, Texas A&M University, College Station, Texas 77843, USA

³Division of Animal Sciences, Animal Science Research Center, University of Missouri, Columbia, Missouri 65211, USA

Correspondence
should be addressed
to G L Williams
Email
glwilliams@tamu.edu

Abstract

Feeding a high-concentrate diet to heifers during the juvenile period, resulting in increased body weight (BW) gain and adiposity, leads to early-onset puberty. In this study, we tested the hypothesis that the increase in GnRH/LH release during nutritional acceleration of puberty is accompanied by reciprocal changes in circulating leptin and central release of neuropeptide Y (NPY). The heifers were weaned at 3.5 months of age and fed to gain either 0.5 (Low-gain; LG) or 1.0 kg/day (High-gain; HG) for 30 weeks. A subgroup of heifers was fitted surgically with third ventricle guide cannulas and was subjected to intensive cerebrospinal fluid (CSF) and blood sampling at 8 and 9 months of age. Mean BW was greater in HG than in LG heifers at week 6 of the experiment and remained greater thereafter. Starting at 9 months of age, the percentage of pubertal HG heifers was greater than that of LG heifers, although a replicate effect was observed. During the 6-h period in which CSF and blood were collected simultaneously, all LH pulses coincided with or shortly followed a GnRH pulse. At 8 months of age, the frequency of LH pulses was greater in the HG than in the LG group. Beginning at 6 months of age, concentrations of leptin were greater in HG than in LG heifers. At 9 months of age, concentrations of NPY in the CSF were lesser in HG heifers. These observations indicate that increased BW gain during juvenile development accelerates puberty in heifers, coincident with reciprocal changes in circulating concentrations of leptin and hypothalamic NPY release.

Key Words

- cerebrospinal fluid
- heifers
- neuropeptide Y
- nutritional programming
- puberty

Journal of Endocrinology
(2014) **223**, 289–298

Introduction

The pubertal initiation of high-frequency, episodic secretion of gonadotropin-releasing hormone (GnRH) is largely dependent upon metabolic cues throughout prepubertal development. Growth restriction during the juvenile period has been demonstrated to delay puberty (Kennedy & Mitra 1963, Kennedy 1969, Foster & Olster 1985, Kile *et al.* 1991), whereas increased adiposity accelerates reproductive

maturation in mammals (Frisch & McArthur 1974, Lee *et al.* 2007, Castellano *et al.* 2011). In cattle, feeding a high-concentrate diet to heifers during the juvenile period, resulting in increased body weight (BW) gain and adiposity, leads to early maturation of the reproductive neuroendocrine system and earlier puberty (Gasser *et al.* 2006a,b, Cardoso *et al.* 2014). Although the mechanisms involved in this

process have not been completely elucidated, leptin and leptin-sensitive cells in the CNS appear to play a critical role (Garcia *et al.* 2003, Maciel *et al.* 2004a, Zieba *et al.* 2005). Leptin, a hormone secreted mainly by adipocytes and positively correlated with body fat mass (Ahren *et al.* 1997), does not acutely affect the secretion of luteinizing hormone (LH) in adequately fed ewes (Henry *et al.* 1999) and cows (Amstalden *et al.* 2002). Nevertheless, leptin prevents a fasting-induced reduction in LH pulsatility in prepubertal heifers (Maciel *et al.* 2004b) and increasing prepubertal secretion of leptin appears to play a permissive yet critical role in timing of the onset of puberty (Garcia *et al.* 2002, Cardoso *et al.* 2014). As several studies have demonstrated that leptin does not act directly on GnRH neurons (Finn *et al.* 1998, Hakansson *et al.* 1998, Quennell *et al.* 2009), leptin's influence on the metabolic control of reproductive maturation is probably relayed by intermediate pathways.

Neuropeptide Y (NPY) neurons in the arcuate nucleus (ARC) contain the leptin receptor (Baskin *et al.* 1999), play an orexigenic role in energy homeostasis (Wang *et al.* 1997), and are responsive to changes in nutritional status (Kalra & Kalra 2003). In ovariectomized ewes, i.c.v. infusion of leptin for 3 days reduces the expression of NPY mRNA in the ARC (Henry *et al.* 1999). Furthermore, NPY has been proposed to mediate the inhibitory effects of under-nutrition on reproductive function (Kalra & Crowley 1984). Contrary to observations in rats in which NPY can have either stimulatory or inhibitory effects on LH release dependent on the gonadal steroid milieu (Sahu *et al.* 1987), NPY has predominantly inhibitory actions on the release of LH in ruminants irrespective of steroidal influences (Barker-Gibb *et al.* 1995, Gazal *et al.* 1998, Estrada *et al.* 2003, Morrison *et al.* 2003). These effects of NPY are largely due to inhibition of GnRH release (Gazal *et al.* 1998) and may be mediated by a direct action on GnRH neurons (Klenke *et al.* 2010). In addition, central infusion of NPY in prepubertal rats is followed by a delay of pubertal maturation similar to that observed in food-restricted animals (Pralong *et al.* 2000). Thus, we reasoned that increased leptin secretion may facilitate pubertal maturation of the hypothalamic–adenohypophyseal–gonadal axis by suppressing hypothalamic NPY release. To test this hypothesis, we examined whether feeding a high-concentrate diet during the juvenile period, a strategy known to accelerate BW gain, increase circulating concentrations of leptin and advance the onset of puberty (Gasser *et al.* 2006a,b, Allen *et al.* 2012, Cardoso *et al.* 2014), would result in lower concentrations of NPY in third-ventricle (3V) cerebrospinal fluid (CSF) concomitantly with increased secretion of GnRH and LH.

Materials and methods

All animal-related procedures used in this study were approved by the Institutional Agricultural Animal Care and Use Committee (IAACUC) of the Texas A&M University System (AUP# 2009-151).

Animals and nutritional model

Twenty-five crossbred heifers ($\frac{1}{2}$ Angus, $\frac{1}{4}$ Hereford, $\frac{1}{4}$ Brahman) were utilized in two replicates over a 2-year period (one replicate/year). Within each year, heifers were weaned at approximately 3.5 months of age (age at weaning = 106 ± 3 days), stratified by date of birth, and assigned randomly to be fed individually until 11 months of age either to achieve a relatively low rate of BW gain (Low-gain (LG), 0.5 kg/day; $n=6$ /replicate) or a high rate (High-gain (HG), 1.0 kg/day; replicate 1, $n=6$; replicate 2, $n=7$). Diets were balanced using the level 2 solution of the Large Ruminant Nutrition System (LRNS; <http://nutrition-models.tamu.edu/lrns.htm>), which is based on the Cornell Net Carbohydrate and Protein System as described by Fox *et al.* (2004). Targeted average daily gain was attained by adjustments in the dry matter intake based on BW gain determined every 2 weeks. Ingredients and diet chemical composition are given in Table 1.

Heifers were allocated individually and fed an acclimation diet for 2 weeks post-weaning. After the acclimation period, heifers were fed solely diet A until 6.5 months of

Table 1 Ingredients and chemical composition of diets fed to prepubertal heifers in the current study. Diet A was provided to heifers during the first 10 weeks of the experiment and diet B was fed to heifers from week 11 until the end of the study (30 weeks)

Item	Diet A ^a	Diet B ^a
Ingredients ^b		
Alfalfa hay (%)	22.42	17.88
Cottonseed hulls (%)	6.71	10.67
Rolled corn (%)	40.37	53.68
Cane molasses (%)	4.48	3.58
Cottonseed meal (%)	6.71	7.14
Corn gluten feed (%)	17.91	5.29
Urea (%)	–	0.58
Producers 12:12 premix (%)	0.46	0.44
Calcium carbonate (%)	0.94	0.74
Chemical composition ^b		
Metabolizable energy (Mcal/kg)	2.59	2.62
Crude protein (%)	15.00	14.8
Digestible intake protein (%)	10.65	10.80

^aDiets were balanced using the Large Ruminant Nutrition System (LRNS).

^bDry matter basis.

age, afterwards they received diet B until the end of the experiment (11 months of age). Blood samples from the coccygeal vasculature were collected twice a month for the duration of the experiment for determination of serum concentrations of leptin. Starting at 7.5 months of age, blood samples were collected twice weekly until the end of the experiment or until puberty was determined (at least three consecutive samples with concentrations of progesterone ≥ 1 ng/ml), whichever occurred first. Serum was collected from blood samples by centrifugation (2200 *g* for 20 min at 4 °C) and stored at -20 °C until assessment for hormone concentration.

Surgical cannulation of the 3V and intensive sampling

Starting at 6 months of age, heifers were acclimated to handling to minimize stress effects on the variables measured. At 7.5 months of age (age = 228 ± 3 days), a subgroup of heifers ($n=3$ per group per replicate) was selected randomly to be surgically fitted with 3V cannulas according to our laboratory procedure, as described previously (Gazal *et al.* 1998). This well-established experimental paradigm has been utilized to measure the release of hypothalamic neuropeptides in several species, including rats (Szczepanska-Sadowska *et al.* 1983), sheep (Skinner *et al.* 1995), cattle (Gazal *et al.* 1998), and nonhuman primates (Van Vugt *et al.* 1985). The location and function of the cannulas were verified by radiography and continuous flow of CSF. A period of at least 2 weeks was allowed for heifers to recover from surgery before CSF was sampled.

At approximately 8 months of age (age = 248 ± 5 days), heifers were fitted with jugular catheters on the day before the intensive sampling. At the same time, the animals were treated prophylactically with antibiotics as reported previously (Gazal *et al.* 1998). On the day of sampling, heifers were placed in stanchions and 20 cm of polyethylene tubing (0.58 mm i.d. $\times$ 0.96 mm o.d.; Intramedic Clay Adams Brand, Becton Dickinson, Sparks, MD, USA) were inserted using aseptic techniques through the guide cannula. The position of the tubing was adjusted until CSF withdrawal was easily accomplished using a blunt 22-gauge needle and tuberculin syringe. The end of the tubing was connected to another 60-cm section of polyethylene tubing. The collection end of the tubing was secured approximately 40–50 cm away from the heifer's head to facilitate semi-remote sampling. The blood samples (6 ml) were collected via a remotely secured extension connected to the jugular catheter, simultaneously with CSF samples (600 μ l) at 15- (replicate 1: $n=3$ /group) or 10-min intervals (replicate 2: $n=3$ /group) for 6 h, and intensive blood

sampling continued for an additional 6 h. The basis of the slight difference in intensive sampling intervals between the two replicates was to ensure accurate detection of GnRH and LH pulses; however, the sensitivity of pulse detection between replicates did not differ. In all cases, void volumes of CSF and blood created by the indwelling tubing and the extensions were discarded before every sample collection. The blood samples were dispensed in tubes containing 100 μ l of heparin solution (10 000 IU/ml) and 5% EDTA, and placed on ice immediately. Plasma was separated by centrifugation (2200 *g* for 20 min at 4 °C) and stored at -20 °C until LH determination. The CSF samples were placed on ice immediately and, within 30 min, stored at -20 °C until NPY analysis. Intensive blood and CSF sampling procedures were repeated at approximately 9 months of age (age = 276 ± 3 days) in heifers that had not achieved puberty yet (LG, $n=6$; HG, $n=4$). One HG heifer did not have a functional 3V cannula for CSF sampling at 9 months of age; thus only jugular blood samples for LH determination were collected at this time point.

RIA

To confirm the pubertal status of heifers, circulating concentrations of progesterone were determined using a commercial RIA kit (Coat-A-Count, Siemens Healthcare, Malvern, PA, USA) as reported previously (Fajersson *et al.* 1999). Sensitivity was 0.1 ng/ml with intra- and inter-assay coefficients of variation (CV) of 5.5 and 13% respectively. Circulating concentrations of leptin were determined in triplicates, in a single assay, using a highly specific ovine leptin RIA validated for use in bovine serum (Delavaud *et al.* 2000). The sensitivity of the assay was 0.1 ng/ml with an intraassay CV of 6%. The plasma concentrations of LH were determined in duplicate 200- μ l aliquots with a validated RIA (McVey & Williams 1991). The sensitivity of the assay averaged 0.1 ng/ml, and average intra- and inter-assay CV were 5 and 9.5%, respectively. Concentrations of GnRH were determined in duplicate 200- μ l CSF samples as described by Ellinwood *et al.* (1985). Antiserum BDS-037 (Dr Alain Caraty, INRA Centre de Tours, Nouzilly, France) was used as the source of primary antibody at a working dilution of 1:50 000. The sensitivity of the assay was 0.5 pg/ml, and average intra- and inter-assay CV were 5 and 16% respectively. Finally, concentrations of NPY were determined directly in duplicate 100- μ l CSF samples using a commercial bovine RIA kit (Peninsula Laboratories, Belmont, CA, USA). No improvements in the detection of NPY in CSF samples were observed when methanol or C18 Sep-Column extraction methods were tested. Recovery of

added mass and parallelism between serial dilutions of samples with the standard curve were performed to validate the use of the RIA kit directly on bovine CSF samples. The sensitivity of the assay averaged 0.25 ng/ml, with intra- and inter-assay CV of 4.5 and 11.5% respectively.

Pulse detection and statistical analysis

BW gain, concentrations of leptin and NPY, and mean concentrations, amplitude, and frequency of GnRH and LH pulses were analyzed by mixed-model analyses for repeated measures using the MIXED procedure of SAS (version 9.3; SAS Institute, Inc., Cary, NC, USA). The sources of variation were treatment, replicate, time and their interactions. Time was used as the repeated variable, and heifer was used as the subject. The frequency and amplitude of LH pulses were determined using a pulse-detection algorithm, Pulsefit 1.2 (Kushler & Brown 1991). Temporal coincidences between GnRH and LH pulses within heifers were determined as defined by Gazal *et al.* (1998). The percentage of pubertal heifers was analyzed using the CATMOD procedure of SAS (SAS Institute, Inc.). Main effects were considered significant when $P \leq 0.05$.

Results

BW gain and circulating concentrations of leptin

As no replicate effect was observed for BW gain and circulating concentrations of leptin, data were pooled by treatment. Mean ($\pm$ S.E.M.) BW at the beginning of the experiment did not differ between groups (140.9 ± 3.2 kg). BW increased linearly in both dietary groups (Fig. 1). As expected based on the experimental design, HG heifers had greater ($P < 0.05$) BW than LG heifers starting at week 6 of the experiment and continuing throughout the study ($P < 0.05$). Beginning at week 8 of the experiment, circulating concentrations of leptin were greater ($P < 0.05$) in HG than that in LG heifers and remained greater ($P < 0.05$) throughout the study (Fig. 2). Body condition score, assessed according to a scale from 1 to 9 as described by Herd & Sprott (1986), was greater ($P < 0.05$) in HG (6.0 ± 0.2) than that in LG heifers (4.9 ± 0.1) at week 16 of the experiment (approximately 8 months of age).

Pulsatile patterns of GnRH and LH release, and central concentrations of NPY relative to peripheral circulating leptin

No replicate effect was observed for the mean concentrations or pattern of secretion of GnRH, LH, and NPY;

Figure 1

Mean ($\pm$ S.E.M.) BW of heifers weaned at approximately 3.5 months of age and fed for 30 weeks to achieve either a low rate of BW gain (LG, $n = 12$ (0–30 weeks)) or a relatively high rate of BW gain (HG, $n = 13$ (0–16 weeks), 12^a (17–20 weeks), 9^a (21–24 weeks), 8^a (25–28 weeks), 6^a (29–30 weeks)). BW of HG heifers was greater ($P < 0.05$) than that in LG heifers beginning at week 6 and continuing throughout the study. ^aHeifers were excluded from the study as they reached puberty.

therefore, data were pooled by treatment. At 8 and 9 months of age, circulating concentrations of leptin were greater ($P < 0.05$) in HG than that in LG heifers subjected to CSF sampling (Fig. 3A). The concentrations of NPY in the CSF did not exhibit a pulsatile pattern of secretion during the 6-h sampling period; thus, overall mean concentrations were used to compare the effects of dietary treatments. At 8 months of age, the concentrations of NPY in the 3V CSF tended to be less ($P = 0.17$) in HG than that in LG heifers (Fig. 3B). At 9 months of age, the concentrations of NPY in the 3V CSF were lower ($P \leq 0.02$) in HG prepubertal heifers than that in LG heifers (Fig. 3B).

Individual secretion patterns for GnRH (CSF) and LH (plasma) in representative LG ($n = 2$) and HG ($n = 2$) heifers are shown in Fig. 4. GnRH was secreted in the 3V CSF in a pulsatile pattern, consistent with previous reports on mature cows (Gazal *et al.* 1998, Zieba *et al.* 2004). High concordance in the patterns of CSF GnRH and plasma LH secretion was observed. During the simultaneous CSF and blood sampling periods (totaling 126 h), 16 LH pulses were identified and 13 (81.3%) occurred in exact temporal synchrony or within one sampling period after a GnRH pulse. Furthermore, all pulses of LH (100%) occurred within two sampling points after the onset of a GnRH pulse. Within the same sampling period, 19 GnRH pulses were detected. Mean GnRH concentrations (8 months

Figure 2

Mean ($\pm$ S.E.M.) circulating concentrations of leptin in heifers weaned at approximately 3.5 months of age and fed for 30 weeks to achieve either a low rate of BW gain (LG, $n=12$ (0–30 weeks)) or a relatively high rate of BW gain (HG, $n=13$ (0–16 weeks), 12^a (17–20 weeks), 9^a (21–24 weeks), 8^a (25–28 weeks), 6^a (29–30 weeks)). The concentrations of leptin were greater ($P<0.05$) in HG heifers than that in LG heifers beginning at week 8 of the experiment and remained greater ($P<0.05$) until the end of the study. ^aHeifers were excluded from the study as they reached puberty.

of age, 0.9 ± 0.4 pg/ml; 9 months of age, 1.5 ± 0.8 pg/ml), amplitude (8 months of age, 2.8 ± 1.3 pg/ml; 9 months of age, 2.2 ± 1.0 pg/ml), and pulse frequency (8 months of age, 1.1 ± 0.4 pulses/6 h; 9 months of age, 2.0 ± 0.9 pulses/6 h) did not differ between experimental groups within the 6-h sampling period. However, at 8 months of age, mean frequency of LH pulses monitored over 12 h was greater ($P<0.01$) in the HG (2.8 ± 0.6 pulses/12 h) than that in the LG group (1.0 ± 0.0 pulses/12 h) before puberty occurred in any heifer. At 9 months of age, the frequency of LH pulses in HG (3.8 ± 1.4 pulses/12 h) and LG heifers (2.0 ± 0.8 pulses/12 h) did not differ. No differences in LH pulse amplitude (8 months of age, 0.9 ± 0.3 ng/ml; 9 months of age, 0.8 ± 0.4 ng/ml) or mean concentrations (8 months of age, 0.4 ± 0.1 ng/ml; 9 months of age, 0.3 ± 0.1 ng/ml) were observed between experimental groups at 8 and 9 months of age. It should be noted that two HG heifers reached puberty before 9 months of age; therefore, they were not included in any of the analyses at this time point.

Pubertal onset

Relatively high rates of BW gain during the juvenile period advanced the onset of puberty in the current study;

however, a replicate effect and a treatment $\times$ replicate interaction ($P<0.03$) were observed. The percentage of pubertal heifers in the HG group in replicate 1 was greater ($P<0.05$) than that in the LG group beginning at 9 months of age, with 50% (3/6), 67% (4/6), and 100% (6/6) of HG heifers achieving puberty by 9, 10, and 11 months of age respectively. For replicate 2, only one (age at puberty: 8.8 months of age) of the HG heifers was pubertal by 11 months of age (1/7, 14%) and the percentage of heifers reaching puberty during the experimental period did not differ significantly between dietary groups. None of the LG heifers (0/12) in either replicate had attained puberty by the end of the experiment (11 months of age).

Discussion

Previous studies have revealed that transcriptional and morphological alterations within the hypothalamic NPY network are involved in the sexual maturation of heifers (Alves *et al.* 2011, Allen *et al.* 2012). Results from the current study provide further evidence that the

Figure 3

Mean ($\pm$ S.E.M.) concentrations of leptin in peripheral blood (A) and NPY in 3V CSF (B) of LG and HG heifers at 8 (LG, $n=6$; HG, $n=6$) and 9 months of age (LG, $n=6$; HG, $n=3^a$). The concentrations of leptin were greater in HG heifers than that in LG heifers at 8 and 9 months of age ($*P<0.05$). Mean concentration of NPY in the 3V CSF was lower in the HG group than that in the LG group at 9 months of age ($**P\leq 0.02$). ^aTwo HG heifers had already reached puberty and one HG heifer did not have a functional 3V cannula at 9 months of age; thus, data from these three animals were not used in the analyses at 9 months.

Figure 4

Patterns of secretion of GnRH in 3V CSF and of LH in plasma of representative LG (#568 and #594) and HG heifers (#587 and #600) at 8 and 9 months of age. Pulses of GnRH and LH are denoted by arrows and

asterisks respectively. Pulses of LH detected within the 12-h sampling period are enumerated in parentheses.

hypothalamic NPY system is a key metabolic-sensing system involved in the nutritionally mediated acceleration of puberty in heifers. Herein, it was demonstrated that elevated BW gain, achieved by feeding a high-concentrate diet to heifers during the juvenile period, leads to greater circulating concentrations of leptin and decreased central release of NPY, characterized by lower concentrations of the peptide in 3V CSF. Furthermore, prepubertal heifers that gained BW at greater rates exhibited increase in pulsatile secretion of LH and an overall advancement of pubertal onset. Because NPY has been demonstrated to have inhibitory effects on GnRH/LH release in ruminants (Barker-Gibb *et al.* 1995, Gazal *et al.* 1998, Estrada *et al.* 2003, Morrison *et al.* 2003), we propose that the acceleration of reproductive maturation seen in this study is mediated, at least in part, by a diminished NPY inhibitory effect on GnRH episodic secretion.

The pubertal transition in females is characterized by an attenuation of estradiol-negative feedback and increased pulsatile release of LH that provides support for final maturation of ovarian follicles and enhanced ovarian steroidogenesis (Gasser *et al.* 2006a). As anticipated, heifers fed a high-concentrate diet to promote accelerated BW gain during the juvenile period, a strategy known to advance the onset of puberty, presented a greater frequency of LH pulses during the peripubertal period. Mean concentrations of LH did not differ between groups. Similar relationships have been reported in adult

cows in which mean concentrations of LH were similar during the early and mid-luteal periods even though the frequency of pulses was markedly greater during the early-luteal phase (Rawlings *et al.* 1980, Williams *et al.* 1983). The limited duration (6 h) of CSF sampling in this study did not allow substantial comparison of the frequency of GnRH pulses between dietary groups. However, the very high correlation observed between GnRH and LH pulses during the initial 6 h of sampling, together with increased pulsatile release of LH in HG heifers over a 12-h sampling period, reinforce the notion that the pubertal 'escape' from estradiol negative feedback is regulated mainly at the hypothalamic level (I'Anson *et al.* 2000).

Nutritional and metabolic signals are perceived largely by the hypothalamus (Cunningham *et al.* 1999, Schneider 2004), and a major limiting factor for increased episodic secretion of LH during the prepubertal period is the lack of appropriate stimulation of the gonadotrophs by GnRH (reviewed by Amstalden *et al.* (2014)). This concept is supported by observations that ovarian function is stimulated in immature female monkeys treated with GnRH (Wildt *et al.* 1980) and in lambs treated with LH (Foster *et al.* 1984). Our group has demonstrated that leptin has a critical permissive role in controlling pubertal maturation in heifers (Garcia *et al.* 2002, Maciel *et al.* 2004a), and this regulation occurs mainly as a result of leptin's effects on GnRH secretion (Zieba *et al.* 2004). Nonetheless, several studies have failed to demonstrate

the presence of leptin receptors in GnRH neurons in rodents (Hakansson *et al.* 1998) and primates (Finn *et al.* 1998), indicating that leptin's actions on GnRH release are probably mediated by intermediate pathways.

NPY, a potent stimulator of food intake (Clark *et al.* 1985) found abundantly in the hypothalamus (Allen *et al.* 1983), has been proposed to be a major mediator of the inhibitory effects of undernutrition on reproductive function (Crown *et al.* 2007). Chronic feed restriction markedly increases the abundance of NPY mRNA in the ARC and decreases secretion of LH in ewe lambs (McShane *et al.* 1993). Furthermore, central infusion of NPY suppresses GnRH and LH release in cattle (Gazal *et al.* 1998) and sheep (Barker-Gibb *et al.* 1995, Estrada *et al.* 2003, Morrison *et al.* 2003). In primates, NPY mRNA and peptide concentrations in the hypothalamus are diminished during the juvenile–pubertal transition (El Majdoubi *et al.* 2000). Thus, we reasoned that NPY may play an important role in the metabolic programming of puberty in heifers. Confirming our hypothesis, prepubertal heifers in the HG group exhibited greater concentrations of leptin and reduced concentrations of NPY in 3V CSF at 9 months of age compared with those in the LG group. Because at 8 months of age there was only a tendency for reduced concentrations of NPY in CSF in HG heifers, we presume that the nutritionally mediated suppression of hypothalamic NPY release occurs shortly before the onset of puberty. In the current study, among the HG heifers that achieved puberty during the experiment ($n=7$), the majority (86%; 6/7) reached sexual maturation around or shortly after 9 months of age (7.9–10.2 months of age).

Using similar nutritional models, it has been demonstrated previously that the expression of NPY mRNA in the ARC was decreased in prepubertal heifers that gained BW at a relatively high rate (Allen *et al.* 2012). Furthermore, the proportion of GnRH neurons in close proximity to NPY fibers in the preoptic area and mediobasal hypothalamus is reduced in heifers with increased BW gain (Alves *et al.* 2011). However, it remains to be determined whether these changes in NPY projections to GnRH neurons involve NPY cells from the ARC in heifers, because in the female sheep there is no significant direct input originating from the ARC to GnRH neurons located in the preoptic area (Pompolo *et al.* 2001). Taken together, these observations indicate that increased hypothalamic NPY activity in juvenile heifers may play a role in restraining the pulsatile release of GnRH and LH necessary for final reproductive development. As heifers approach a more positive state of body energy reserves, characterized by increased BW and body condition score and greater

circulating concentrations of leptin, NPY inhibitory tone is suppressed, culminating in reproductive competence.

Although no replicate effects were observed on BW gain and circulating concentrations of leptin, the proportion of HG heifers that achieved precocious puberty (puberty ≤ 300 days) differed between replicates. In a recent study (Cardoso *et al.* 2014), the majority of the heifers subjected to a similar nutritional regimen reached puberty between 11 and 14 months of age. As this study evaluated puberty only through 11 months of age, the number of heifers attaining puberty between 11 and 14 months of age was not determined. Variability in age at puberty in heifers of similar genetic background and fed diets of analogous formulation to promote similar rates of BW gain has been previously observed by our group, and has been reported by others (Gasser *et al.* 2006a,b,c). Hence, it is possible that factors other than postnatal nutrition and genetics may influence the maturation of the reproductive neuroendocrine system. For example, maternal nutrition during fetal development has been shown to cause adverse changes in brain functions that regulate nutrient intake, energy expenditure, and endocrine physiology. Some of these changes are characterized by alterations in expression of NPY and the number of NPY cells within the ARC (Warnes *et al.* 1998, Plagemann *et al.* 2000), and reduced ability of leptin to signal metabolic status postnatally (Guo & Jen 1995, Levin & Dunn-Meynell 2002, Ford *et al.* 2007). Therefore, further studies are required to determine whether maternal nutrition may influence the responsiveness of the offspring's hypothalamic circuitry to metabolic cues in prepubertal heifers.

Finally, it should also be noted that leptin may influence pubertal activation of GnRH neurons through additional neuronal pathways, such as the hypothalamic proopiomelanocortin system (Amstalden *et al.* 2014). Thus, it is possible that, despite the observed decrease in central release of NPY in HG heifers in replicate 2, reciprocal changes in other important neuropeptides that control the activity of GnRH neurons (e.g., α -melanocyte-stimulating hormone) may not have been fully activated. Moreover, because NPY neurons located in the ARC appear to regulate GnRH cells via interneuronal pathways, at least in female sheep (Pompolo *et al.* 2005), changes in the responsiveness to the nutritional acceleration of puberty in heifers may require alterations in these neuronal connections.

In summary, the results of the current study, in conjunction with those described in previous reports, support the hypothesis that the hypothalamic NPY

pathway is involved in the nutritional acceleration of puberty in heifers. Favorable metabolic status during early calthood, mainly characterized by increased circulating concentrations of leptin, resulted in lower hypothalamic NPY release, greater pulsatile release of LH, and hastened onset of puberty. Collectively, these results contribute to a better understanding of the mechanisms by which elevated BW gain advances the onset of puberty in female mammals, and may be valuable for the development of nutritional strategies that can exploit postnatal neuroendocrine plasticity to optimally time the onset of puberty in heifers.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This project was supported by the Agriculture and Food Research Initiative Competitive grant number 2009-65203-05678 from the USDA National Institute of Food and Agriculture.

Acknowledgements

The authors would like to acknowledge Dr A F Parlow (NIDDK National Hormone and Peptide Program, Torrance, USA) for providing LH RIA reagents and Dr Alain Caraty (INRA, Nouzilly, France) for providing the GnRH antiserum. They also acknowledge the careful animal care and assistance of Randle Franke, Reynaldo Rodrigues Jr, and Ray Villareal.

References

- Ahren B, Larsson H, Wilhelmsson C, Nasman B & Olsson T 1997 Regulation of circulating leptin in humans. *Endocrine* **7** 1–8. (doi:10.1007/BF02778056)
- Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR & Polak JM 1983 Neuropeptide Y distribution in the rat brain. *Science* **221** 877–879. (doi:10.1126/science.6136091)
- Allen CC, Alves BR, Li X, Tedeschi LO, Zhou H, Paschal JC, Riggs PK, Braga-Neto UM, Keisler DH, Williams GL *et al.* 2012 Gene expression in the arcuate nucleus of heifers is affected by controlled intake of high- and low-concentrate diets. *Journal of Animal Science* **90** 2222–2232. (doi:10.2527/jas.2011-4684)
- Alves BR, Liu S, Stevenson E, Thorson JF, Cardoso RC, Tedeschi LO, Keisler DH, Williams GW & Amstalden M 2011 Accelerated body weight gain during the juvenile period reduced neuropeptide Y close contacts with GnRH neurons in heifers. Conference abstract: Society for the Study of Reproduction Annual Meeting, Portland, USA, 31 July–4 August, 2011. *Biology of Reproduction* **85** abstract 191.
- Amstalden M, Garcia MR, Stanko RL, Nizielski SE, Morrison CD, Keisler DH & Williams GL 2002 Central infusion of recombinant ovine leptin normalizes plasma insulin and stimulates a novel hypersecretion of luteinizing hormone after short-term fasting in mature beef cows. *Biology of Reproduction* **66** 1555–1561. (doi:10.1095/biolreprod66.5.1555)
- Amstalden M, Cardoso RC, Alves BRC & Williams GL 2014 Hypothalamic neuropeptides and the nutritional programming of puberty in heifers. *Journal of Animal Science* **92** 3211–3222. (doi:10.2527/jas.2014-7808)
- Barker-Gibb ML, Scott CJ, Boublik JH & Clarke IJ 1995 The role of neuropeptide Y (NPY) in the control of LH secretion in the ewe with respect to season, NPY receptor subtype and the site of action in the hypothalamus. *Journal of Endocrinology* **147** 565–579. (doi:10.1677/joe.0.1470565)
- Baskin DG, Breininger JF & Schwartz MW 1999 Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. *Diabetes* **48** 828–833. (doi:10.2337/diabetes.48.4.828)
- Cardoso RC, Alves BRC, Prezotto LD, Thorson JF, Tedeschi LO, Keisler DH, Park CS, Amstalden M & Williams GL 2014 Use of a stair-step compensatory gain nutritional regimen to program the onset of puberty in beef heifers. *Journal of Animal Science* **92** 2942–2949. (doi:10.2527/jas.2014-7713)
- Castellano JM, Bentsen AH, Sánchez-Garrido MA, Ruiz-Pino F, Romero M, García-Galiano D, Aguilar E, Pinilla L, Diéguez C, Mikkelsen JD *et al.* 2011 Early metabolic programming of puberty onset: impact of changes in postnatal feeding and rearing conditions on the timing of puberty and development of the kisspeptin system. *Endocrinology* **152** 3396–3408. (doi:10.1210/en.2010-1415)
- Clark JT, Kalra PS & Kalra SP 1985 Neuropeptide Y stimulates feeding but inhibits sexual behavior in rats. *Endocrinology* **117** 2435–2442. (doi:10.1210/endo-117-6-2435)
- Crown A, Clifton DK & Steiner RA 2007 Neuropeptide signaling in the integration of metabolism and reproduction. *Neuroendocrinology* **86** 175–182. (doi:10.1159/000109095)
- Cunningham MJ, Clifton DK & Steiner RA 1999 Leptin's actions on the reproductive axis: perspectives and mechanisms. *Biology of Reproduction* **60** 216–222. (doi:10.1095/biolreprod60.2.216)
- Delavaud C, Bocquier F, Chilliard Y, Keisler DH, Gertler A & Kann G 2000 Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep. *Journal of Endocrinology* **165** 519–526. (doi:10.1677/joe.0.1650519)
- Ellinwood WE, Ronnekleiv OK, Kelly KJ & Resko JA 1985 A new antiserum with conformational specificity for LHRH: usefulness for radio-immunoassay and immunocytochemistry. *Peptides* **6** 45–52. (doi:10.1016/0196-9781(85)90075-0)
- El Majdoubi M, Sahu A, Ramaswamy S & Plant TM 2000 Neuropeptide Y: a hypothalamic brake restraining the onset of puberty in primates. *PNAS* **97** 6179–6184. (doi:10.1073/pnas.090099697)
- Estrada KM, Pompolo S, Morris MJ, Tilbrook AJ & Clarke IJ 2003 Neuropeptide Y (NPY) delays the oestrogen-induced luteinizing hormone (LH) surge in the ovariectomized ewe: further evidence that NPY has a predominant negative effect on LH secretion in the ewe. *Journal of Neuroendocrinology* **15** 1011–1020. (doi:10.1046/j.1365-2826.2003.01087.x)
- Fajersson P, Stanko RL & Williams GL 1999 Distribution and repeatability of anterior pituitary responses to GnRH and relationship of response classification to the *postpartum* anovulatory interval of beef cows. *Journal of Animal Science* **77** 3043–3049.
- Finn PD, Cunningham MJ, Pau KY, Spies HG, Clifton DK & Steiner RA 1998 The stimulatory effect of leptin on the neuroendocrine reproductive axis of the monkey. *Endocrinology* **139** 4652–4662. (doi:10.1210/endo.139.11.6297)
- Ford SP, Hess BW, Schwoppe MM, Nijland MJ, Gilbert JS, Vonnahme KA, Means WJ, Han H & Nathanielsz PW 2007 Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. *Journal of Animal Science* **85** 1285–1294. (doi:10.2527/jas.2005-624)
- Foster DL & Olster DH 1985 Effect of restricted nutrition on puberty in the lamb: patterns of tonic luteinizing hormone (LH) secretion and

- competency of the LH surge system. *Endocrinology* **116** 375–381. (doi:10.1210/endo-116-1-375)
- Foster DL, Ryan KD & Papkoff H 1984 Hourly administration of luteinizing hormone induces ovulation in prepubertal female sheep. *Endocrinology* **115** 1179–1185. (doi:10.1210/endo-115-3-1179)
- Fox DG, Tedeschi LO, Tylutki TP, Russell JB, Van Amburgh ME, Chase LE, Pell AN & Overton TR 2004 The Cornell net carbohydrate and protein system model for evaluating herd nutrition and nutrient excretion. *Animal Feed Science and Technology* **112** 29–79. (doi:10.1016/j.anifeedsci.2003.10.006)
- Frisch RE & McArthur JW 1974 Menstrual cycles: fatness as a determinant of minimum weight for height necessary for their maintenance or onset. *Science* **185** 949–951. (doi:10.1126/science.185.4155.949)
- Garcia MR, Amstalden M, Williams SW, Stanko RL, Morrison CD, Keisler DH, Nizielski SE & Williams GL 2002 Serum leptin and its adipose gene expression during pubertal development, the estrous cycle, and different seasons in cattle. *Journal of Animal Science* **80** 2158–2167.
- Garcia MR, Amstalden M, Morrison CD, Keisler DH & Williams GL 2003 Age at puberty, total fat and conjugated linoleic acid content of carcass, and circulating metabolic hormones in beef heifers fed a diet high in linoleic acid beginning at four months of age. *Journal of Animal Science* **81** 261–268.
- Gasser CL, Bridges GA, Mussard ML, Grum DE, Kinder JE & Day ML 2006a Induction of precocious puberty in heifers III: hastened reduction of estradiol negative feedback on secretion of luteinizing hormone. *Journal of Animal Science* **84** 2050–2056. (doi:10.2527/jas.2005-638)
- Gasser CL, Grum DE, Mussard ML, Fluharty FL, Kinder JE & Day ML 2006b Induction of precocious puberty in heifers I: enhanced secretion of luteinizing hormone. *Journal of Animal Science* **84** 2035–2041. (doi:10.2527/jas.2005-636)
- Gasser CL, Burke CR, Mussard ML, Behlke EJ, Grum DE, Kinder JE & Day ML 2006c Induction of precocious puberty in heifers II: advanced ovarian follicular development. *Journal of Animal Science* **84** 2042–2049. (doi:10.2527/jas.2005-637)
- Gazal OS, Leshin LS, Stanko RL, Thomas MG, Keisler DH, Anderson LL & Williams GL 1998 Gonadotropin-releasing hormone secretion into third-ventricle cerebrospinal fluid of cattle: correspondence with the tonic and surge release of luteinizing hormone and its tonic inhibition by suckling and neuropeptide Y. *Biology of Reproduction* **59** 676–683. (doi:10.1095/biolreprod59.3.676)
- Guo F & Jen KL 1995 High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. *Physiology & Behavior* **57** 681–686. (doi:10.1016/0031-9384(94)00342-4)
- Hakansson ML, Brown H, Ghilardi N, Skoda RC & Meister B 1998 Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. *Journal of Neuroscience* **18** 559–572.
- Henry BA, Goding JW, Alexander WS, Tilbrook AJ, Canny BJ, Dunshea F, Rao A, Mansell A & Clarke IJ 1999 Central administration of leptin to ovariectomized ewes inhibits food intake without affecting the secretion of hormones from the pituitary gland: evidence for a dissociation of effects on appetite and neuroendocrine function. *Endocrinology* **140** 1175–1182. (doi:10.1210/endo.140.3.6604)
- Herd DB & Sprott LR 1986 Body condition, nutrition and reproduction of beef cows. *Texas Agricultural Experiment Station Bulletin* **B-1526** 1–11.
- l'Anson H, Manning JM, Herbosa CG, Pelt J, Friedman CR, Wood RI, Bucholtz DC & Foster DL 2000 Central inhibition of gonadotropin-releasing hormone secretion in the growth-restricted hypogonadotropic female sheep. *Endocrinology* **141** 520–527. (doi:10.1210/en.141.2.520)
- Kalra SP & Crowley WR 1984 Norepinephrine-like effects of neuropeptide Y on LH release in the rat. *Life Science* **35** 1173–1176. (doi:10.1016/0024-3205(84)90187-5)
- Kalra SP & Kalra PS 2003 Neuropeptide Y: a physiological orexigen modulated by the feedback action of ghrelin and leptin. *Endocrine* **22** 49–56. (doi:10.1385/ENDO:22:1:49)
- Kennedy GC 1969 The relation between the central control of appetite, growth and sexual maturation. *Guy's Hospital Reports* **118** 315–327.
- Kennedy GC & Mitra J 1963 Body weight and food intake as initiating factors for puberty in the rat. *Journal of Physiology* **166** 408–418.
- Kile JP, Alexander BM, Moss GE, Hallford DM & Nett TM 1991 Gonadotropin-releasing hormone overrides the negative effect of reduced dietary energy on gonadotropin synthesis and secretion in ewes. *Endocrinology* **128** 843–849. (doi:10.1210/endo-128-2-843)
- Klenke U, Constantin S & Wray S 2010 Neuropeptide Y directly inhibits neuronal activity in a subpopulation of gonadotropin-releasing hormone-1 neurons via Y1 receptors. *Endocrinology* **151** 2736–2746. (doi:10.1210/en.2009-1198)
- Kushler RH & Brown MB 1991 A model for the identification of hormone pulses. *Statistics in Medicine* **10** 329–340. (doi:10.1002/sim.4780100305)
- Lee JM, Appugliese D, Kaciroti N, Corwyn RF, Bradley RH & Lumeng JC 2007 Weight status in young girls and the onset of puberty. *Pediatrics* **119** 624–630. (doi:10.1542/peds.2006-2188)
- Levin BE & Dunn-Meynell AA 2002 Maternal obesity alters adiposity and monoamine function in genetically predisposed offspring. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **283** 1087–1093. (doi:10.1152/ajpregu.00402.2002)
- Maciel MN, Zieba DA, Amstalden M, Keisler DH, Neves JP & Williams GL 2004a Leptin prevents fasting-mediated reductions in pulsatile secretion of luteinizing hormone and enhances its gonadotropin-releasing hormone-mediated release in heifers. *Biology of Reproduction* **70** 229–235. (doi:10.1095/biolreprod.103.021345)
- Maciel MN, Zieba DA, Amstalden M, Keisler DH, Neves JP & Williams GL 2004b Chronic administration of recombinant ovine leptin in growing beef heifers: effects on secretion of LH, metabolic hormones, and timing of puberty. *Journal of Animal Science* **82** 2930–2936.
- McShane TM, Petersen SL, McCrone S & Keisler DH 1993 Influence of food restriction on Neuropeptide-Y, proopiomelanocortin, and luteinizing hormone-releasing hormone gene expression in sheep hypothalamus. *Biology of Reproduction* **49** 831–839. (doi:10.1095/biolreprod49.4.831)
- McVey WR Jr & Williams GL 1991 Mechanical masking of neurosensory pathways at the calf-teat interface: endocrine, reproductive and lactational features of the suckled anestrous cow. *Theriogenology* **35** 931–941. (doi:10.1016/0093-691X(91)90304-V)
- Morrison CD, Daniel JA, Hampton JH, Buff PR, McShane TM, Thomas MG & Keisler DH 2003 Luteinizing hormone and growth hormone secretion in ewes infused intracerebroventricularly with neuropeptide Y. *Domestic Animal Endocrinology* **24** 69–80. (doi:10.1016/S0739-7240(02)00206-0)
- Plagemann A, Harder T, Rake A, Melchior K, Rohde W & Dörner G 2000 Hypothalamic nuclei are malformed in weanling offspring of low protein malnourished rat dams. *Journal of Nutrition* **130** 2582–2589.
- Pompolo S, Rawson JA & Clarke IJ 2001 Projections from the arcuate/ventromedial region of the hypothalamus to the preoptic area and bed nucleus of stria terminalis in the brain of the ewe; lack of direct input to gonadotropin-releasing hormone neurons. *Brain Research* **904** 1–12. (doi:10.1016/S0006-8993(01)02372-1)
- Pompolo S, Ischenko O, Pereira A, Iqbal J & Clarke IJ 2005 Evidence that projections from the bed nucleus of the stria terminalis and from the lateral and medial regions of the preoptic area provide input to gonadotropin releasing hormone (GnRH) neurons in the female sheep brain. *Neuroscience* **132** 421–436. (doi:10.1016/j.neuroscience.2004.12.042)
- Pralong FP, Voirol M, Giacomini M, Gaillard RC & Grouzmann E 2000 Acceleration of pubertal development following central blockade of the Y1 subtype of neuropeptide Y receptors. *Regulatory Peptides* **95** 47–52. (doi:10.1016/S0167-0115(00)00130-0)
- Quennell JH, Mulligan AC, Tups A, Liu X, Phipps SJ, Kemp CJ, Herbison AE, Grattan DR & Anderson GM 2009 Leptin indirectly regulates gonadotropin-releasing hormone neuronal function. *Endocrinology* **150** 2805–2812. (doi:10.1210/en.2008-1693)

- Rawlings NC, Weir L, Todd B, Manns J & Hyland J 1980 Some endocrine changes associated with the postpartum period of the suckling beef cow. *Journal of Reproduction and Fertility* **60** 301–308. (doi:10.1530/jrf.0.0600301)
- Sahu A, Crowley WR, Tatemoto K, Balasubramaniam A & Kalra SP 1987 Effects of neuropeptide Y, NPY analog (norleucine⁴-NPY), galanin and neuropeptide K on LH release in ovariectomized (ovx) and ovx estrogen, progesterone-treated rats. *Peptides* **8** 921–926. (doi:10.1016/0196-9781(87)90081-7)
- Schneider JE 2004 Energy balance and reproduction. *Physiology & Behavior* **81** 289–317. (doi:10.1016/j.physbeh.2004.02.007)
- Skinner DC, Malpoux B, Delaleu B & Caraty A 1995 Luteinizing hormone-releasing hormone in third ventricular cerebrospinal fluid of the ewe: correlation with LH pulses and the LH surge. *Endocrinology* **136** 3230–3237. (doi:10.1210/endo.136.8.7628356)
- Szczepanska-Sadowska E, Gray D & Simon-Oppermann C 1983 Vasopressin in blood and third ventricle CSF during dehydration, thirst, and hemorrhage. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **245** 549–555.
- Van Vugt DA, Diefenbach WD, Alston E & Ferin M 1985 Gonadotropin-releasing hormone pulses in third ventricular cerebrospinal fluid of ovariectomized rhesus monkeys: correlation with luteinizing hormone pulses. *Endocrinology* **117** 1550–1558. (doi:10.1210/endo-117-4-1550)
- Wang Q, Bing C, Al-Barazanji K, Mossakowska DE, Wang XM, McBay DL, Neville WA, Taddayon M, Pickavance L, Dryden S *et al.* 1997 Interactions between leptin and hypothalamic neuropeptide Y neurons in the control of food intake and energy homeostasis in the rat. *Diabetes* **46** 335–341. (doi:10.2337/diab.46.3.335)
- Warnes KE, Morris MJ, Symonds ME, Phillips ID, Clarke IJ, Owens JA & McMillen IC 1998 Effects of increasing gestation, cortisol and maternal undernutrition on hypothalamic neuropeptide Y expression in the sheep fetus. *Journal of Neuroendocrinology* **10** 51–57. (doi:10.1046/j.1365-2826.1998.00172.x)
- Wildt L, Marshall G & Knobil E 1980 Experimental induction of puberty in the infantile female rhesus monkey. *Science* **207** 1373–1375. (doi:10.1126/science.6986658)
- Williams GL, Talavera F, Peterson BJ, Kirsch JD & Tilton JE 1983 Coincident secretion of follicle-stimulating hormone and luteinizing hormone in early postpartum beef cows. Effects of suckling and low-level increases of systemic progesterone. *Biology of Reproduction* **29** 363–373. (doi:10.1095/biolreprod29.2.362)
- Zieba DA, Amstalden M, Morton S, Maciel MN, Keisler DH & Williams GL 2004 Regulatory roles of leptin at the hypothalamic–hypophyseal axis before and after sexual maturation in cattle. *Biology of Reproduction* **71** 804–812. (doi:10.1095/biolreprod.104.028548)
- Zieba DA, Amstalden M & Williams GL 2005 Regulatory roles of leptin in reproduction and metabolism: a comparative review. *Domestic Animal Endocrinology* **29** 166–185. (doi:10.1016/j.domaniend.2005.02.019)

Received in final form 9 October 2014

Accepted 17 October 2014

Accepted Preprint published online 17 October 2014