NUTRITIONAL PROGRAMMING OF NEUROENDOCRINE PATHWAYS CONTROLLING THE ONSET OF PUBERTY IN HEIFERS

A Dissertation

by

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ABSTRACT

Accelerated body weight gain during the juvenile period leads to early maturation of the reproductive neuroendocrine system and puberty by advancing the onset of high-frequency release of gonadotropin-releasing hormone (GnRH). In a series of studies conducted as part of this dissertation, it was hypothesized that nutritional regimens that accelerate pubertal development alter the function of kisspeptin and neuropeptide Y (NPY) neurons, known modulators of the release of GnRH. In addition, alterations in the pattern of methylation of target genome sequences were investigated in DNA collected from the arcuate nucleus, a hypothalamic region well-characterized for its role in mediating the metabolic control of reproductive function. Early-weaned crossbred heifers were fed diets to promote an average daily gain of 0.5 Kg (Low Gain; n=12) or 1.0 Kg (High Gain; n=12) from approximately 4 to 8 mo of age. This nutritional regimen has been demonstrated to facilitate early onset of puberty in heifers. At eight mo of age, heifers were slaughtered and a block of tissue containing the preoptic area and hypothalamus was collected and processed for examining the expression of *NPY* and *KISS1*, investigating the innervation of GnRH and kisspeptin neurons by NPY fibers, and characterizing the methylation profile of target genomic DNA sequences isolated from the arcuate nucleus. Mean body weight and circulating concentrations of leptin were greater in High-gain than in Low-gain heifers. Elevated rate of body weight gain was associated with reduced expression of *NPY* in the arcuate nucleus, reduced proportion of GnRH neurons in apposition to NPY-containing

varicosities, particularly in GnRH neurons located in the mediobasal hypothalamus, and a reduced number of *KISS1*-expressing cells in the mid portion of the arcuate nucleus. The rate of body weight gain during the juvenile period did not affect NPY innervation of kisspeptin neurons. Differential methylation of target genomic DNA sequences isolated from the arcuate nucleus was observed in association with rate of body weight gain in juvenile heifers, and genes affected included *GHR, IGF2, IGF1R, LEPR, PEG3, LIN28B* and *HMGA2*. Overall, results from these studies support the hypothesis that accelerated body weight gain during the juvenile period promotes alterations in cellular functions within the hypothalamus that facilitate early onset of puberty in heifers.

This dissertation is dedicated to the memory of my grandparents Diva and Edson, who inspired my choices, supported my decisions and were always present in my heart and mind.

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TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION..........iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS viii

LIST OF FIGURES..........xi

LIST OF TABLES xii

CHAPTER I INTRODUCTION 1

CHAPTER II LITERATURE REVIEW 5

Reproductive maturation in heifers 5
Gonadotropin-releasing hormone neuron: the central coordinator of reproductive function..........7
Afferent circuitry to GnRH neurons..........11
Gamma Aminobutyric Acid (GABA) 12
Endogenous opioid peptides..........13
Neuropeptide Y 13
Glutamate 16
Kisspeptin..........17
Neurokinin B 22
Insulin-like growth factor-1 (IGF-1) 23
Transcriptional control of female puberty..........24
Influence of nutrition on the pubertal process..........26
Role of leptin in signaling metabolic status 27
Hypothalamic neurocircuitry linking metabolism and reproduction: roles of NPY and kisspeptin 29
Nutritional programming of hypothalamic neuronal circuitry..........32
Role of epigenetics in hypothalamic programming of reproduction..........36

CHAPTER III ELEVATED BODY WEIGHT GAIN DURING THE JUVENILE PERIOD ALTERS NEUROPEPTIDE Y NEURONAL CIRCUITRY IN THE HYPOTHALAMUS OF PREPUBERTAL HEIFERS 38

Overview 38
Introduction 39

Material and methods ... 41
Animals and nutritional regimens ... 41
Hormone assays ... 44
Experiment 1a: Expression of *NPY* in the arcuate nucleus ... 44
Experiment 1b: Functional structure of the NPY-GnRH circuitry ... 46
Statistical analyses ... 49
Results ... 50
Experiment 1a: Expression of *NPY* in the arcuate nucleus ... 52
Experiment 1b: Functional structure of the NPY-GnRH circuitry ... 54
Discussion ... 58

CHAPTER IV INFLUENCE OF BODY WEIGHT GAIN DURING THE JUVENILE PERIOD ON KISSPEPTIN NEURONAL PATHWAY IN HEIFERS ... 65

Overview ... 65
Introduction ... 66
Material and methods ... 68
Animals and experimental procedures ... 68
Experiment 1: *KISS1* expression in the POA and ARC of juvenile heifers ... 70
Experiment 2: Kisspeptin-immunoreactive cells in the POA and ARC and their NPY afferent projections in juvenile heifers ... 71
Statistical analysis ... 74
Results ... 74
Experiment 1: *KISS1* expression in the POA and ARC of juvenile heifers ... 74
Experiment 2: Kisspeptin-immunoreactive cells in the POA and ARC and their NPY afferent projections in juvenile heifers ... 76
Discussion ... 78

CHAPTER V PATTERN OF DNA METHYLATION IN THE ARCUATE NUCLEUS IN PREPUBERTAL HEIFERS GROWING AT HIGH AND LOW RATES ... 86

Overview ... 86
Introduction ... 87
Material and methods ... 89
Genomic DNA isolation and methylated DNA enrichment assay ... 90
Optimization and validation of the methylated DNA enrichment assay and whole-genome amplification of bovine hypothalamic DNA ... 91
Pattern of methylation of target DNA in the ARC nucleus of juvenile heifers ... 93
Oligonucleotide array ... 94
DNA labeling and hybridization ... 96
Data analyses ... 96

Page

Results 98
Optimization and validation of the methylated DNA enrichment assay and whole-genome amplification of bovine hypothalamic DNA 98
Pattern of methylation of target DNA in the ARC nucleus of juvenile heifers 101
Discussion 111

CHAPTER VI SUMMARY AND CONCLUSION 123

REFERENCES 126

LIST OF FIGURES

Page

Figure 3.1 Body weight of heifers fed for a target average daily gain of 1 Kg (High Gain) or 0.5 Kg (Low Gain) …………..……………………..…..…. 51

Figure 3.2 Circulating concentrations of leptin in heifers fed for a target average daily gain of 1 Kg (High Gain) or 0.5 Kg (Low Gain)………………….. 52

Figure 3.3 Expression of NPY in the arcuate nucleus (ARC) of 8-mo-old heifers fed to gain body weight at high (1 Kg/d; High Gain) or low (0.5 Kg/d; Low Gain) rate, beginning at approximately 4.5 mo of age. …………… 53

Figure 3.4 NPY innervation of GnRH neurons in 8-mo-old heifers fed to gain body weight at high (1 Kg/d; High Gain) or low (0.5 Kg/d; Low Gain) rate beginning at approximately 4.5 mo of age ...………………………. 56

Figure 4.1 Expression of *KISS1* in the arcuate nucleus (ARC) of 8-mo-old heifers fed to gain body weight at high (1 Kg/d; High Gain) or low (0.5 Kg/d; Low Gain) rate beginning at approximately 4.5 mo of age.……..……… 75

Figure 4.2 Number of kisspeptin-immunoreactive cells detected in the rostral, mid and caudal arcuate nucleus (ARC) in Low-gain and High-gain heifers.... 77

Figure 4.3 Neuropeptide Y-containing fibers are in close proximity to kisspeptin cells in 8-mo-old heifers fed to gain body weight at high (1 Kg/d; High Gain) or low (0.5 Kg/d; Low Gain) rates during the juvenile period.….. 77

Figure 5.1 PCR amplification of GAPDH and OCT4 from bovine hypothalamic DNA. …………………………………………………………….……....... 100

Figure 5.2 Distribution of methylated-enriched DNA intervals in genomic regions encompassing DLK1-DIO3 and IGF2-H19 among individual Low-gain and High-gain heifers………………………………………………… 101

Figure 5.3 Genomic location of sequences within the imprinted gene cluster in the bovine chromosome 29…..…………………………………..…….. 111

LIST OF TABLES

Page

Table 3.1 Composition and nutrient concentration of diets ……..………….…….. 43

Table 3.2 Percentage of area covered by NPY fibers. ……………..…...……..…… 58

Table 5.1 Sequences of primers used for PCR .....…………………………….…….. 92

Table 5.2 Genes and target intervals used for designing probes included in array .. 95

Table 5.3 Number of regions targeted by individual probes that were differently methylated in High-gain heifers……….…………………………….. 102

Table 5.4 Greatest differences in log2 ratio between High-gain and Low-gain groups determined by the Sliding *t-test* method……………..………. 103

Table 5.5 Greatest differences in log2 ratio between High-gain and Low-gain groups determined by the Three-point Average method………..……… 104

Table 5.6 Genes and spanned regions detected to be hypomethylated in High-gain heifers concurrently by the Sliding *t-test* and Three-point Average methods 105

Table 5.7 Genes and spanned regions detected to be hypermethylated in High-gain heifers concurrently by the Sliding *t-test* and Three-point Average methods ……………………………………………….……. 106

Table 5.8 Genes and spanned regions exhibiting both hypo- and hypermethylated sequences in High-gain heifers concurrently by the Sliding *t-test* and Three-point Average methods …………………….………………… 107

Table 5.9 Genes and spanned regions detected to be hypomethylated in High-gain heifers by the Categorical Comparison method…………………... 108

Table 5.10 Genes and spanned regions detected to be hypermethylated in High-gain heifers by the Categorical Comparison method……………………. 109

Table 5.11 Genes and spanned regions exhibiting both hypo- and hypermethylated sequences in High-gain heifers by the Categorical Comparison method.. 110

CHAPTER I

INTRODUCTION

Reproductive efficiency is one of the most important constrains in livestock production systems (Dickerson, 1970). Optimal lifetime productivity of cows is attained when heifers calve for the first time as two-year-olds (Short and Bellows, 1971; Lesmeister et al., 1973; Patterson et al., 1992). In order to achieve this goal, heifers should reach puberty at approximately 12 to 13 mo of age for enhanced chances of successful pregnancy to be established by 14 to 15 mo of age (Byerley et al., 1987). However, in a large number of beef herds in the US (Lucy et al., 2001), and in other prominent cattle industries around the world (Nogueira, 2004; Albuquerque et al., 2006; Johnston et al., 2009), heifers attain puberty at older ages. This scenario is particularly relevant to *Bos indicus* breeds and its crosses (Chenoweth, 1994), which are raised extensively in the southern US and encompass the majority of the beef cattle herds in the tropical and subtropical world (Cundiff, 2012). Therefore, reducing age at puberty in heifers is greatly needed to improve the efficiency of beef production systems worldwide.

It is well known that nutrition has a pivotal role in influencing age at puberty in mammals, including cattle (Short and Bellows, 1971). Nutritional status during the earlier developmental period appears to be particularly important for timing the pubertal process (Kirkwood et al., 1987). Indeed, high-energy diets fed to heifers early in life (from approximately 3 to 5 mo of age) advanced puberty (Gasser et al., 2006a; Gasser et

al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d). These findings indicate that maturation of the reproductive neuroendocrine system is programmed during a critical period of juvenile development in response to nutritional and metabolic cues. However, it is still not well understood how nutritional signals time puberty. Elucidating the physiological mechanisms that interconnect nutritional status and reproductive function is fundamental for optimizing managerial strategies that promote puberty in heifers consistently at a desirable age.

Puberty in female mammals involves maturation of the hypothalamic-pituitary-ovarian axis, and is dependent on development of adequate secretion of gonadotropin-releasing hormone (GnRH) and, consequently luteinizing hormone (LH). Final stages of pubertal maturation include follicle maturation and first ovulation (Foster and Jackson, 2006). The neurons that secrete GnRH are located within the preoptic area (POA) and hypothalamus (Dees and McArthur, 1981; Lehman et al., 1986). These neurons receive inputs from several neuronal systems originated in various regions of the brain (Chen et al., 1990; Turi et al., 2003; Herbison, 2008). Although not fully understood, the mechanism that leads to the pubertal increase in the frequency of GnRH release involves alterations in the excitatory and inhibitory neurocircuitry upstream to GnRH neurons (Ojeda and Skinner, 2006; Clarkson et al., 2009). Notably, some populations of those afferent neurons are located in the arcuate nucleus (ARC) of the hypothalamus and are known to be responsive to peripheral metabolic signals (McShane et al., 1993; Castellano et al., 2005; Elias and Purohit, 2013). The populations of neuropeptide Y- (NPY) and kisspeptin-producing neurons are of particular interest to this dissertation.

Neuropeptide Y negatively influences GnRH release in cows (Gazal et al., 1998) and delays puberty in rats (Pierroz et al., 1995). Neuropeptide Y gene expression and peptide synthesis are inhibited by the adipokine leptin (Stephens et al., 1995; Wang et al., 1997). Leptin is known as an important peripheral signal for the control of reproductive function and appears to have a permissive role in facilitating pubertal development in heifers (Garcia et al., 2003; Zieba et al., 2005). In addition, leptin regulates neurite outgrowth in the ARC and may play a role in inducing neuronal plasticity during early postnatal development (Bouret et al., 2004). Therefore, nutritional programming of puberty may involve increased circulating concentrations of leptin associated with growth and adiposity, and alterations in the hypothalamic NPY system.

Kisspeptin controls GnRH neuronal activity and influences the secretion of GnRH (Messager et al., 2005). Kisspeptin has been shown to induce secretion of LH in prepubertal ewes (Redmond et al., 2011b) and heifers (Kadokawa et al., 2008; Urias et al., 2011). Although the physiological relevance for direct influence of leptin in kisspeptin neurons remains unclear, kisspeptin neurons are clearly responsive to metabolic cues (Castellano et al., 2005). Hence, kisspeptin may also be involved in the pathway by which nutrition programs puberty. Therefore, it is proposed that the expression of the gene encoding kisspeptin (*KISS1*) is influenced by nutritional status during the juvenile period. In addition, because NPY neurons appear to establish functional synapses with kisspeptin neurons (Amstalden et al., 2011), it is plausible to inquire if the rate of body weight gain during the juvenile period alters NPY inputs onto kisspeptin neurons.

The nutritional programming of reproductive maturation may expand beyond the neurocircuitry discussed earlier and include additional neuronal pathways and interactions with glia cells. Indeed, it has been observed previously that several genes within the ARC are differentially expressed in prepubertal heifers fed diets that promote distinct rates of body weight gain (Allen et al., 2012). Epigenetic modifications, such as DNA methylation, lead to altered patterns of gene expression (Jirtle and Skinner, 2007). Thus, it is possible that nutritional status during the juvenile period may influence the pattern of DNA methylation in cells within the hypothalamus leading to long-lasting effects in the neurocircuitry that controls various neuroendocrine functions and the onset of puberty in heifers.

The overall objective of the work presented in this dissertation is to investigate the mechanisms by which dietary regimens shown previously to facilitate early onset of puberty in heifers affect the neuroendocrine pathways believed to integrate metabolic and reproductive functions. The specific objectives are to determine whether accelerated body weight gain during calfhood influences 1) *NPY* gene expression in the ARC, 2) NPY projections in the hypothalamus and towards GnRH neurons, 3) *KISS1* gene expression in the POA and ARC, 4) NPY projections towards kisspeptin neurons and 5) the methylation pattern of target genes within the ARC in heifers.

CHAPTER II

LITERATURE REVIEW

Reproductive maturation in heifers

Puberty is defined as a maturation process that culminates with the acquisition of the capacity to reproduce. In heifers, the ability to conceive and carry a pregnancy until term depends upon ovulation followed by a luteal phase of normal duration (Kinder et al., 1987; Moran et al., 1989). Ovulation is triggered by a surge of LH, which is induced by the surge of GnRH released in the hypothalamic-pituitary portal circulation (Foster and Jackson, 2006). Thus, the initiation of ovarian ciclicity is intrisically dependent on events that occur within the central nervous system and which, ultimatelly, results in a surge of GnRH release.

Puberty occurs in heifers most often at the age of 10 to 15 mo in *Bos taurus* and at 15 to 27 mo in *Bos indicus* and *Bos indicus*-influenced breeds (Chenoweth, 1994). Although first ovulation followed by a luteal phase of normal length is accepted as an indicator for puberty, maturation of the reproductive system occurs throughout the juvenile period (Kinder et al., 1994). The development of the ovarian follicles starts at the fetal life (Tanaka et al., 2001) and an adult-like pattern of follicular waves can be observed in two week-old heifers (Evans et al., 1994). Secretion of LH and follicle stimulating hormone (FSH) has been observed in heifer calves, and both gonadotropins stimulate follicle development and steroidogenesis at this early age (Gonzalez-Padilla et

al., 1975b; Dodson et al., 1988). Still, the final development of ovarian follicles, which is dependent on the enhanced frequency of LH stimulation, does not occur until puberty.

A small gradual increase in the frequency and amplitude of LH pulses occurs in heifers from 3 to 39 weeks of age (Dodson et al., 1988). A more consistent elevation in the frequency of LH release is observed only approximately 30 to 60 days prior to first ovulation. During this period, the size of the largest ovarian follicle increases (Day et al., 1986; Bergfeld et al., 1994) concurrent with the enlargement of the tubular genitalia (uterus, cervix and vagina; Honaramooz et al., 2004). Uterine growth occurs markedly under the influence of ovarian steroids, especially by 50 days before first ovulation (Day et al., 1987). The corpus luteum (CL) formed after the first ovulation is frequently of short lifespan (Berardinelli et al., 1979; Evans et al., 1994). The mechanisms leading to the short luteal phase involve enhanced prostaglandin F2α (PGF2α) bioshynthesis by the CL itself (Hu et al., 1990) and precocious production of PGF2α by the still imature uterus (Taponen et al., 2003). Final uterine maturation depends on the alternate stimulation by estrogen and progesterone, which occurs only after the first CL is formed (Spencer and Bazer, 2004). Full fertitlity of a heifer is usually achieved only after the third ovulation (Byerley et al., 1987). Therefore, reproductive capacity in heifers relies on the maturation of the hypothalamic-pituitary-ovarian-uterine axis, which starts early in life and is completed after the first ovulation.

Ovulation can be induced early in 2-month-old heifers by the administration of equine chorionic gonadotropin, to stimulate follicular development (Seidel et al., 1971). Gonadotropin-releasing hormone secretagoges can also elicit LH release and ovulation

in heifers (Gonzalez-Padilla et al., 1975a; Swanson and McCarthy, 1978; Schillo et al., 1983; Honaramooz et al., 1998). Despite that, ovarian cyclicity does not persist after cessation of the treatment. Hence, it is generaly accepted that inadequate GnRH release is the most limiting factor for pubertal progression. However, the mechanisms that lead to increased episodic release of GnRH are still unclear.

Gonadotropin-releasing hormone neuron: the central coordinator of reproductive function

The neurohormone GnRH is a decapeptide that plays a central role in regulation of reproduction in vertebrates. There are three known subtypes of GnRH molecules: I, II and III. The type I, referred to here as GnRH, is the most studied subtype in mammals due to its major relevance in the control of the reproductive function (Herbison, 2006). Gonadotropin-releasing hormone is produced by neurons that originate in the olfactory placode and migrate to the forebrain during embryonic life (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). The postnatal localization of those neurons follows a scattered continuum, from the diagonal band of Broca (DBB) to the premammillary region of the hypothalamus. The distribution patterns vary accordingly to species. In rodents, cell bodies of GnRH neurons are located mainly in the POA and anterior hypothalamus (Bennett-Clarke and Joseph, 1982), whereas in primates GnRH neurons extend to the medial basal hypothalamus (MBH) and are found in large numbers in the infundibular nucleus (a region anatomically similar to the ARC) and premammillary

nucleus (Silverman et al., 1982). In sheep, the greatest portion of GnRH cell bodies is located in the POA and anterior hypothalamic area (AHA; Lehman et al., 1986; Caldani et al., 1988). Similarly to humans, GnRH neurons in sheep extend to the MBH and are also found in the ARC and ventromedial nucleus, except that in fewer numbers compared to humans (Lehman et al., 1986). In cattle, GnRH cell bodies were detected by immunocytochemistry in the DBB, medial and lateral POA, and AHA (Dees and McArthur, 1981; Leshin et al., 1988). Although Dees and McArthur (1981) observed immunoreactive GnRH neurons in the ARC, Leshin and collaborators (1988) did not find GnRH cell bodies caudal to the AHA. Therefore, there is still uncertainty about the presence of GnRH cell bodies within the MBH in cattle.

Gonadotropin-releasing hormone neurons project to the median eminence (ME). There, GnRH is released from the neuronal terminals and, through the hypothalamic-pituitary portal vasculature, reaches the pituitary gland (Antunes et al., 1978). Gonadotropin-releasing hormone receptors are present within the gonadotropes located in the anterior pituitary, and their activation by GnRH stimulates the synthesis and release of LH and FSH (Jeong and Kaiser, 2006). There are two characterized modes of GnRH release: surge and episodic. The surge mode is observed only in females (Herbosa et al., 1996). In sheep and rodents, a GnRH surge is required for the pre-ovulatory surge of LH and ovulation, whereas in primates a GnRH surge appears to be permissive, but not deterministic for the LH surge (Knobil et al., 1980). The episodic mode, observed in both males and females, stimulates the pulsatile secretion of LH and FSH, which promotes the development of ovarian follicles and steroidogenesis (Fortune and Quirk,

1988). It has been demonstrated that pulses of LH occur concomitantly to the episodic release of GnRH in various mammals including sheep (Caraty et al., 1982; Clarke and Cummins, 1982), cattle (Gazal et al., 1998; Yoshioka et al., 2001), horses (Irvine and Alexander, 1997) and primates (Knobil, 1980).

Extensive research has been conducted to understand the mechanisms that control GnRH release during pubertal maturation. The mechanisms that drive the episodic and surge modes of GnRH secretion are likely different (Moenter et al., 1992; Tanaka et al., 1995). Studies conducted using immortalized, mouse GnRH-secreting GT1 cells (Martinez de La Escalera et al., 1992) and embryonic GnRH neurons isolated from the olfactory placode (Terasawa et al., 1999; Duittoz and Batailler, 2000) indicate that GnRH neurons exhibit an intrinsic ability to release GnRH in a pulsatile manner. It is also proposed that synaptic transmission between GnRH neurons is involved in the synchronization of GnRH secretion (Herbison, 2006). However, inputs from other neuronal types are also believed to be involved in the synchronization of GnRH neuronal activity leading to a detectable episode of GnRH release. The interneuronal regulation of GnRH neuronal activation may occur at the level of the cell bodies and/or at the level of nerve terminals in the ME (Maeda et al., 2010).

In domestic ruminants, there is evidence that the pulsatile secretion of LH is driven by GnRH neurons located in the MBH. Studies using an electrophysiological approach were able to record a high correlation between volleys of multiple unit activity (MUA) in the MBH and LH pulses in goats (Mori et al., 1991). However, the cellular source of those volleys is unclear. Studies in sheep indicated that episodic LH secretion

induced in rams and ewes is coupled with induction of Fos immunoreactivity (a marker for neuronal activation) in GnRH neurons located in the MBH, but not in the POA (Boukhliq et al., 1999). In contrast, activation of a GnRH surge in sheep does not appear to be associated with a particular subanatomical group (Moenter et al., 1993).

Gonadal steroids have a large influence on the control of GnRH secretion and pubertal maturation. It is well established that the preovulatory GnRH surge is induced by the positive feedback action of estradiol. In contrast, tonic secretion is controlled by the negative feedback promoted by estradiol and progesterone (Goodman et al., 1981; Herbison, 1998). In domestic ruminants, the low frequency of release of GnRH/LH during the infantile and juvenile period in females is proposed to be a consequence of the high sensitivity of the reproductive neuroendocrine axis to the inhibitory effect of estradiol. This hypothesis is supported by the observation that post-gonadectomy enhancement of pulsatile secretion of LH in prepubertal ewe lambs is restrained by estradiol replacement (Foster and Ryan, 1979). Therefore, pubertal progression appears to involve the reduction in the sensitivity of the reproductive neuroendocrine axis to the estradiol negative feedback (Day et al., 1984; Ebling et al., 1990) followed by increased frequency of release of LH and enhanced steroidogenesis in ovarian follicles (Day et al., 1987). High concentrations of estradiol elicit a stimulatory effect on the hypothalamic pituitary axis, promoting the preovulatory surges of GnRH and LH (Yoshioka et al., 2001). Although the mechanism that leads to the peripubertal change in sensitivity to estradiol negative feedback is not understood, it might involve reduction in the number of estradiol receptors in the hypothalamus (Day et al., 1987).

Despite the important role of estradiol in the control of GnRH secretion, GnRH neurons do not contain estrogen receptor alpha (ERα; Lehman and Karsch, 1993). Although GnRH neurons contain ERβ (Hrabovszky et al., 2001) and the membrane form of estrogen receptor GPR30 (Noel et al., 2009), the role of those estrogen receptor forms in mediating estradiol control of GnRH neuronal activity is unclear (Ábrahám et al., 2003; Sun et al., 2010). Indeed, evidence indicates that ERα is a major mediator of estradiol effects in the control of the reproductive neuroendocrine axis (Dorling et al., 2003; Wintermantel et al., 2006). It is generally accepted that the influence of estradiol on GnRH release is mediated through afferent cellular components that contain ERα. In sheep, the positive feedback action of estradiol on GnRH release involves estradiol-sensitive cells located in the MBH (Caraty et al., 1998). Such observation emphasizes the role of estradiol-sensitive effector cells upstream to GnRH neurons. These effectors likely involve both neurons and glial cells that convey regulatory signals to GnRH neurons.

Afferent circuitry to GnRH neurons

A variety of inhibitory and excitatory signals have been described to control GnRH neuronal activity (Maeda et al., 2010). They may affect GnRH neuronal function by synaptic and non-synaptic mechanisms (Ojeda and Terasawa, 2002). Inhibitory molecules that directly influence GnRH secretion include gamma-aminobutiric acid (GABA), endogenous opioid peptides and NPY; whereas, stimulatory molecules include

glutamate, kisspeptin, neurokinin B, and insulin-like growth factor 1 (IGF-1), among others. It is proposed that an orchestrated reduction of inhibitory inputs and enhancement of excitatory inputs to GnRH neurons are involved in elevation of frequency of GnRH release characteristic of pubertal transition (Ojeda and Skinner, 2006).

Gamma Aminobutyric Acid (GABA)

Gamma-aminobutyric acid is considered a major inhibitory neurotransmitter to GnRH secretion during pubertal development in primates (Mitsushima et al., 1994). In rodents, GABA's role in puberty onset is ambiguous because GABA can both inhibit and stimulate release of GnRH (Han et al., 2002). The influence of GABA in inhibiting GnRH release during pubertal development occurs mainly through $GABA_A$ receptors, which have been demonstrated to be present in GnRH neurons (Han et al., 2004). In ewes, $GABA_A$ and $GABA_B$ receptors have been detected in GnRH neurons (Tomaszewska-Zaremba and Przekop, 2006). Stimulation of $GABA_A$ receptors in ewes during the follicular phase attenuates GnRH release, an effect that appears to occur via GABA actions in GnRH cell bodies in the POA, and in GnRH nerve terminals at the ME (Tomaszewska-Zaremba et al., 2003). In sheep, GABA appears to suppress both GnRH synthesis and release (Ciechanowska et al., 2009). There is also evidence that GABA is involved in the negative feedback of estradiol in ewes during the non-breeding season (Bogusz et al., 2008).

Endogenous opioid peptides

Endogenous opioid peptides (enkephalin, dynorphin, endorphin and orphanin) also serve as key inhibitory inputs to GnRH neurons (Smith and Jennes, 2001; Terasawa and Fernandez, 2001). In rodents, a reduction in the opioid-mediated inhibition of LH release was observed during pubertal development (Wilkinson and Bhanot, 1982). The administration of the opioid inhibitor naloxone stimulates LH release in prepubertal sheep (Ebling et al., 1989) and heifers (Byerley et al., 1992; Wolfe et al., 1992), and opioid inhibition of LH secretion declines during the prepubertal period in heifers (Wolfe et al., 1992).

Neuropeptide Y

Neuropeptide Y, a 36-amino acid peptide highly abundant in the mammalian central nervous system, is known to be involved in the control of various hypothalamic functions, including feed intake and reproduction (Li et al., 1994; Morton et al., 2006). Cell bodies of NPY-producing neurons are located in multiple brain areas, including the ARC (Antonopoulos et al., 1989). The population of NPY neurons in the ARC exclusively co-expresses agouti-related protein (AGRP) (Hahn et al., 1998). Axons containing NPY are observed throughout the brain (Morris, 1989; Fetissov et al., 2004), and most NPY projections observed in ARC, PVN, dorsomedial hypothalamus and lateral hypothalamic area (LHA) also contain AGRP (Grove et al., 2001). This indicates that these projections originate from NPY neuronal cell bodies localized in the ARC.

Therefore, NPY neurons in the ARC are believed to play a major role in the control of hypothalamic functions, including reproduction.

Neuropeptide Y has an inhibitory role on LH secretion in ovariectomized rats (Kalra and Crowley, 1984a) and monkeys (Kaynard et al., 1990). However, there is evidence indicating that NPY can also exert stimulatory effects in intact and ovariectomized, estrogen-primed adult rats (Levine et al., 1991), and in intact, adult female monkeys (Terasawa and Fernandez, 2001). In ruminants, NPY has an inhibitory effect on the release of GnRH and LH in the presence and absence of estradiol. Intracerebroventricular (ICV) infusions of NPY reduce the frequency of pulses and mean concentrations of GnRH and LH in ovariectomized, estradiol-implanted cows (Gazal et al., 1998). Neuropeptide Y also reduces release of LH in ovariectomized ewes (McShane et al., 1992b) and cows (Thomas et al., 1999; Garcia et al., 2004) regardless of estradiol replacement. In addition, NPY blocks the preovulatory LH surge in intact ewes (Estrada et al., 2003) and inhibits the pulse generator activity (measured by MUA volleys) in ovariectomized goats receiving estradiol replacement or not (Ichimaru et al., 2001).

Four G protein-coupled receptors (Y1, Y2, Y4 and Y5) have been identified as cellular mediators of NPY function (Fetissov et al., 2004). The control of reproductive function by NPY may occur through direct actions on GnRH neurons (Sullivan and Moenter, 2004) via activation of Y1 receptors (Klenke et al., 2010; Roa and Herbison, 2012). Y1 receptors have been demonstrated to be coupled with inhibitory G protein (Gi), and its signal appears to involve the activation of G-protein coupled inward rectifier potassium channels (Klenke et al., 2010). Immunocytochemistry and electron

microscopy evidence also support a direct action of NPY on GnRH neurons (Li et al., 1994; Grove et al., 2003). It has been observed that NPY neurons located in the ARC send projections within close proximity to GnRH cell bodies in the POA and to GnRH fibers in the ME (Li et al., 1999). In addition, GnRH nerve fibers and terminals in both the vascular organ of the lamina terminalis (OVLT) and ME contain Y1-immunoreactivity, indicating that Y1 may be a major receptor mediating NPY actions on GnRH neurons.

Other NPY receptors have also been proposed to mediate NPY effects on reproductive function (Sainsbury et al., 2002a; Toufexis et al., 2002; Schneider, 2004). In rats, Y5 receptor is present in GnRH neurons (Campbell et al., 2001), and a Y2 receptor agonist suppresses the secretion of LH in sheep (Barker-Gibb et al., 1995) and delays the estradiol-induced LH surge (Clarke et al., 2005). Therefore, it is plausible to speculate that in cattle, the negative effect of NPY on GnRH release may also occur via direct effects of NPY on GnRH neurons, and these actions may occur at the level of the cell body and nerve terminals.

There is evidence that NPY has a role in pubertal maturation. Expression of NPY as well as its receptors (Y1, Y2, and Y5) is down-regulated during development, from early life to adulthood (Naveilhan et al., 1998). In ewes, it was observed that pubertal transition involves decreased activity of NPY neurons in the ARC, characterized by a reduction of *NPY* mRNA and an increase in NPY immunoreactivity. The latter may indicate a reduction in NPY release and consequent peptide accumulation in terminals and dendrites (Tillet et al., 2010). Daily ICV injections of NPY for 7 to 12 days in

female rats inhibited the onset of puberty (Pierroz et al., 1995), while chronic exposure to Y1 antagonist accelerated pubertal progression defined as an earlier increase in the LH content in the pituitary (Pralong et al., 2000). A study using castrated monkeys (El Majdoubi et al., 2000) indicated that *NPY* mRNA abundance in the MBH decreased at the onset of puberty. In that study, ICV infusion of a Y1 antagonist increased LH release. This effect was abolished by prior treatment with a GnRH receptor antagonist. These data support the idea that the negative influence of NPY on GnRH release is reduced during pubertal progression and allows for an elevation in the frequency of GnRH release.

Glutamate

Glutamate is well known as a potent stimulator of GnRH release in several mammalian species, acting through the metabotropic N-methyl-D-aspartate (NMDA) receptor and the ionotropic kainate receptors (Terasawa and Fernandez, 2001). The sensitivity to glutamatergic stimulation has been demonstrated to increase at puberty in rats (Bourguignon et al., 1992) and monkeys (Claypool et al., 2000). The stimulatory effect of glutamate is proposed to occur via direct action on GnRH neurons (Gore, 2001;Ottem et al., 2002) or through other regulatory neuronal networks (van den Pol et al., 1990). Glutamate has also been demonstrated to induce LH release in prepubertal ewes (I'Anson et al., 1993) and to induce puberty in female goats (Meza-Herrera et al., 2011). Moreover, an NMDA agonist induces LH secretion and ovulation in prepubertal heifers (Honaramooz et al., 1998).

Kisspeptin

Kisspeptin, a product of the *KISS1* gene, is as a potent stimulator of GnRH release (Messager et al., 2005) and has been considered an important gatekeeper of reproductive maturation in mammals (Kauffman, 2010). Kisspeptin is transcribed as a long precursor peptide processed into biologically-active shorter peptides (10 to 54 amino-acids). The 16- and 13-amino acid forms (Kp16 and Kp13) appear to be the major forms detectable in the sheep hypothalamus (Caraty et al., 2012). The decapeptide (Kp-10) corresponds to the C-terminus common to other forms of kisspeptin and has been demonstrated to have full biological activity (Gottsch et al., 2004b).

Loss-of-function mutations in the *KISS1* gene and in the gene that encodes kisspeptin receptor (*KISS1R*), are associated with hypogonadotropic hypogonadism in rodents and humans (de Roux et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007; Silveira et al., 2010). These observations indicate that normal function of the kisspeptin system is required for proper reproductive function (Seminara et al., 2003). However, it was demonstrated that ablation of kisspeptin neurons during fetal development does not cause infertility, indicating that redundant or compensatory mechanisms overcome the lack of kisspeptin- and kisspeptin receptor- signaling (Mayer and Boehm, 2011).

There is evidence that the effects of kisspeptin on stimulation of GnRH and LH release occur by direct actions on GnRH neurons (Navarro et al., 2004b; Castellano et al., 2005; Messager et al., 2005; Smith et al., 2008). This includes the observation that kisspeptin fibers project to GnRH cell bodies (Yeo and Herbison, 2011), *KISS1R* is

present in GnRH cells (Herbison et al., 2010; Smith et al., 2011) and kisspeptin increases excitability of GnRH neurons (Han et al., 2005b). Kisspeptin may also affect GnRH secretion indirectly by acting on other afferents to GnRH neurons, such as GABA and glutamate neurons (Pielecka-Fortuna and Moenter, 2010). The action of kisspeptin in regulating GnRH secretion may also occur at the nerve terminals located in the ME, because close apposition between kisspeptin and GnRH fibers was observed in that region (Smith et al., 2011; Uenoyama et al., 2011). Moreover, *in vitro* experiments indicate that kisspeptin stimulates LH release by acting directly at the pituitary (Suzuki et al., 2008). However, this remains controversial because kisspeptin treatment did not alter GnRH-stimulated LH secretion in hypothalamo-pituitary-disconnected ewes (Smith et al., 2008).

Kisspeptin neuronal cell bodies are observed in two anatomically-distinct populations: the POA/periventricular (PeV) area (or the anteroventral periventricular area (AVPV) population characterized in rodents), and the ARC (Oakley et al., 2009) population. Kisspeptin cells in these populations are phenotypically different as they express different sets of genes. For instance, in mice, kisspeptin neurons located in the AVPV are mainly glutamatergic, whereas kisspeptin neurons located in the ARC are mainly GABAergic (Cravo et al., 2011). Also, other studies with rodents have demonstrated that met-enkephalin (Porteous et al., 2011), and galanin (Kalló et al., 2012) are co-expressed in a subset of the AVPV kisspeptin population, but not in the ARC population. Conversely, kisspeptin neurons located in the ARC co-express neurokinin B and dynorphyn, as demonstrated in rodents (Burke et al., 2006; Navarro et al., 2009),

sheep (Goodman et al., 2007), goats (Wakabayashi et al., 2010) and primates (Ramaswamy et al., 2013).

Kisspeptin neurons contain receptors for estradiol (ERα and ERβ), progesterone and androgens, and are responsive to gonadal steroids (Smith et al., 2005a; Smith et al., 2005b; Franceschini et al., 2006; Smith et al., 2007). Estradiol exhibits distinct regulatory effects on the two populations of kisspeptin neurons (Smith et al., 2007; Xu et al., 2012): it has an inhibitory effect on the kisspeptin population in the ARC (observed by number of kisspeptin-immunoreactive cells or *KISS1*-expressing cells), and a stimulatory effect on *KISS1* expression in the POA (Smith et al., 2005a; Smith et al., 2005b; Smith et al., 2007; Smith et al., 2008). Moreover, kisspeptin neurons within the POA/PeV are sexually dimorphic. In this area, a greater number of kisspeptin neurons are found in females than in males (Smith et al., 2005a; Smith et al., 2005b; Adachi et al., 2007; Xu et al., 2012). Interestingly, this sexual dimorphism does not appear to be present in the ARC population (Gottsch et al., 2004a).

In both sheep and rodents, kisspeptin neurons have been implicated in mediating the estradiol-induced GnRH/LH surge that is present only in females (Herbosa et al., 1996). Constant infusion of kisspeptin produces sustained LH release and synchronizes the timing of the LH surge in adult ewes (Caraty et al., 2007), whereas administration of kisspeptin antagonist reduces the magnitude of the estradiol-induced LH surge in mice (Pineda et al., 2010b) and sheep (Smith et al., 2011). In rodents, the control of the GnRH surge appears to involve kisspeptin neurons located in the AVPV exclusively. Evidence for sexual dimorphism in the POA/PeV population of kisspeptin neurons (Smith et al.,

2005a) and the stimulatory effects of estradiol on *KISS1* expression in the POA/PeV (Smith et al., 2006b) supports this hypothesis. In sheep, both the POA/PeV and ARC populations are likely involved in the generation of the GnRH surge. Evidence to support the latter is from studies demonstrating an increase in the expression of *KISS1* and kisspeptin immunoreactivity in the ARC (particularly in the caudal portion of this nucleus) and POA during the late follicular phase preceding ovulation (Estrada et al., 2006; Smith et al., 2009). In addition, transcriptional activation (determined by the detection of c-fos) is enhanced in kisspeptin neurons located in the ARC and POA/PeV populations during the LH surge (Merkley et al., 2012). However, studies by Hoffman and collaborators (Hoffman et al., 2011) indicate a major role for the POA/PeV population in the generation of GnRH surge in sheep.

The ARC population of kisspeptin neurons has been proposed to mediate the estradiol negative feedback present in males and females. Observations in support of this hypothesis include: 1) the lack of sexual dimorphism in the ARC population (Gottsch et al., 2004a); 2) inhibition of *KISS1* expression in the ARC by estradiol (Smith et al., 2005b;Smith et al., 2008); 3) recording of multiunit electrical activity in the ARC associated with GnRH pulses (Maeda et al., 1995; Ohkura et al., 2009); and 4) suppression of LH pulsatility after administration of kisspeptin antagonist into the ARC, but not in the POA (Li et al., 2009).

The neuronal kisspeptin system appears to be developmentally regulated. An earlier study demonstrated that in female rats, the expression of *KISS1* in the hypothalamus decreases with the transition from the infantile to the juvenile period, but

increases thereafter, reaching highest levels during the pubertal phase (Navarro et al., 2004a). Later, it was demonstrated that the reduction in *KISS1* expression during the infantile to juvenile transition occurs specifically in the neurons located within the ARC, but not in the AVPV (Cao and Patisaul, 2011). In fact, *KISS1* expression increases in the AVPV during the infantile to juvenile transition (Cao and Patisaul, 2011). Indeed, the developmental pattern of *KISS1* expression observed from infantile to adult life was also observed in kisspeptin-immunoreactive fibers within the ARC. As puberty approaches, the number of kisspeptin neurons, as well as kisspeptin fiber projections in close proximity to GnRH neurons, increase (Navarro et al., 2004b; Clarkson and Herbison, 2006;Clarkson et al., 2009). Experiments using push-pull perfusion and microdialysis in the ME of monkeys indicate that the kisspeptin content in the ME and pulsatile release of kisspeptin also increase with the onset of puberty (Keen et al., 2008). In ovariectomized, estradiol-implanted ewe lambs, the increase in *KISS1* expression in the ARC occurs in association with the increase in the frequency of LH release during the juvenile period (Redmond et al., 2011a). Furthermore, exogenous kisspeptin treatment advances puberty in rats (Navarro et al., 2004b) and mice (Han et al., 2005a), and a kisspeptin antagonist delays the onset of puberty (Pineda et al., 2010a). Kisspeptin has also been demonstrated to induce secretion of LH in young heifers (Kadokawa et al., 2008; Ezzat Ahmed et al., 2009; Urias et al., 2011). In this latter study, it was also reported that the responsiveness to kisspeptin increased with age (Urias et al., 2011). Overall, these studies indicate that maturation of the kisspeptin system, which includes increases in *KISS1* gene expression, kisspeptin synthesis and release, as well as

kisspeptin inputs onto GnRH neurons, occurs concurrently with the onset of the pubertal elevation in frequency of LH release. However, whether kisspeptin is required to trigger or amplify GnRH neuronal activity for the onset of puberty is still unclear.

Neurokinin B

Neurokinin B (NKB), encoded by the *TAC3* gene, has also been involved in the regulation of GnRH release. Loss-of-function mutations in *TAC3* and NKB receptor (NK3R) gene (*TACR3*) in humans has been identified as a cause for hypogonadotropic hypogonadism (Topaloglu et al., 2008). Neurokinin B stimulates LH release in rats, sheep and goats (Billings et al., 2010; Wakabayashi et al., 2010; Navarro et al., 2011) and NKB neurons are detected in various areas of the central nervous system (Rance et al., 2010), including the ARC, where they co-express kisspeptin and dynorphin A (Goodman et al., 2007). Evidence supports a mechanism of autoregulation among NKB neurons in the ARC. Neurokinin B neurons in the ARC contain NK3R receptor (Amstalden et al., 2010) and NKB axons originating from the ARC population are in close-proximity to NKB neurons in the ARC (Krajewski et al., 2010; Wakabayashi et al., 2012). Indeed, central administration of NKB and NK3R agonist activates ARC kisspeptin neurons in ewes (Sakamoto et al., 2012) and rats (Navarro et al., 2011). In addition, central administration of NKB in combination with kisspeptin in mice promotes a significant increase in LH concentrations compared to kisspeptin alone (Corander et al., 2010). Although NK3R was not observed in GnRH neurons in sheep (Amstalden et al., 2010), NK3R is observed in GnRH nerve terminals at the ME of rats

(Krajewski et al., 2005), indicating a potential action of NKB on the GnRH neuron in this species.

Neurokinin B has also been implicated in the negative feedback of estradiol and control of episodic release of GnRH. This is supported by the observation that estradiol inhibits the expression of the genes encoding NKB and NK3R (Pillon et al., 2003; Gill et al., 2012), and that ovariectomy leads to increased NKB cell numbers in pre- and postpubertal ewes (Nestor et al., 2012). Nestor et al. reported an increase in NKB fiber density in the ARC of mature ewes compared to prepubertal ewe lambs. In addition, the treatment of prepubertal ewes with a NK3R agonist stimulated LH pulsatility (Nestor et al., 2012). In mice, *TAC3* and *TAC*R3 mRNA increases with puberty (Gill et al., 2012). Therefore, it is likely that NKB participates in concert with kisspeptin to mediate the estradiol negative feedback control of LH release and regulate pubertal progression.

Insulin-like growth factor-1 (IGF-1)

Insulin-like growth factor-1 (IGF-1) is a hormone that has growth-promoting and insulin-like activities (Veldhuis et al., 2006). It is proposed to be one of the glial-derived factors that stimulate GnRH release (Ojeda et al., 2008). In addition to being synthesized in astrocytes, IGF-1 is also synthesized in GnRH neurons (Daftary and Gore, 2003). Circulating IGF-1, produced mainly in the liver (Murphy et al., 1987), can cross the blood-brain barrier (Reinhardt and Bondy, 1994) and reach the central nervous system (CNS) via complex and not well-characterized mechanisms that include transportation of IGF-1 by binding proteins (IGFBPs; Jones and Clemmons, 1995). GnRH neurons

contain IGF-1 receptors (Olson et al., 1995) and IGF-1 has been demonstrated to stimulate *GNRH* expression and GnRH release, and facilitate the puberty process (Hiney et al., 1991; Hiney et al., 1996). The expression of *IGF1* in the brain is elevated during puberty (Sullivan and Feldman, 1994). In addition, it has been observed that IGF-1 content in the ARC of female rats is increased during the onset of puberty, an effect regulated by estradiol (Duenas et al., 1994). Insulin-like growth factor-1 receptor activation has been demonstrated to be involved in remodeling of synapses within the ARC that occur during the estrous cycle in rats (Fernandez-Galaz et al., 1999). Therefore, the mechanisms by which IGF-1 facilitates pubertal progression may involve direct stimulation of GnRH neuronal activity and GnRH release, as well as remodeling of synaptic input on GnRH neurons.

Transcriptional control of female puberty

Ojeda and collaborators have proposed a model in which the rheostat of GnRH neuronal activation for pubertal progression relies on concerted regulation of a plethora of regulatory genes organized in a network in hierarchical mode (Ojeda et al., 2006). According to this model, the pubertal process is coordinated by genes that encode factors involved in the control of GnRH release. Those genes encode peptides and enzymes that participate in the process of synthesis of neurotransmitters or their receptors, and have been designated as subordinate genes. Another subset of genes, the so-called integrative genes, influences the communication of neuronal and glia networks by mediating

synaptic connectivity and signal transduction pathways and, therefore, may integrate inputs to GnRH neurons. An example in this category is the gene encoding cell adhesion molecule 1(*SynCAM*), which is required for synapses formation (Biederer et al., 2002; Sandau et al., 2011).

The higher hierarchical level of regulatory genes is comprised of a group of genes that control the transcriptional activity of subordinate and integrative genes. *LIN28B* is one of the genes considered relevant at higher hierarchical regulation. The protein encoded by *LIN28B* is an RNA binding protein known to control expression of other genes via posttranscriptional regulation (Moss et al., 1997). One of LIN28B target is the micro RNA (miRNA) *let-7*. Upregulation of *LIN28B* is associated with a reduction in *let-7* miRNA (Viswanathan and Daley, 2010). The miRNA *let-7* controls the expression of genes such as *HMGA2* (High Mobility Group A2; Lee and Dutta, 2007). The *HMGA2* gene encodes a non-histone chromosomal protein that influences chromatin structure and regulates expression of several genes (Pfannkuche et al., 2009). Single nucleotide polymorphism analysis indicated an association of *LIN28B* with earlier onset of puberty in girls (Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009; Elks et al., 2010; Ong et al., 2011). Similar analysis in cattle indicated an association of *HMGA2* with age at puberty in heifers (Fortes et al., 2011). Studies using transgenic mice indicated that overexpression of *LIN28A*, a homologue of *LIN28B*, delays the onset of puberty in females (Zhu et al., 2010). Interestingly, a reduction of *LIN28B* RNA in the hypothalamus is observed during pubertal development in female rats (Sangiao-Alvarellos et al., 2013). It was also observed that the expression of *let-7a* and *let-7b* in

rat hypothalamus had an inverse relationship with *LIN28B* mRNA abundance (Sangiao-Alvarellos et al., 2013). These observations indicate that the *LIN28B-let-7-HMGA2* pathway may play an important role in controlling pubertal progression.

Influence of nutrition on the pubertal process

The influence of nutritional status on the maturation of reproductive function is well established in various species, including cattle (Short and Bellows, 1971; Day et al., 1986; Garcia et al., 2002). Nutrient utilization is coupled to rate of growth, and pre- and post-weaning rate of growth is inversely related to age at puberty in heifers (Menge et al., 1960; Wiltbank et al., 1969). High-energy diets fed to heifers during the juvenile period leads to precocious puberty (defined by puberty by 300 d of age; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d). The effect of this nutritional strategy was associated with an earlier reduction in the sensitivity to estradiol negative feedback (Gasser et al., 2006b), a hastening of the peripubertal increase in the frequency of episodic release of LH (Gasser et al., 2006d), and an acceleration of the development of large dominant follicles on the ovary (Gasser et al., 2006c). A recent study performed in our laboratory indicated that heifers fed a high-concentrate diet, formulated to promote high rates of body weight gain, beginning at 4 mo of age, exhibited a greater frequency of LH pulses at 8 mo of age than heifers fed to gain at a lower rate of gain (Cardoso et al., 2012). This dietary regimen was observed to increase the proportion of heifers attaining puberty by 11 mo of age. Although the molecular

mechanisms that link nutrition and reproductive function are not fully understood, they may involve signaling by hormones that include glucose, insulin (Adam and Findlay, 1998), growth hormone, IGF-1(Simpson et al., 1991b; Yelich et al., 1996; Adam et al., 1998), ghrelin (Barreiro and Tena-Sempere, 2004; Farifteh et al., 2008; Harrison et al., 2008), adiponectin (Cheng et al., 2011) and leptin (Garcia et al., 2003; Barb and Kraeling, 2004).

Role of leptin in signaling metabolic status

In the 1980s, Frisch (Frisch, 1984) proposed that a minimum degree of adiposity is critical for pubertal development. Later, leptin, a hormone produced mainly in the adipose tissue, was shown to play a major role in regulating food intake, energy expenditure, and reproductive function (Chehab et al., 1996; Friedman and Halaas, 1998). Leptin has been shown to rescue infertility in leptin-deficient mice (Chehab et al., 1996), and to advance puberty in mice (Ahima et al., 1997) and rats (Zeinoaldini et al., 2006); therefore, leptin was considered a metabolic gate for pubertal progression in rodents (Cheung et al., 1997). In developing heifers, circulating concentrations of leptin are positively correlated with adiposity and increases in body weight (Garcia et al., 2003). Leptin gene expression in adipose tissue and circulating concentrations of leptin are reduced by 48 h of fasting concomitantly with reductions in the frequency of LH pulses (Amstalden et al., 2000b). Leptin treatment prevents the reduction in the frequency of LH pulses in fasted prepubertal intact heifers (Maciel et al., 2004). These observations indicate that the hypothalamic-pituitary axis of immature heifers is

particularly sensitive to acute perturbations in nutrient intake and that leptin signals nutritional status to the control of reproductive function. However, leptin treatment did not accelerate the development of mature patterns of gonadotropin secretion in normal fed or growth-restricted-heifers (Maciel et al., 2004). Therefore, at least in cattle, leptin signaling appears to be permissive, but not sufficient for the onset of puberty (Zieba et al., 2005).

Circulating leptin reaches the CNS via leptin transporters located in hypothalamic endothelium and choroid plexus and, to a lesser magnitude, by crossing the blood-brain barrier by diffusion (Banks et al., 1996; Zlokovic et al., 2000; Adam and Findlay, 2010). Leptin receptors are members of the type I cytokine receptor family, and six alternative splicing forms have been identified (LepRa-LepRf). The only isoform that contains full intracellular signaling capability is the LepRb (Baumann et al., 1996). The binding of leptin to LepRb involves phosphorylation of janus kinase-2 (JAK2) and signal transducer and activator of transcription-3 (STAT3), and also an increase in suppressor of cytokine signaling-3 (SOCS-3), which is a negative regulator of this cascade (Elias et al., 1999; Hegyi et al., 2004). Activation of LepRb also triggers other signaling cascades (Bates et al., 2003; Myers, 2004), including the phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR). The latter has recently been proposed to be involved in mediating leptin’s actions on the regulation of reproductive function (Roa and Tena-Sempere, 2010; Yang et al., 2010; Tena-Sempere, 2012).

The LepRb receptor has been identified in various areas of the hypothalamus, but the highest concentrations are observed in the ventral premammilary (PMv), dorsomedial, and ARC nuclei (Elmquist et al., 1999; Donato et al., 2009; Patterson et al., 2011). The ARC is considered the primary site for leptin action in the CNS (Satoh et al., 1997). Leptin receptor has not been found in GnRH neurons (Finn et al., 1998; Woller et al., 2001; Quennell et al., 2009), and conditional deletion of leptin receptor in GnRH neurons does not lead to impaired reproductive function (Quennell et al., 2009). Therefore, leptin is proposed to regulate reproductive function via intermediate neurons, which in turn, control GnRH neuronal function and the release of GnRH (Barb and Kraeling, 2004; Crown et al., 2007). Neurons located within the ARC are likely intermediate players in this neural communication (Ahima et al., 1996; Barb and Kraeling, 2004).

Hypothalamic neurocircuitry linking metabolism and reproduction: roles of NPY and kisspeptin

Neuropeptide Y neurons in the ARC express leptin receptor (Hakansson et al., 1998) and *NPY* expression is highly influenced by nutritional status (Williams et al., 2000). Expression of *NPY* in the ARC is increased in leptin-deficient (*ob/ob)* mice and leptin treatment reduces *NPY* expression (Wilding et al., 1993; Stephens et al., 1995) and NPY release (Stephens et al., 1995). In feed-restricted, ovariectomized ewes hypothalamic *NPY* mRNA and NPY concentrations in the cerebrospinal fluid are increased compared to control animals (McShane et al., 1992b; McShane et al., 1993;

Thomas et al., 2009). A reduction in the expression of *NPY* in the ARC was observed in prepubertal heifers fed high-concentrate diets that induced high rates of body weight gain and greater levels of circulating leptin compared to heifers gaining weight at low rates (Allen et al., 2012). Nutritional regimens similar to that adopted in the experiment by Allen et al. (2012) were observed to advance puberty in heifers (Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d). Thus, a reduction in NPY signaling may be involved in the nutritional programing of accelerated puberty in heifers.

Electrophysiological studies demonstrated that leptin hyperpolarizes NPY neurons (Cowley et al., 2001; Qiu et al., 2011), diminishes release of NPY from terminals (Cowley et al., 2001) and reduces NPY gene expression (Qiu et al., 2011). In rats, leptin decreases *NPY* mRNA in the ARC (Ahima et al., 1996; Schwartz et al., 1996b) and prevents NPY-induced feed intake (Sahu et al., 1998). Interestingly, approximately 20% of the females born from the crossing of *ob/ob* with NPY deficient mice are fertile (Erickson et al., 1996). Similarly, fertility was reestablished in approximately 50% of the females following deletion of Y4 receptor in *ob/ob* mice (Sainsbury et al., 2002b). The deletion of Y4 in *ob/ob* mice also induced increased GnRH expression. These observations indicate that the infertility of leptin deficient mice requires NPY signaling through Y4 receptor. Collectively, these data indicates that leptin targets directly NPY neurons and inhibits *NPY* gene expression and release. Reduction of NPY inhibitory tone may facilitate the increased episodic release of GnRH and LH that occurs during of the pubertal transition.

The expression of *KISS1* is also regulated by metabolism (Castellano et al., 2005; Forbes et al., 2009), indicating that kisspeptin may play a role in mediating the nutritional control of reproductive function. Hypoleptinemia is associated with a reduction in hypothalamic *KISS1* mRNA in rodents, and administration of leptin prevents the fasting-induced decrease in *KISS1* expression (Castellano et al., 2006; Smith et al., 2006a). At least a subset of kisspeptin neurons in mice (Smith et al., 2006a) and sheep (Backholer et al., 2010b) contains leptin receptor, indicating that leptin may directly influence kisspeptin production and release. Indeed, leptin was demonstrated to directly depolarize kisspeptin neurons from guinea pigs (Qiu et al., 2011). However, in mice, it has been proposed that the action of leptin in facilitating the onset of puberty may not require direct actions of leptin on kisspeptin neurons, but may involve interneurons located in the PMv (Donato Jr et al., 2011).

Kisspeptin neurons may also be regulated by other metabolic-sensing pathways. Central and peripheral administration of IGF-1 to immature rats induces *KISS1* expression in the AVPV, but not in the ARC (Hiney et al., 2009). In addition, adiponectin, an adipocyte-specific secreted factor that regulates energy metabolism, was demonstrated to decrease *KISS1* expression in GT1-7 hypothalamic cells (Wen et al., 2012). Ghrelin, a hormone released by the stomach at increased amounts during periods of negative energy, was also shown to reduce *KISS1* in the AVPV (Forbes et al., 2009). Furthermore, it has been reported that kisspeptin neurons are in close proximity to NPY-containing fibers in rats (True et al., 2011a) and sheep (Amstalden et al., 2011), indicating that NPY may regulate kisspeptin neuronal function.

Nutritional programming of hypothalamic neuronal circuitry

Gasser and collaborators (Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d) observed that a high percentage of beef heifers weaned at 3 to 4 mo of age and fed a high-concentrate diet to promote high rates of body weight gain exhibited precocious puberty. An interesting outcome from those studies is that the nutritional regimen that advanced puberty was applied for only 70 days between 4 and 7 mo of age. Puberty was not advanced at the same rate in heifers fed high-concentrate diets later during juvenile development. This indicates that the nutritional programming of puberty occur early during calfhood. Therefore, it is reasonable to propose that timing of the onset of puberty involves regulation of hypothalamic neurocircuitries that control GnRH release.

Important hypothalamic neural pathways involved in the control of feed intake are established during the early developmental period (Ahima et al., 1998). Nutritional status during pre- and/or early postnatal life can program development of axonal projections of neurons associated with the control of energy balance (Remmers and Delemarre-van de Waal, 2011). Therefore, it is generally accepted that nutritional programming involves the establishment of hypothalamic circuitries that control metabolic functions during the adult life (Coupe et al., 2010; Bouret, 2012). Interestingly, functional changes in areas of the brain that control energy balance include modifications of NPY neurons (Bouret et al., 2004).

Leptin may have an integral role in programming hypothalamic pathways controlling neuroendocrine functions. Leptin induces neurite outgrowth in the hypothalamus (Bouret et al., 2004), influences the production of glial proteins that are involved in synapse formation (García-Cáceres et al., 2011), and regulates the establishment of NPY neuronal projections to various hypothalamic areas, as well as synapse formation between NPY and efferent neurons (Bouret et al., 2004; Pinto et al., 2004). Adult *ob/ob* mice have reduced NPY fiber density in the PVN (Bouret et al., 2004), indicating that leptin deficiency during development impairs development of normal neuronal projections to this hypothalamic nucleus. It has been observed that a surge of leptin release occurs during the perinatal period in laboratory rodents (Ahima et al., 1998) and sheep (Long et al., 2011). This elevation in leptin appears to be critical for the establishment of hypothalamic neuronal projections (Ahima et al., 1998; Elmquist et al., 1998a; Delahaye et al., 2008). Maternal nutrient restriction during pregnancy and lactation delays the leptin surge in neonates, and this nutritional insult has been associated with altered expression of hypothalamic genes related to cell cycle proliferation, neuronal development, cytoskeleton organization, axon elongation and synaptogenesis (Coupe et al., 2010). Hence, leptin has a direct influence on the development of hypothalamic circuitries, including those involving NPY neurons. Therefore, leptin is proposed to play an important role in nutritional programming of reproduction as well.

Although the development of hypothalamic projections that control energy metabolism occurs to a large extent during the perinatal and neonatal periods in rodents

(Bouret, 2012), there is evidence that these circuitries remain plastic during adulthood (Pinto et al., 2004; Kokoeva et al., 2005). A study conducted using leptin-deficient, *ob/ob* adult mice indicates that NPY neurons receive more excitatory and less inhibitory inputs when compared to wild type animals (Pinto et al., 2004). In that study, leptin treatment in leptin-deficient mice normalized the patterns of inhibitory and excitatory inputs onto NPY neurons, indicating that the neurotropic action of leptin in the hypothalamus can also occur during adulthood. Interestingly, we have previously observed that genes related to the control of axonal growth and neuronal remodeling were also differentially expressed in the ARC of six-month-old heifers fed to gain body weight at a high rate during the prepubertal period (Allen et al., 2012). Therefore, plasticity within the ARC neurocircuitry may occur in response to dietary regimens offered to heifers during the juvenile period. Because projections of NPY neurons are influenced by metabolic status during hypothalamic development (Pinto et al., 2004), and NPY controls GnRH secretion (Kalra and Crowley, 1984b; Gazal et al., 1998) and the onset of puberty (Pierroz et al., 1995), plasticity within the NPY system may play a role in facilitating early onset of puberty in females.

The programming of reproductive function by nutritional status during early development may also involve kisspeptin neurons. Results reported recently support this hypothesis. Castellano et al. (2011) manipulated litter size of rat dams to control rate of body weight gain of pups during the suckling period. In this study it was observed that placing pups in small litters increased body weight during the suckling period and resulted in earlier onset of puberty, increased circulating leptin and increased *KISS1*

expression in the AVPV (Castellano et al., 2011). In contrast, limited growth induced by placing rat pups in large litters resulted in delayed puberty and reduction in *KISS1* expression in the ARC. In another study (Caron et al., 2012), undernutrition resulted in delayed puberty, attenuation of LH increases in response to ovariectomy and disrupted development of axonal projections from the ARC to the AVPV. These axonal projections may include afferent fibers from kisspeptin and NKB neurons because the density of kisspeptin and NKB fibers in the middle POA was reduced. Interestingly, these alterations persisted during adulthood. It was also observed that the fertility index (determined by the number of litters per month) was reduced in the adult females underfed during early life (Caron et al., 2012). Similarly to undernutrition, overnutrition during pre- and postnatal periods may also influence the establishments of neuronal projections later in life. Although overnutrition associated with placing rat pups in small litters did not alter age of puberty, neuronal projections within the ARC were altered. These projections included NKB axons, but not axonal projections containing kisspeptin.

Changes in nutritional status and growth rate after weaning also alter reproductive function and involve the kisspeptin neuronal system. A high fat diet fed to female rats after weaning resulted in increased circulating leptin and estradiol, early onset of peripubertal increases in LH pulse frequency and greater expression of *KISS1* in the POA and ARC (Feng Li et al., 2012). In this study, it was also observed that the expression of *TAC3* in the ARC was elevated in females receiving high-fat diet. Altogether, these observations indicate that kisspeptin neurons, including those that co-express NKB, are sensitive to metabolic status during juvenile period. Therefore,

alterations in *KISS1* gene expression and kisspeptin neuronal projections in response to decreased or increase rate of weight gain early in life may affect the onset of puberty and lifetime fertility in females.

Role of epigenetics in hypothalamic programming of reproduction

One mechanism by which environmental inputs lead to persistent alterations in gene expression is through epigenetic modifications (Jirtle and Skinner, 2007). Epigenetics involves regulation of cellular activity and gene expression that is not dependent on DNA sequence. Such alterations can be long lasting and potentially heritable. Epigenetic traits include methylation of cytosine residues in cytosine-guanine dinucleotides (CpGs of DNA) and covalent modifications to histone proteins (Goldberg et al., 2007). Modifications in the chromatin structure interfere with the accessibility of cellular machinery to the DNA, resulting in changes in the transcriptional activity of the gene (Chen and Pikaard, 1997; Jones and Takai, 2001; Li, 2002). Indeed, epigenetic modifications in genes involved in the control of energy homeostasis have been reported and appear to have a role in programming neuroendocrine functions regulating feed intake and energy expenditure (Gallou-Kabani and Junien, 2005). The role of epigenetic modifications in programming time at puberty has been proposed, and evidence to support its relevance has recently been demonstrated (Ojeda et al., 2010; Tena-Sempere, 2012). It has been observed that inhibition of the activity of the enzymes that transfer methyl groups to cytosine residues (DNA methyl-transferases, or DNMTs) delays puberty in female rats (Coupe et al., 2010). This effect is suggested to be due to a

disruption in gene silencing mechanisms that would, otherwise, occur normally during pubertal development (Lomniczi et al., 2010). Interestingly, disruption of the postnatal leptin surge in neonatal rats due to maternal undernutrition during pregnancy has been associated with differential expression of DNMTs in the hypothalamus (Coupe et al., 2010).

Differential methylation of *POMC*, the gene encoding the anorexigenic peptide α-MSH, and glucocorticoid receptor was observed in rats (Plagemann et al., 2009) and sheep (Stevens et al., 2010; Begum et al., 2012) exposed to undernutrition during perinatal life. Expression of these genes was observed to be differently regulated by nutrition in heifers that were fed to gain body weight at a higher rate during juvenile period (Allen et al., 2012). Because both α-MSH (Backholer et al., 2010a; Israel et al., 2012; Roa and Herbison, 2012) and glucocorticoids (Tilbrook et al., 2000) are known to influence reproductive neuroendocrine function, alterations in the methylation profile of these genes may alter GnRH neuronal activity and control pubertal progression in heifers. Therefore, alteration in methylation of key genes involved in the process of pubertal development may be involved in the mechanisms by which nutrition programs the timing of pubertal onset.

CHAPTER III

ELEVATED BODY WEIGHT GAIN DURING THE JUVENILE PERIOD ALTERS NEUROPEPTIDE Y NEURONAL CIRCUITRY IN THE HYPOTHALAMUS OF PREPUBERTAL HEIFERS

Overview

Increased body weight gain during the juvenile period leads to early maturation of the reproductive neuroendocrine system. We hypothesized that nutritional regimens that accelerate the onset of puberty lead to functional alterations in the hypothalamic neuropeptide Y (NPY) circuitry that are permissive for enhanced GnRH secretion during the peripubertal period. Crossbred heifers were weaned at approximately 4 mo of age and fed diets to promote average daily gains of 0.5 (Low Gain, n=6/replicate) or 1.0 Kg (High Gain, n=6/replicate). At eight mo of age, heifers were slaughtered and a block of tissue containing the preoptic area (POA) and hypothalamus was collected and processed for analysis of *NPY* mRNA (replicate 1) and for double immunofluorescence detection of NPY and GnRH (replicate 2). Mean body weight and circulating concentrations of leptin were greater in High- than in Low-gain heifers. Expression of *NPY* in the arcuate nucleus (ARC) was reduced in High-gain heifers as determined by a decreased number of *NPY* mRNA-containing cells in the middle ARC and decreased *NPY* expression per cell throughout the ARC. Although the number of immunoreactive GnRH neurons did not differ between groups, the proportion of GnRH neurons in apposition to NPY-

containing varicosities was reduced in High-gain heifers. Interestingly, the number of NPY close contacts to GnRH cell bodies and proximal dendrites was reduced in the population of GnRH neurons in the mediobasal hypothalamus (MBH), but not in the POA or anterior hypothalamus (AHA) of High-gain heifers. Similarly, the proportion of neurons highly innervated by NPY fibers (10 or more close-appositions/cell body and proximal dendrites) was reduced by 50% in MBH GnRH neurons of High-gain heifers. Results indicate that functional alterations in the hypothalamic NPY circuitry occur in response to elevated body weight gain during the juvenile period and include a reduction of NPY innervation of GnRH neurons in the MBH. These alterations may facilitate early onset of puberty.

Introduction

Maturation of the reproductive neuroendocrine axis includes the initiation of a high-frequency pattern of episodic release of gonadotropin-releasing hormone (GnRH) (Foster et al., 1985; Sisk and Foster, 2004). The episodic pattern of GnRH secretion is regulated mainly by inputs from afferent neurons, although signals from glial and endothelial cells contribute to GnRH exocytosis from neuronal terminals located at the median eminence (Terasawa and Fernandez, 2001; Prevot et al., 2007; Ojeda et al., 2008). Nutrition has a major impact on timing the onset of puberty in mammalian species by controlling the onset of high-frequency episodic GnRH and luteinizing hormone (LH) release (I'Anson et al., 2000).

It has been documented that elevated growth and adiposity during the juvenile period advances the onset of puberty in humans (Lee et al., 2007; Rosenfield et al., 2009), rats (Castellano et al., 2011) and heifers (Garcia et al., 2003; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d). In heifers, an early increase in the frequency of episodic release of LH and precocious puberty, defined as puberty before 10 mo of age, are induced by a high-energy diet consumed during the period between 4 and 7 mo of age (Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d). Therefore, the early juvenile period appears to be critical for nutrition to sensitize the neuroendocrine reproductive system and facilitate the early onset of puberty.

Metabolic cues, such as the adipokine leptin (Cheung et al., 1997), are perceived by the central nervous system, particularly in cells located in the hypothalamic arcuate nucleus (ARC; Elmquist et al., 1998b; Hakansson et al., 1998; Wang et al., 2004). Neuropeptide Y neurons located in the ARC contain the leptin receptor, (Hakansson et al., 1998; Williams et al., 1999) and are part of the metabolic sensing neuronal network that mediate the effects of the nutrition on the central control of reproduction (Li et al., 1994; Kalra and Kalra, 1996; Li et al., 1999; Crown et al., 2007). The expression of the *NPY* gene is readily responsive to changes in nutritional status (Brady et al., 1990; McShane et al., 1993; Allen et al., 2012) and undernutrition increases *NPY* expression (Brady et al., 1990; McShane et al., 1993). Increased NPY inhibition is believed to mediate the negative effects of undernutrition on reproductive cyclicity in females (Kalra and Kalra, 1996). Central administration of NPY delays the onset of puberty in rats

(Pierroz et al., 1995), while treatment with a NPY antagonist advances pubertal progression in rats (Pralong et al., 2000) and monkeys (El Majdoubi et al., 2000). In cows, cerebroventricular administration of NPY inhibits the episodic release of GnRH and LH (Gazal et al., 1998; Thomas et al., 1999) in the presence and absence of estradiol. Because NPY neurons project to GnRH cell bodies and nerve terminals (Li et al., 1999) and NPY inhibits GnRH neuronal activity (Klenke et al., 2010), the inhibition of GnRH release by NPY is proposed to involve transsynaptic communication.

In the studies reported herein, we investigated the involvement of NPY neurocircuitry on the nutritional acceleration of puberty in heifers. We used a well-established animal model in which puberty is advanced in early-weaned heifers fed high-energy diets during the juvenile period (Garcia et al., 2003; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d). Using this model, we tested the hypothesis that *NPY* expression, and NPY projections within the hypothalamus and to GnRH neurons, are differentially regulated in juvenile heifers growing at an accelerated rate.

Material and methods

Animals and nutritional regimens

All animal-related procedures used in this study were approved by the Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System. Twenty-four spring-born crossbred (¾ *Bos taurus*, ¼ *Bos indicus*) beef heifers were used

in two replicates. Heifers were weaned at approximately 3.5 mo of age, stratified by date of birth and assigned randomly to either High Gain (n=6/replicate) or Low Gain (n=6/replicate) groups. Heifer calves were allocated to pens (n=6/pen) equipped with the Calan gate feeding system and fed individually. Heifers were acclimated to experimental conditions for 3 to 4 weeks before the initiation of the dietary treatment. A total mixed diet was offered once a day and feed intake was controlled to promote an average daily gain (ADG) of 1 Kg in High-gain heifers or 0.5 Kg in Low-gain heifers. Individual feed intake was monitored daily, and the amount of diet offered to each heifer was adjusted biweekly according to individual requirements to achieve the target body weight gain. The Large Ruminant Nutrition System, which is based on the Cornell Net Carbohydrate and Protein System (Fox et al., 2004), was used to formulate diets. In order to accommodate changes in the nutrient requirements of heifers throughout the experiment, two diets were formulated. Diet 1 was used for the first 8 weeks, and Diet 2 was used for the remaining of the experiment. To minimize the potential impact of changes in diet formulation, the transition from Diet 1 to 2 was made by gradually increasing the proportion of Diet 2 in the total diet offered for a period of 4 days. Ingredients and nutritional compositions of diets are presented in **Table 3.1**.

Table 3.1 | Composition and nutrient concentration of diets

	Diet 1	Diet 2
Diet Composition (%DM)		
Alfalfa hay	22.4	17.9
Cottonseed hulls	6.7	10.7
Corn, rolled	40.4	53.7
Cane molasses	4.5	3.6
Corttonseed meal	6.7	7.1
Corn gluten feed	17.9	5.3
Urea	0.0	0.6
Mineral mix (Producers 12:12)	0.5	0.4
Calcium carbonate	0.9	0.7
Diet Concentrations		
Crude protein (%DM)	15.0	14.8
Metabolizable energy (Mcal/Kg DM)	2.5	2.6

Diet 1 was used was used for the first 8 weeks, and Diet 2 was used for the remaining of the experiment.

Body weight and blood samples (10 ml) were obtained every other week from each heifer before the morning feeding for the duration of the experimental period. During the last four weeks of the experiment, blood samples were obtained from heifers twice a week for analysis of circulating concentrations of progesterone. At the completion of the experimental dietary period (between 8 and 8.5 mo of age), heifers were slaughtered by humane procedures after an overnight fasting. A blood sample was obtained at slaughter. The brain was removed from the cranium and a block of tissue containing the septum, the preoptic area (POA), and hypothalamus was dissected and

processed for histological procedures. Ovaries were examined postmortem for the presence of luteal tissue.

Hormone assays

Solid phase RIA (Coat-A-Count Progesterone Kit; Siemens, Los Angeles, CA) was used to determine concentrations of progesterone in serum, as described previously (Fajersson et al., 1999). Sensitivity of the assay was 0.1 ng/ml, intra-assay and inter-assay CV averaged 3.5% and 9.2%, respectively. Circulating concentrations of leptin were determined in serum by RIA as described previously (Amstalden et al., 2000;Delavaud et al., 2000). Minimum detectable concentration was 0.1 ng/ml, intra-assay CV averaged 9.5% and inter-assay CV averaged 10%.

Experiment 1a: Expression of NPY in the arcuate nucleus

Tissue collected from heifers in Replicate 1 (n=6/dietary group) was used for this experiment. After slaughter, the blocks of tissue dissected from the brains were frozen in dry ice and stored at -80° C until further processing. Blocks were cut in series of 20-μm coronal sections using a cryostat. Sections were thaw-mounted onto SuperFrost Plus slides and stored at -80° C. One series of sections were used for detection of *NPY* mRNA by isotopic *in situ* hybridization (ISH).

Complementary RNA radiolabeled probe for detection of *NPY* mRNA was synthesized by *in vitro* transcription of a DNA template containing a partial sequence of the ovine NPY cDNA. Originally, the pKS Bluescript plasmid containing a partial ovine

NPY cDNA was provided graciously by Dr. Gilles Bruneau, INRA, France. A DNA sequence was produced by PCR from the linearized plasmid. The standard reaction contained 60 ng of linearized plasmid, 1.25 U of DNA polymerase (Ex Taq, Fisher Scientific, Fair Lawn, NJ), 5 μl of 10x transcription buffer (ExTaq Buffer, Fisher Scientific, Fair Lawn, NJ), 10 μM dNTP and 20 pmol of forward and reverse primers. Primers used in this reaction were: 5'-GCCAGCACGATGCTGGGTA-3'and 5'-GAGAGCAAGTCTCATTTCCCATCAC-3'. The PCR was carried out in 30 cycles of 94°C for 30 sec, 50°C for 1 min and 70°C for 1 min. A second PCR was performed using the product of the first reaction (5 μl of 1:10 dilution) as template and primers tagged with the T3 and T7 RNA polymerase promoter sequences. Primers used in the second reaction were: 5'-**AATTAACCCTCACTAAAGGG**GCCAGCACGATGCTGGGTA-3' (T3 promoter in bold) and 5'**TAATACGACTCACTATAGGG**AGAGAGCAAGT CTCATTTCCCATCAC-3' (T7 promoter in bold). The product obtained was sequenced and the sequence obtained was aligned to the available *Bos taurus* genome database using the BLAST tool. The partial ovine cDNA obtained is 98% identical to the bovine *NPY* gene. This DNA sequence was used subsequently as template for *in vitro* transcription.

Sense and antisense radiolabeled cRNA probes were synthesized by *in vitro* transcription using the DNA template describe above and procedures described previously (Redmond et al., 2011a). Before processing for ISH, sections were dried at 55°C for 10 min and fixed in fresh 4% paraformaldehyde for 15 min at room temperature. Additional processes were performed similarly as described previously

(Redmond et al., 2011a) except that slides were incubated in pre-hybridization solution (50% deionized formamide in 4X sodium chloride-sodium citrate) for one hour before application of hybridization solution containing the radiolabeled probe. After completion of ISH, sections were air dried, dipped in photographic emulsion (NBT – Kodak Inc., Rochester, NY), and exposed in the dark at $4^{o}C$ for 28 days. After developing, sections were counterstained with cresyl violet, dehydrated, and covered with a glass coverslip using DPX. Controls included incubation of sections with sense probe.

The number of *NPY*-expressing cells in the ARC and the *NPY* expression per cell, determined as the area covered by silver grains, were determined in sections observed under dark- and bright-field microscopy (Nikon eclipse 80i - Nikon Inc., Melville, NY) using procedures described previously (Redmond et al., 2011a). For determining expression per cell, images of cells selected randomly were captured using a 40X objective and the Nikon DS-Qi1 digital camera (Nikon Inc., Melville, NY). The NIS-Elements software (Nikon Inc., Melville, NY) was used for image analysis. Images of 10, 30 and 5 cells were analyzed for the rostral, mid and caudal subdivisions of the ARC, respectively. Anatomical subdivisions of the ARC were determined as described previously in sheep (Lehman et al., 1993; Redmond et al., 2011a).

Experiment 1b: Functional structure of the NPY-GnRH circuitry

For Experiment 1b, blocks of hypothalamic tissue collected after slaughter from heifers in Replicate 2 were sliced into approximately 3 to 5 mm coronal sections, placed

in embedding cassettes and incubated in 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde, for 48 h at 4°C. Paraformaldehyde solution was replaced after 24 h of incubation. Tissue was then incubated in PB containing 30% sucrose at 4° C for 7 to 10 days to allow for sucrose infiltration. Tissue blocks were cut at 50 µm coronal sections in series of four using a freezing microtome. Sections were placed in a cryopreservative solution (Watson Jr et al., 1986) and stored at -20° C until processed for immunohistochemistry.

One series of sections (200 µm apart) was processed for double immunofluorescent detection of NPY and GnRH, using procedures similar to those reported previously (Amstalden et al., 2010). Primary antibodies used included the rabbit antiserum against NPY (1:50,000; Sigma, St Louis, MO, Cat # N9528) and the mouse monoclonal antibody against GnRH (1:10,000, Covance, San Diego, CA, Cat # SMI-41R). Detection of NPY immunoreactivity was performed using biotinylated goat anti-rabbit (1:400; Vector Laboratories, Burlingame, CA, Cat # BA-1000) and Alexa 555-conjugated streptavidin (1:250, Invitrogen, Carlsbad, CA, Cat # S-21381). Detection of GnRH immunoreactivity was performed using Alexa 488-conjugated goat anti-mouse IgG (1:200; Invitrogen, Carlsbad, CA, Cat # A-11001). Controls for the dual immunofluorescence procedure included the omission of each primary antibody and pre-absorption of anti-NPY with 0.8 µM of a synthetic sequence corresponding to the ovine NPY (Sigma, St Louis, MO, Cat # N6269). These procedures eliminated fluorescent signal for the corresponding antigen.

Tissue sections were observed using an epifluorescent microscope (Nikon Eclipse 80i - Nikon Inc., Melville, NY). To determine the number of GnRH neurons in the POA and anterior hypothalamus, the left side of each section was analyzed. Because the number of GnRH neurons located in the mediobasal hypothalamus (MBH) is limited (Dees and McArthur, 1981; Leshin et al., 1988; Weesner et al., 1993), both the left and right sides of sections were used to determine the number of GnRH cells in the MBH. The number of NPY varicosities in close proximity to each identified GnRH neuron was determined by visualization of direct apposition between NPY-containing varicosities and GnRH-immunoreactive cell bodies and proximal dendrites observed in the same focal plane using a 40X objective. To determine the accuracy of this analysis, images of sections containing 10 representative GnRH neurons were captured using a Zeiss 510 Meta NLO laser-scanning confocal microscope (Zeiss, Heidelberg, Germany). Images were acquired in Z-stacks of 1-μm optical sections. The comparision of close aposition data obtained by visual counts using the epifluorescent microscope and by confocal image analysis resulted in 98.5% concordance (total of 68 and 67 NPY close-contacts in 10 GnRH neurons observed using the epifluorescent and confocal methods, respectively). Gonadotropin-releasing hormone neurons in close apposition to 10 or more varicosities were classified as highly innervated neurons and the maximum number of contacts recorded per cell was 10.

The density of NPY-containing fibers was analyzed in digital images captured with a 10X objective and the Nikon DS-Qi1 camera (Nikon Inc., Melville, NY) connected to the Nikon Eclipse 80i epifluorescent microscope. Procedures for

quantification of fiber density followed a method described previously (Leibowitz et al., 1998). Comparable areas within the POA, periventricular area (PeV), ventromedial (VMH), dorsomedial (DMH) and lateral hypothalamic (LHA) areas, paraventricular nucleus (PVN), ARC and median eminence (ME) were selected. The POA was subdivided into anterior, mid and posterior portions, corresponding to the regions anterior to, at the level of and posterior to the organum vasculosum of the lamina terminalis (OVLT). The subdivisions of the ARC were determined as described in experiment 1. Images of three sections were obtained for each area and analyzed using the NIS-Elements software (Nikon Inc.). A threshold signal was established and used to normalize all images within the anatomical division of interest before density analysis. A region of interest (ROI) of 200 μm in diameter was placed randomly over the image and the proportional area containing detectable signal was determined. A total of 3 ROI were used for each image of the ME and 6 ROI were used for each image of all other regions.

Statistical analyses

The Statistical Analysis System (SAS- SAS Inst. Inc., Cary, NC) software was used for analyses. Body weight and serum concentrations of leptin were analyzed using the PROC MIXED procedure of SAS. The main effects of treatment, replicate, experimental week and appropriate interactions were tested using mixed models for repeated measures. Week was used as the repeated variable and heifer within dietary group was used as the subject.

For Experiment 1a, the mean number of cells containing *NPY* mRNA in the ARC and the area covered by silver grains (in pixels) per cell were compared between dietary treatments (High Gain and Low Gain) using the *t*-test procedure. For Experiment 1b, the proportion of GnRH neurons in close proximity to NPY-containing fibers, and the proportion of GnRH neurons highly-innervated by NPY fibers were normalized using the arcsine-square root transformation method. The normalized proportions, as well as the total number of GnRH cells, the mean number of NPY close-contacts per GnRH cell body and proximal dendrite, and the area covered by NPY fibers in the POA and various hypothalamic regions were compared between dietary groups using the *t*-test procedure. Each hypothalamic area investigated was analyzed independently.

Results

One heifer in the Low-gain group of Replicate 1 exhibited elevated concentrations of progesterone (1.7 ng/ml) and presence of luteal tissue in the ovary on the day of slaughter. Therefore, data obtained from this heifer were excluded from all analysis.

Mean body weight at the beginning of the study did not differ between groups (**Figure 3.1**). Body weight increased in both groups, but as expected, the ADG was greater ($p < 0.01$) in the High-gain (1.05 ± 0.02 Kg) than in the Low-gain (0.52 ± 0.02 Kg) group. Beginning at Week 6, average body weight was greater ($p < 0.01$) in the

High-gain group than in Low-gain group and remained greater until the end of the experiment (**Figure 3.1**). No effect of replicate was observed.

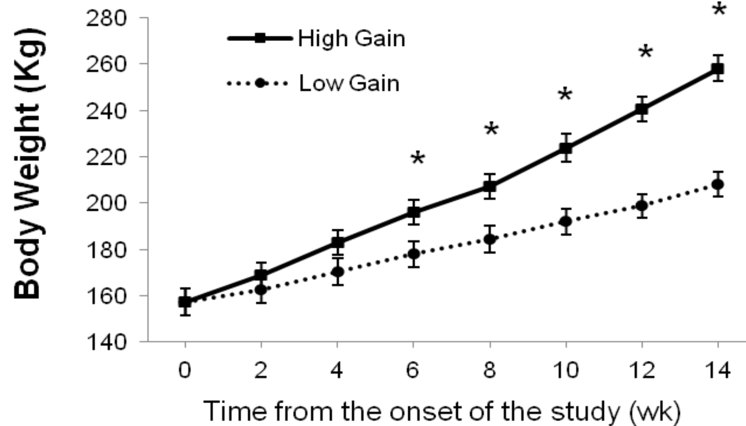


Figure 3.1 | Body weight of heifers fed for a target average daily gain of 1 Kg (High Gain) or 0.5 Kg (Low Gain). Dietary treatments started at approximately 4.5 mo of age. Mean body weight was greater (treatment X week; $p < 0.01$) in High-gain heifers compared to Low-gain heifers beginning at Week 6 from the onset of the study. Values are mean ± SEM. * $p < 0.05$ within week of the study.

Mean circulating concentrations of leptin did not differ between treatments at the beginning of the experiment (**Figure 3.2**). Similar to changes in body weight, mean concentrations of leptin were greater ($p < 0.01$) in the High Gain than in the Low-gain group beginning at Week 6 of the experiment. With the exception of Week 8, when no significant difference was detected, mean concentrations of leptin were greater throughout the remainder of the experiment (**Figure 3.2**).

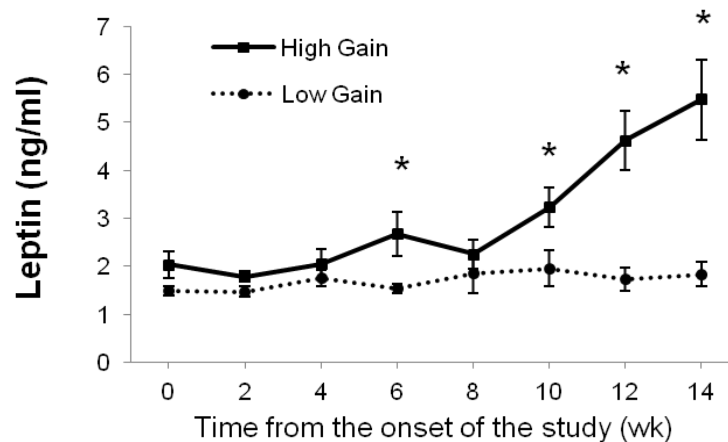


Figure 3.2 | Circulating concentrations of leptin in heifers fed for a target average daily gain of 1 Kg (High Gain) or 0.5 Kg (Low Gain). Dietary treatments started at approximately 4.5 mo of age. Circulating concentrations of leptin were greater (treatment X week; $p < 0.01$) in High-gain heifers than in Low-gain heifers. Values are mean ± SEM. * $p < 0.05$ within week of the study.

Experiment 1a: Expression of NPY in the arcuate nucleus

Cells expressing *NPY* were observed in the entire extent of the ARC, with most cells observed in the middle portion of the ARC (**Figure 3.3A-C**). The number of cells containing *NPY* mRNA in the rostral and caudal regions of the ARC did not differ between heifers gaining body weight at high or low rates (**Figure 3.3C**). However, the number of *NPY*-expressing cells in the mid ARC was approximately three times lower ($p< 0.01$) in the High-gain group than in the Low-gain group (**Figure 3.3C**). Although changes in the number of cells expressing *NPY* was not consistent throughout the various sub-regions of the ARC, the abundance of *NPY* mRNA per cell was consistently lower ($p < 0.05$) in High-gain heifers in all three sub-regions of the ARC examined (**Figure 3.3D**).

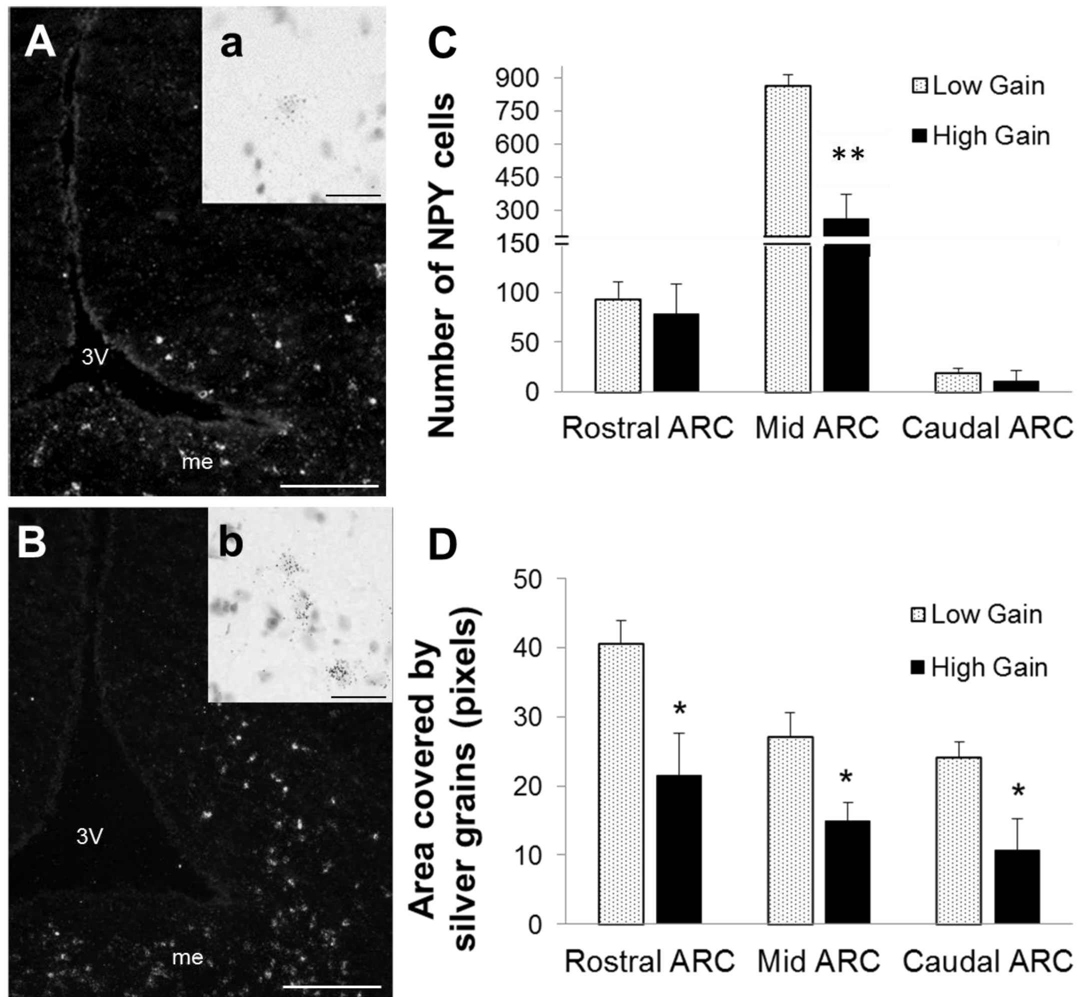


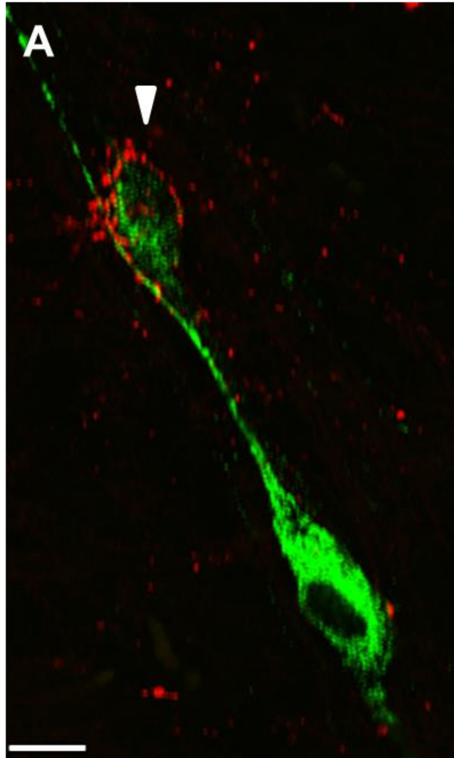
Figure 3.3 | Expression of *NPY* in the arcuate nucleus (ARC) of 8-mo-old heifers fed to gain body weight at high (1 Kg/d; High Gain) or low (0.5 Kg/d; Low Gain) rate, beginning at approximately 4.5 mo of age. Images of coronal sections processed for *in situ* hybridization detection of *NPY* mRNA depicting cells in the mid ARC of representative High-gain (**A**,**a**) and Low-gain (**B**,**b**) heifers. Low-magnification (**A**, **B**), dark-field images showing accumulation of silver grains (white clusters) in sections at the level of the mid ARC. Insets (**a**, **b**) represent high-magnification, bright-field images depicting silver grain accumulation in Cresyl violet-stained cells. (**C**) Number of *NPY*-expressing cells in the rostral, mid and caudal portions of the ARC. (**D**) Area covered by silver grains per cell in the rostral, mid and caudal portions of the ARC. The mean number of cells containing *NPY* mRNA was reduced in the mid ARC of High-gain heifers compared to Low-gain heifers, but it did not differ in the rostral and caudal ARC (**C**). However, the *NPY* expression per cell was reduced throughout all subregions of the ARC in High-gain heifers (**D**). Values are mean ± SEM. ** $p < 0.01$ and * $p < 0.05$ between High Gain and Low Gain. me, median eminence; 3V, third ventricle. Scale bar: A-B, 250µm; a-b, 25µm.

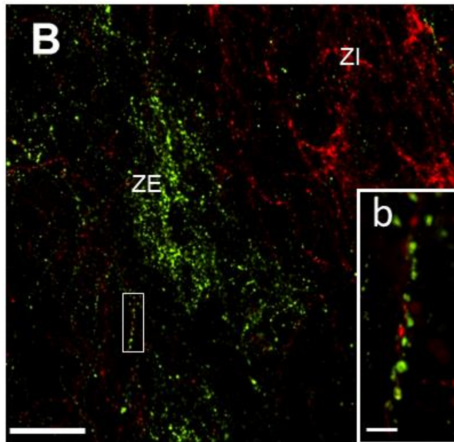
Experiment 1b: Functional structure of the NPY-GnRH circuitry

Immunoreactive GnRH neurons were observed scattered in a rostral to caudal continuum extending from the septum to the MBH. Numerous GnRH neurons were observed in the medial POA at the level of the OVLT, but a smaller proportion of GnRH neurons was observed to locate caudally from the anterior hypothalamic area (AHA). Because studies in the sheep have indicated that GnRH neurons located in the MBH are activated distinctly from GnRH neurons in rostral aspects of the hypothalamus during pulsatile LH release, we have examined the POA and AHA population separate from the MBH population. The average number of immunoreactive GnRH neurons detected in the POA/AHA population (167 ± 15) and in the MBH population (26 ± 6) did not differ between dietary groups.

Gonadotropin-releasing hormone and NPY immunoreactivity did not colocalize in any of the areas investigated in the current study. However, a considerable proportion of GnRH cell bodies and proximal dendrites located in the POA and hypothalamus were observed in close proximity to NPY-containing varicosities (**Figure 3.4A**). Likewise, GnRH fibers were observed to intermingle with NPY-containing fibers in the external zone of the median eminence (**Figure 3.4B, b**). Elevated body weight gain during the juvenile period reduced ($p < 0.05$) the proportion of GnRH neurons in close-proximity to NPY fibers in the POA/AHA and MBH populations (**Figure 3.4C**). The relative reduction in the proportion of GnRH neurons innervated by NPY in the High-gain group was more prominent in the MBH (approximately 25%) than in the POA/AHA (approximately 10%).

Figure 3.4 | NPY innervation of GnRH neurons in 8-mo-old heifers fed to gain body weight at high (1 Kg/d; High Gain) or low (0.5 Kg/d; Low Gain) rate beginning at approximately 4.5 mo of age.(A) Confocal microscope image of a section processed for double-label immunofluorescent detection of GnRH (green) and NPY (red) depicting NPY-containing varicosities in close apposition to two GnRH cell bodies and proximal dendrites. Note that the GnRH cell body on the top is surrounded by numerous NPY-containing varicosities and represents a highly-innervated neuron (arrow head). **(B)** Epifluorescent image of a section processed for detection of GnRH (green) and NPY (red) depicting GnRH- and NPY-containing fibers in the median eminence. The inset (b) depicts a high-magnification image of GnRH and NPY fibers intermingled in the external zone of the median eminence (boxed area in B). **(C-E)** Percentage of GnRH neurons observed in close apposition to NPY fibers **(C)**, number of NPY close contacts to GnRH cell bodies and proximal dendrites **(D)**, and percentage of GnRH neurons highly innervated (more than 10 close contacts) by NPY fibers **(E)** in the preoptic area and anterior hypothalamus (POA+AHA), and mediobasal hypothalamus (MBH). High rate of body weight gain during the juvenile period leads to an overall reduction of NPY innervation of GnRH neurons, particularly in the GnRH neuron population in the MBH. Values are mean ± SEM. * $p < 0.05$ between High Gain and Low Gain. ZI: internal zone of the median eminence; ZE: external zone of the median eminence. Scale bar: A, 25 µm; B, 100 µm; b, 10 µm.


A


B
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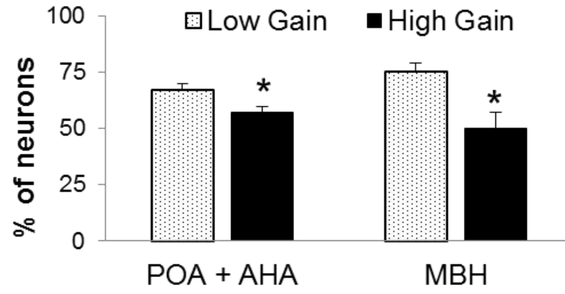
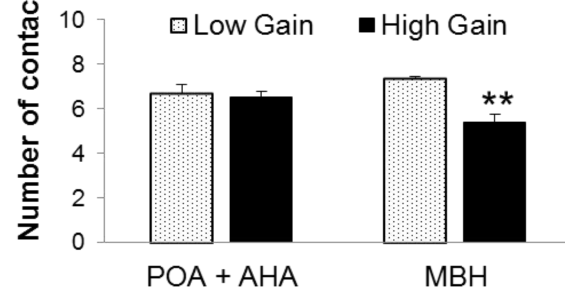
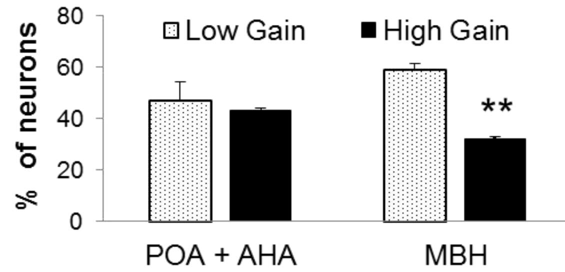
ZE
b


C
GnRH neurons in close-apposition to NPY fibers
Low Gain
High Gain
% of neurons
100
75
50
25
0
*
*
POA + AHA
MBH


D
NPY close-contacts per GnRH neuron
Low Gain
High Gain
Number of contacts
10
8
6
4
2
0
**
POA + AHA
MBH


E
Highly-innervated GnRH neurons
Low Gain
High Gain
% of neurons
80
60
40
20
0
**
POA + AHA
MBH

In addition to a reduction in the proportion of GnRH neurons innervated by NPY, a decrease ($p < 0.01$) in the mean number of NPY close contacts per GnRH cell body and proximal dendrite in the High-gain group was also observed. However, this alteration was observed only in the GnRH neuronal population located in the MBH (**Figure 3.4D**). Unique to GnRH neurons in the MBH was also an approximate 50% reduction ($p < 0.01$) in the percentage of neurons highly innervated by NPY fibers in High-gain heifers (**Figure 3.4E**).

Neuronal fibers containing NPY immunoreactivity were observed throughout the POA and hypothalamus. The proportional area covered by NPY signal did not differ between the Low Gain and High Gain groups in most hypothalamic areas investigated, except in the caudal portion of the ARC and in the DMH. In these areas, NPY fiber density was reduced by 37% in the caudal ARC and 40% in the DMH in High-gain heifers (**Table 3.2**).

Table 3.2 | Percentage of area covered by NPY fibers

	Low Gain	High Gain	p-value
Anterior POA	3.3 ± 0.4	3.7 ± 0.7	0.87
Medial POA	6.5 ± 0.8	7.2 ± 0.3	0.76
Posterior POA	7.1 ± 0.4	8.4 ± 0.3	0.33
VMH	4.9 ± 0.2	4.7 ± 0.2	0.75
DMH	4.7 ± 0.1	2.8 ± 0.2	0.01
LHA	1.6 ± 0.1	1.1 ± 0.1	0.06
PVN	7.8 ± 0.1	8.4 ± 0.6	0.74
ME	11.0 ± 0.7	10.9 ± 0.4	0.96
PeV	9.1 ± 0.5	7.3 ± 0.6	0.37
Rostral ARC	8.3 ± 0.6	6.1 ± 0.5	0.27
Mic ARC	7.5 ± 0.5	6.7 ± 0.1	0.55
Caudal ARC	9.6 ± 0.4	6.1 ± 0.4	0.03

Neuropeptide Y fiber immunoreactivity was assessed in 8-mo-old heifers fed to gain body weight at high (1 Kg/d; High Gain, n=6) or low (0.5 Kg/d; Low Gain, n=6) rate beginning at approximately 4.5 mo of age. Density of NPY fibers was reduced ($p < 0.05$) in the DMH and in the caudal ARC in High Gain heifers. Values are mean ± SEM. POA: preoptic area, VMH: ventromedial hypothalamus; LHA: lateral hypothalamus; PVN: paraventricular nucleus; ME: median eminence; PeV: periventricular region, ARC: arcuate nucleus.

Discussion

Nutritional status during the juvenile period has a critical role in timing the onset of puberty in females. Results of the studies reported herein support the hypothesis that alterations in NPY-GnRH neurocircuitry are involved in the process by which increased rates of body weight gain during the juvenile period accelerate the maturation of the

reproductive neuroendocrine system in heifers. These alterations include a reduction in *NPY* expression in the ARC and in NPY innervation of GnRH neurons. Most noticeable was a considerable decrease in NPY innervation of GnRH neurons located in the MBH. Such changes may contribute to the nutritional programming of pubertal development reported in earlier studies in cattle (Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d) and other species (Castellano et al., 2011; Caron et al., 2012).

As expected, the nutritional regimen used in the current study promoted successfully an average daily body weight gain that has been shown to advance puberty in cattle (Garcia et al., 2003; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d). Importantly, the rate of body weight gain achieved in the Low-gain group did not promote states of negative-energy balance. In fact, an ADG of 0.5 Kg is compatible with recommended growth rate for heifers to reach puberty by 15 mo of age (Fox et al., 1988; National Research Council, 1996). Thus, this experimental model permits the investigation of the effects of a high rate of body weight gain during the juvenile period, a strategy that advances pubertal onset in heifers, without compromising individual nutrient requirements for target growth.

The influence of nutrition on pubertal development has been well documented in several mammalian species (Kennedy and Mitra, 1963; Ferrell, 1982; Kirkwood et al., 1987; Schillo et al., 1992; Kaplowitz et al., 2001; Baptiste et al., 2005). Although the specific physiological mechanisms by which nutrition influences the timing of pubertal onset have not been fully elucidated, an increase in the frequency of release of GnRH

occurs during pubertal development (Foster and Jackson, 2006). This developmental increase in the frequency of GnRH release is proposed to occur as a result of alterations in inhibitory and excitatory inputs that control the synthesis and release of GnRH (Ojeda et al., 2010). Nutritional status may affect the onset of puberty by interfering with the balance of positive and negative inputs to GnRH neurons, and NPY appears to be a major player mediating these effects (Gazal et al., 1998; Klenke et al., 2010; Roa and Herbison, 2012). In cattle and sheep, NPY inhibits the release of GnRH/LH in the presence and absence of estradiol negative feedback (McShane et al., 1992a; Gazal et al., 1998; Thomas et al., 1999). Neuropeptide Y inhibition of GnRH neuronal activity occurs likely by direct transsynaptic mechanisms. Synapses between NPY axons and GnRH neurons have been demonstrated in sheep and mice (Norgren Jr and Lehman, 1989; Turi et al., 2003). Moreover, studies in laboratory rodents indicate that NPY receptors in GnRH neurons mediate the inhibitory effects (Li et al., 1999; Klenke et al., 2010; Roa and Herbison, 2012). Hence, the magnitude of NPY innervation of GnRH neurons reported herein provides further support for a direct inhibition of GnRH release by NPY. In this study, we observed a consistent reduction in NPY innervation of GnRH neurons throughout the POA and hypothalamus in High-gain heifers. However, the origin of the NPY projections innervating GnRH neurons is unclear. In a study using anterograde-label tracing of neuronal projections in sheep, only few GnRH neurons were observed in close proximity to fibers originating in the VMH/ARC (Pompolo et al., 2001). In that study analyses were limited to GnRH cell bodies localized in the diagonal band of Broca and POA. In another study (Goubillon et al., 2002), approximately 50% of GnRH

neurons distributed throughout the POA and hypothalamus were adjacent to neuronal fibers originating in the MBH. Moreover, approximately 50% of the NPY fibers in close proximity to GnRH cell bodies originate in the ARC (co-localize agouti-related protein) and approximately 25% originate in the brainstem (co-localize dopamine hydroxylase) in mice (Turi et al., 2003). This indicates that NPY projections from ARC neurons are major afferent inputs to GnRH neurons. Nevertheless, the origin of the NPY projections that target GnRH neurons, and are prone to be altered by rate of body weight gain during the juvenile period remains to be determined.

In the current study, changes in NPY innervation of GnRH neurons occurred more markedly in the GnRH cell bodies/proximal dendrites localized in the MBH. There is evidence that the MBH population of GnRH neurons may be functionally-distinct from those located in the POA and AHA. Studies have demonstrated that experimental conditions that stimulate the episodic release of LH in ewes and rams are associated with activation of GnRH neurons in the MBH, but not in the POA (Boukhliq et al., 1999). Moreover, during pubertal transition in monkeys, the number of synapses onto GnRH neurons decreased only in the GnRH neurons located in the MBH, but not in the POA (Perera and Plant, 1997). Those findings corroborate the proposal that the population of GnRH neurons located within the MBH is particularly relevant for changes that support pubertal maturation. Hence, the decrease in NPY innervation of GnRH neurons in the MBH observed in the current study indicates that elevated body weight gain during the juvenile period alters NPY inputs in a key population of GnRH neurons. Because increased pulsatility in the release of LH is a major neuroendocrine event driving

pubertal maturation, diminished NPY innervation of GnRH neurons in the MBH may represent a critical step in the mechanisms leading to the onset of puberty.

The observation of decreased *NPY* expression reported herein expands the observations of our earlier studies reporting decreased *NPY* mRNA in the ARC of heifers fed a high-concentrate diet and gaining weight at a high rate (Allen et al., 2012) by indicating that reduction occur largely in the mid portion of the ARC. Altered *NPY* expression in the mid ARC (but not in the rostral or caudal portions) has been reported in rats during lactation (a physiological state of negative-energy balance; Smith, 1993; Li et al., 1998). This indicates that neurons originating at this location of the ARC may have a more pronounced sensitivity to variations in energy balance. Furthermore, the reduction of *NPY* expression appears to be coupled with reduced release of NPY because diminished NPY content in the cerebrospinal fluid of the third ventricle was observed in high-gain heifers submitted to the same nutritional regimen used in the experiment reported herein (Cardoso et al., 2012).

Despite a considerable decrease in *NPY* expression in the ARC, diminished density of NPY fibers in High Gain heifers was limited to the DMH and caudal portion of the ARC. Neuropeptide Y fibers originating in non-ARC neurons contribute to the NPY immunoreactivity in the POA and hypothalamus and may mask potential changes occurring in NPY fibers emanating from the ARC. However, expression of *NPY* in the DMH (Li et al., 1998) and in brainstem neurons that innervate the hypothalamus (Li and Ritter, 2004) increases in states of metabolic deficiency. Alternatively, decreased release of NPY during physiological states of elevated nutritional status (McShane et al., 1992a;

Cardoso et al., 2012) may result in peptide accumulation in fibers. Therefore decreased release of NPY may contribute to the lack of clearly consistent differences in fiber density in the hypothalamus.

The remarkable decrease in the proportion of GnRH neurons highly innervated by NPY fibers selectively in the MBH, in the absence of changes in overall NPY fiber density in proximal areas, indicates that plasticity of NPY projections is specific to target neuronal populations. Plasticity in NPY innervation has been documented in response to metabolic challenges (Pinto et al., 2004; Horvath, 2005) and includes a reduction in synaptic inputs onto GnRH neurons during pubertal transition (Perera and Plant, 1997). In addition, in a study investigating the transcriptome of the ARC in juvenile heifers fed high concentrate diets to promote high rates of body weight gain, we observed changes in expression of several genes involved in synaptic plasticity (Allen et al., 2012). These included *SNCA* (alpha synuclein), *NCAN* (neurocan) and *CPNE6* (copine 6). Therefore, rate of body weight gain during the juvenile period in heifers appear to modulate the plasticity of NPY innervation of GnRH neurons, particularly GnRH neuron cell bodies located in the MBH.

The increase in circulating concentrations of leptin in response to higher rates of body weight gain in heifers is in accordance with previous observations in our laboratory (Allen et al., 2012). Concentrations of leptin in circulation are closely associated with body weight and metabolic status of developing heifers (Amstalden et al., 2000; Garcia et al., 2002) and increase as puberty approaches (Garcia et al., 2002; Garcia et al., 2003). Leptin is considered a permissive, but critical metabolic signal for reproductive

maturation (Cheung et al., 2001; Zieba et al., 2005; Roa et al., 2010; Elias, 2012). Leptin regulates neurite outgrowth in the hypothalamus (Bouret et al., 2004) and influences synapse formation (García-Cáceres et al., 2011). Establishment of NPY neuronal projections in various hypothalamic areas, as well as synapses between NPY and efferent neurons, is also modulated by leptin (Bouret et al., 2004; Pinto et al., 2004). Therefore, elevated concentrations of leptin in the circulation of heifers gaining body weight at high rates may play a role in the plasticity of NPY innervation of GnRH neurons. Leptin is also a potent inhibitor of *NPY* expression (Stephens et al., 1995; Schwartz et al., 1996a). Therefore, in addition to potentially programming NPY projections that target GnRH neurons, sustained elevated concentrations of leptin in circulation may have a primary role in the reduced *NPY* expression in the ARC observed in High-gain heifers.

In conclusion, increased rates of body weight gain during the juvenile period promote functional alterations in the NPY-GnRH neurocircuitry. These alterations are indicative of plastic changes leading to reduced NPY innervation of GnRH neurons and are associated with increased concentrations of leptin in circulation. Therefore, increased body weight gain during prepubertal development facilitates early onset of puberty by programming the neuroendocrine system through mechanisms that appear to include alleviation of NPY inhibition of GnRH release.

CHAPTER IV

INFLUENCE OF BODY WEIGHT GAIN DURING THE JUVENILE PERIOD ON KISSPEPTIN NEURONAL PATHWAY IN HEIFERS

Overview

Kisspeptin has been considered a gatekeeper for pubertal development. Because kisspeptin neurons are responsive to metabolic cues, activation of kisspeptin neuronal function may play a role in the nutritional programming of puberty. In this study we investigated the effect of rate of body weight gain during the juvenile period on the regulation of kisspeptin neurons. Crossbred heifers were fed diets to promote an average daily gain of 0.5 Kg (Low Gain, n=12) or 1.0 Kg (High Gain, n=12) from approximately 4 to 8 mo of age. This nutritional regimen has been demonstrated to elicit early onset of puberty in heifers. Expression of *KISS*, kisspeptin immunoreactivity and neuropeptide Y (NPY) innervation of kisspeptin neurons in the preoptic area (POA) and arcuate nucleus (ARC) were investigated. *KISS1* expression in the POA, rostral and caudal ARC, and number of kisspeptin cells in the rostral ARC were not affected by the nutritional treatment. In the mid portion of the ARC, the number of *KISS1*-expressing cells was reduced in High-gain heifers. In contrast, there was a tendency for increased number of kisspeptin-immunoreactive cells in the mid and caudal portions of the ARC in High-gain heifers. Close-contacts between NPY fibers and kisspeptin cell bodies and proximal dendrites were not affected by treatment in any area investigated. Therefore, although

nutrition during the juvenile period alters *KISS1* gene expression, this alteration is not supportive of a major role of kisspeptin neurons in mediating the nutritional programming of accelerated puberty in heifers.

Introduction

Pubertal maturation of the reproductive neuroendocrine axis is characterized by development of a sustained, high-frequency release of gonadotropin-releasing hormone (GnRH) and, consequently, luteinizing hormone (LH) (Sisk and Foster, 2004). Although the mechanisms that trigger the increased frequency of release of GnRH during the peripubertal period are unclear, it is believed that alterations in inhibitory and excitatory inputs to GnRH neurons are involved. These changes favor greater GnRH neuronal secretory activity (Ojeda and Skinner, 2006). The neuropeptide kisspeptin is a potent stimulator of GnRH release (Messager et al., 2005), and this effect occurs likely by direct actions of kisspeptin on GnRH neurons (Han et al., 2005b; Yeo and Herbison, 2011). Because inactivating mutations in the genes encoding kisspeptin, *KISS1* (de Tassigny et al., 2007; Silveira et al., 2010), and kisspeptin receptor, *KISS1R* (de Roux et al., 2003; Seminara et al., 2003), lead to impaired puberty, kisspeptin has been considered an important gatekeeper of reproductive maturation in mammals (Kauffman, 2010). Kisspeptin is produced in two functionally distinct neuronal populations located in the preoptic (POA)/periventricular (PeV) areas, and in the arcuate nucleus (ARC) (Smith et al., 2005a; Franceschini et al., 2006). In sheep, increased *KISS1* expression in

the ARC has been associated with increased frequency of pulsatile LH release in maturing ewe lambs (Redmond et al., 2011a). Furthermore, increased expression of *KISS1*, as well as kisspeptin immunoreactivity, have been reported during pubertal development in rodents (Navarro et al., 2004a; Clarkson and Herbison, 2006; Clarkson et al., 2009; Cao and Patisaul, 2011) and sheep (Redmond et al., 2011a; Nestor et al., 2012). Such changes are indicative of enhanced kisspeptin-induced stimulation of GnRH neurons. Therefore, activation of kisspeptin neuronal function may be critical for the onset of puberty.

In heifers, high-concentrate diets fed during the early calfhood (4 to 7 mo of age) advances the peripubertal increase in the frequency of pulsatile release of LH (Gasser et al., 2006d) and onset of puberty (Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d). A similar nutritional regimen has been demonstrated to promote changes in the transcriptional profile of the ARC of juvenile heifers that are conducive to earlier pubertal onset (Allen et al., 2012). Those modifications include decreased expression of *NPY*, which encodes the neurotransmitter, neuropeptide Y (NPY) which inhibitory effects on the release of GnRH in cattle (Gazal et al., 1998). Neuropeptide Y neurons in the ARC are major metabolic-sensing cells in the central nervous system and are considered to mediate the inhibitory effects of undernutrition on the reproductive axis (Kalra and Kalra, 1996; Crown et al., 2007). Hence, alleviation of NPY input to hypothalamic neurocircuitries might be a mechanism by wich elevated body weight gain during the juvenile period induces early onset of puberty. The NPY inhibition of GnRH release occurs likely by direct effects on GnRH neurons (Li et al.,

1999; Klenke et al., 2010). However, because NPY neurons have been observed to project to kisspeptin neurons in sheep (Backholer et al., 2010b; Amstalden et al., 2011), NPY may inhibit GnRH secretion also indirectly via inhibition of kisspeptin neuronal function. This dual mode of action would reinforce maintenance of low GnRH secretory activity in states of insufficient nutrient reserves. In the studies reported herein, we hypothesized that increased expression of *KISS1* gene and kisspeptin immunoractivity in the POA and ARC, as well as decreased NPY innervation of kisspeptin neurons, occur in response to nutritional regimens demonstrated previously to advance the onset of puberty in heifers (Garcia et al., 2003; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d).

Material and methods

Animals and experimental procedures

All animal-related procedures used in this study were approved by the Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System. Tissue blocks containing the POA and hypothalamus obtained from heifers used for the experiments described in Chapter III (please, see Animals and experimental procedures in Chapter III) were used for this experiment. Twenty-four crossbred (¾ *Bos taurus*, ¼ *Bos indicus*) heifers were used for the experiment in two replicates (n=12/replicate). Heifers were weaned at approximately 3.5 mo of age, stratified by age, assigned randomly to one of two dietary treatment groups: High Gain

(n=6/replicate) and Low Gain (n=6/replicate). Heifers were fed individually, and under controlled intake, a total mixed diet to promote a daily body weight gain of 1 Kg (High Gain) or 0.5 Kg (Low Gain). Diets were formulated using the Large Ruminant Nutrition System software. Diet 1 contained 15% CP and 2.52 Mcal/Kg, and was offered from Week 1 to 8 of the experiment. Diet 2 contained 14.8% CP and 2.62 Mcal/Kg, and was offered from Week 9 until the end of the experimental period at Week 15 (Replicate 1) or 16 (Replicate 2; please refer to Table 3.1 on Chapter III for composition of diets 1 and 2). The amount of diet offered to each heifer was adjusted as needed to achieve the target body weight gain. As reported in the previous chapter, heifers in the High-gain group exhibited greater average daily gain throughout the experiment and were heavier than Low-gain heifers beginning at Week 6 until slaughter.

To confirm pubertal status, concentrations of circulating progesterone were determined in blood samples obtained twice weekly during the last 4 weeks of the experiment. One heifer in the Low-gain group in Replicate 1 exhibited elevated concentrations of progesterone on the day of slaughter and evidence of luteal tissue upon examination of the ovaries. Therefore, data from this heifer were removed from all analyses. Heifers were humanely slaughtered at approximately 8 to 8.5 mo of age, and brain tissue containing the septum, POA, and hypothalamus was dissected and processed for determination of *KISS1* expression (Replicate 1) or for detection of kisspeptin immunoreactivity (Replicate 2). The infundibular region of the hypothalamus of one heifer in Replicate 2 was damaged during tissue collection and processing. Thus, tissue from this heifer was not used for analysis.

Experiment 1: KISS1 expression in the POA and ARC of juvenile heifers

Brain tissue obtained from heifers in the High Gain (n=6) and Low Gain (n=5) groups in Replicate 1 were used for this experiment. Blocks of tissue containing the POA and hypothalamus were frozen and cut in series of 20-μm coronal sections. Sections were thaw-mounted onto SuperFrost Plus slides and stored at -80° C until processing. Sections that included the POA, AHA and ARC were selected and used for detection of *KISS1* mRNA by isotopic *in situ* hybridization (ISH).

A DNA template was generated by PCR amplification of a linearized plasmid containing the ovine *KISS1* cDNA graciously provided by Dr. Clay Lents (ARS-USDA, Clay Center, NE; GenBank accession #DQ059506). The PCR primers were extended at the 5' end with T3 (sense) and T7 (antisense) RNA polymerase promoter sequences. Primers used in this reaction were: 5'-**AATTAACCCTCACTAAAGGG**G AAGCCCACAGCGGCCGG- 3' (T3 promoter in bold) and 5'-**TAATACGA CTCACTATAGGG**TCCTTTATTGCTTCGGGACA- 3' (T7 promoter in bold). The product obtained was sequenced and the sequence obtained was aligned to the *Bos taurus* genome database using the BLAST tool. The 316 bp DNA obtained is 90% homologous to the bovine mRNA sequence, and spanned bases 43-336 of the bovine *KISS1* gene. Sense and antisense radiolabeled cRNA probes were synthesized by *in vitro* transcription using procedures as described in Chapter III. Slides were exposed to photographic emulsion in the dark at 4°C for 28 days. Negative controls included hybridization of sections with radiolabeled sense probe.

Slides were analyzed using a bright- and dark-field microscope (Nikon eclipse 80i - Nikon Inc., Melville, NY, USA). *KISS1*-expressing cells were identified by accumulation of silver grains 5x above the background. The numbers of *KISS1*-expressing cells were determined in comparable sections that included the POA/PeV (n=5) and the rostral (n=3), mid (n=5) and caudal (n=3) ARC. The density of silver grains per cell was accessed only in the mid portion of the ARC by analysis of images captured from 15 cells selected randomly in at least three different sections. Density of silver grains per cell in the rostral and caudal ARC and in the POA/PeV was not determined because the number of *KISS1*-expressing cells detected in these areas was limited for valid, meaningful comparisons.

Experiment 2: Kisspeptin-immunoreactive cells in the POA and ARC and their NPY afferent projections in juvenile heifers

Brain tissue collected after slaughter from heifers in Replicate 2 (High Gain, n=5 and Low Gain, n=6) were used for this experiment. Briefly, blocks of tissue containing the POA and hypothalamus were dissected, fixed in 4% paraformaldehyde and cut in 50-µm coronal sections. A series of sections 200-µm apart was further cut sagittally in two halves. One series containing coronal halves was processed for detection of kisspeptin and NPY by double-label immunofluorescence. Procedures were carried out on free-floating sections at room temperature under gentle agitation following procedures similar to those described previously (Amstalden et al., 2010). To enhance detection of immunoreactive signals, sections were submitted to antigen retrieval procedure

following the principles of Shi et al. (Shi et al., 1997). Briefly, sections were incubated in 10mM Sodium Citrate solution (pH 6) in a water bath at 90°C for 18 min. Subsequently, sections were allowed to cool to room temperature for 20 min before detection of kisspeptin and NPY. Kisspeptin was detected using a rabbit anti-kisspeptin10 serum (1:250 000; AC#564, graciously provided by Dr. Alain Caraty), followed by biotinylated goat anti-rabbit IgG (1:400; Vector Laboratories, Burlingame, CA) and streptavidin-conjugated horseradish peroxidase (Vectastain® Elite ABC; Laboratories, Burlingame, CA). Tyramide amplification (Hunyady et al., 1996) was used to enhance the signal associated with kisspeptin immunoreactivity and minimize cross reactivity with the secondary antibody. Biotinyl-tyramide (1:400, Perkin Elmer, Boston, MA) amplification followed procedures reported previously (Amstalden et al., 2010). Alexa 555 conjugated-streptavidin (1:250 in PBS, Invitrogen, Carlsbad, CA; Cat. # S-21381) was used to detect kisspeptin immunoreactivity. Detection of NPY was performed using the rabbit anti-NPY antibody (1:50 000; Sigma, St Louis, MO, Cat # N9528) followed by incubation with Alexa 488 conjugated goat anti-rabbit IgG (1:200; Invitrogen, Carlsbad, CA; Cat. # 21235).

Controls for the double-label procedure included omission of first primary antibody (rabbit anti-kisspeptin10), pre-absorption of the first primary antibody with 3µM of ovine kisspeptin decapeptide obtained by custom synthesis (American Peptide Company Inc., Sunnyvale CA, USA), and omission of the amplification procedure. In addition, omission of the second primary antibody (rabbit anti-NPY) and pre-absorption of the second primary antibody solution with 0.8 µM of a peptide corresponding to the

ovine NPY (Sigma, St Louis, MO, Cat. # N6269) were also performed. These procedures eliminated the fluorescent signal for each corresponding antigen.

Sections were observed using an epifluorescent microscope (Nikon Eclipse 80i - Nikon Inc., Melville, NY, USA) for localization of kisspeptin and NPY immunoreactivity. The number of kisspeptin-immunoreactive cell bodies and the proportion of kisspeptin neurons in close proximity to NPY-containing varicosity were determined. Close contacts were defined as direct apposition between NPY-containing varicosities and kisspeptin-immunoreactive soma and proximal dendrites observed in the same focal plane using a 40X objective. In order to access the number of kisspeptin-immunoreactive cells in the ARC, three comparable sections of each subdivision of this area (rostral, mid and caudal ARC) were selected from each animal, and the total number of kisspeptin cells within each subregion was obtained.

The density of kisspeptin-containing fibers in the median eminence was determined in digital images captured with a 10X objective using a Nikon DS-Qi1 camera coupled to the Nikon Eclipse 80i epifluorescence microscope (Nikon Inc., Melville, NY, USA). The procedure for determining the density of kisspeptin fibers was similar to that described in Chapter III. For each heifer, images were obtained from three sections containing the median eminence and analyzed using the NIS-Elements imaging software (Nikon Inc. Melville, NY, USA). A threshold was established and applied to the images. Four regions of interest (ROI) of 200 μm in diameter were placed over the image representing the median eminence, and the area occupied by kisspeptin fibers was determined.

Statistical analysis

The Statistical Analysis System (SAS- SAS Inst. Inc., Cary, NC) software was used for analyses. The proportion of kisspeptin neurons in close proximity to NPY-containing fibers was normalized using the arcsine square root transformation. The number of cells expressing *KISS1*, density of silver grains per cell, mean number of NPY close-contacts with kisspeptin neurons, normalized proportion of kisspeptin neurons in close proximity to NPY fibers and density of kisspeptin fibers in the ME were compared between High-gain and Low-gain groups by the Student's *t*-test. Each hypothalamic area investigated was analyzed independently.

Results

Experiment 1: KISS1 expression in the POA and ARC of juvenile heifers

Cells expressing *KISS1* were detected in the POA/PeV, and throughout the rostral, mid and caudal ARC (**Figure 4.1A, B**). In the mid ARC, the number of cells expressing *KISS1* was greater ($p<0.05$) in the Low-gain heifers. Considerable variability in the number of *KISS1* cells was observed in the POA/PeV, and in the rostral and caudal regions of the ARC, and no differences were detected in these areas. Moreover, no differences were observed in the density of silver grains per cell in the mid ARC (High Gain=23 ± 5 pixels and Low Gain=19 ± 4 pixels, $p>0.05$). Because few to no *KISS1* cells were observed in the POA/PeV, and in the rostral and caudal ARC in heifers of both groups, *KISS1* expression per cell was not analyzed in these areas.

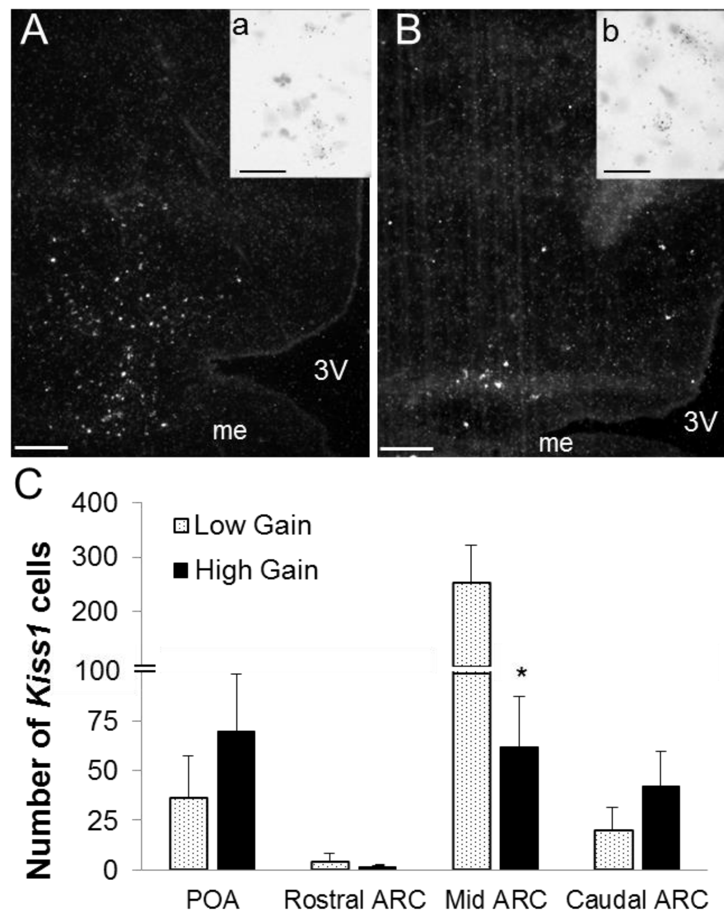


Figure 4.1 | Expression of *KISS1* in the arcuate nucleus (ARC) of 8-mo-old heifers fed to gain body weight at high (1 Kg/d; High Gain) or low (0.5 Kg/d; Low Gain) rate beginning at approximately 4.5 mo of age. (**A, a, B, b**) Images of coronal sections processed for in situ hybridization detection of *KISS1* mRNA depicting cells in the mid portion of the ARC of representative Low-gain (**A,a**) and High-gain (**B,b**) heifers. Low-magnification, dark-field images showing accumulation of silver grains (white clusters) in sections at the level of the mid ARC (**A, B).** Insets represent high-magnification, bright-field images depicting silver grain accumulation (**a, b**). (**C**) Number of *KISS1*-expressing cells in the POA, rostral, mid and caudal portions of the ARC. The mean number of cells containing *KISS1* mRNA was reduced (* $p < 0.05$) in the mid ARC of High-gain heifers compared to Low-gain heifers, but did not differ in the POA/PeV, rostral and caudal ARC. Values are mean ± SEM. me, median eminence; 3V, third ventricle. Scale bars in A and B, 250 µm. Scale bar in **a** and **b,** 25 µm.

Experiment 2: Kisspeptin-immunoreactive cells in the POA and ARC and their NPY afferent projections in juvenile heifers

The detection of kisspeptin-immunoreactive cells in the POA/PeV was highly variable with few or no cells detected among heifers in the current study. Therefore, the number of kisspeptin-immunoreactive cells was not analyzed within the POA. In the ARC, kisspeptin-containing cells were detected throughout the rostral to caudal extension of the nucleus. However, no differences in the number of cells within the rostral ARC was detected, and only a tendency for an increased number of cells in the mid (p=0.10) and caudal (p=0.08) portions of the ARC in heifers of the High-gain group was observed (**Figure 4.2**). In addition, the density of kisspeptin-immunoreactive fibers in the median eminence did not differ between High Gain and Low Gain groups (2.3 ± 0.4 % of the ROI area occupied by kisspeptin fibers).

The percentage of kisspeptin cells in close proximity to NPY fibers did not differ between Low- or High-gain heifers (**Figure 4.3A**). The average number of NPY varicosities in close contact to kisspeptin cells was 4.1 ± 0.2 (**Figure 4.3B**) and did not differ between dietary groups.

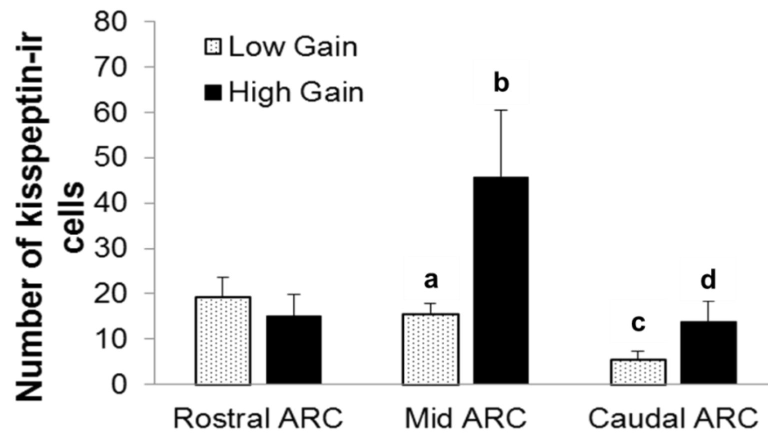


Figure 4.2 | Number of kisspeptin-immunoreactive cells detected in the rostral, mid and caudal arcuate nucleus (ARC) in Low-gain and High-gain heifers. Number of kisspeptin cells in the mid and caudal ARC of High-gain heifers tended to be increased compared to Low-gain heifers. Values are mean ± SEM. a, b: p=0.10; c, d: p=0.08.

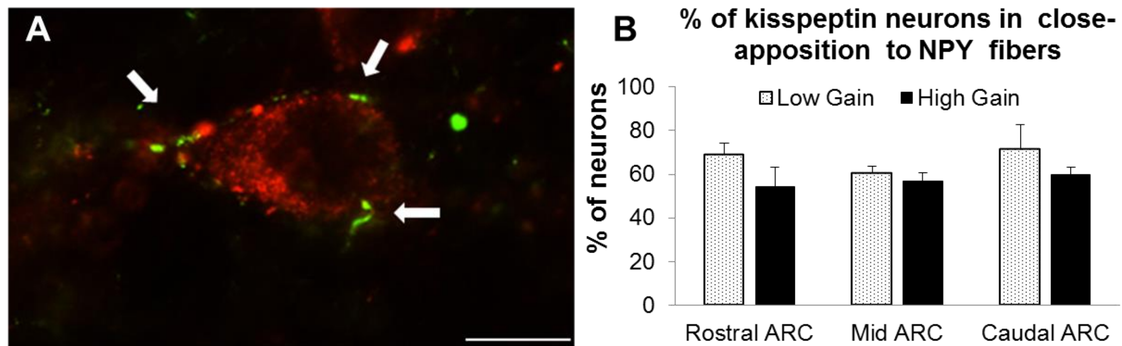


Figure 4.3 | Neuropeptide Y-containing fibers are in close proximity to kisspeptin cells in 8-mo-old heifers fed to gain body weight at high (1 Kg/d; High Gain) or low (0.5 Kg/d; Low Gain) rates during the juvenile period. (**A**) Epifluorescent image of a section processed for detection of kisspeptin (red) and NPY (green) depicting a kisspeptin-immunoreactive cell body in close apposition (arrows) to NPY-containing varicosities. **(B**) Percentage of kisspeptin-immunoreactive cells in close proximity to at least one NPY varicosite in the various subregions of the ARC nucleus. Values are mean ± SEM. Scale bar in A: 10 μ.

Discussion

In the studies reported herein, we investigated whether nutritional regimens shown previously to advance the onset of puberty affect *KISS1* expression, kisspeptin immunoreactivity and NPY innervation of kisspeptin neurons in the POA and ARC of juvenile heifers. Similar to other mammalian species, diencephalic expression of *KISS1* was observed in the POA/PeV and in the ARC of prepubertal heifers. However, contrary to our hypothesis, a decreased number of *KISS1*-expressing cells in the ARC nucleus of heifers fed to gain body weight at high rates was observed. Although a tendency for an increased number of kisspeptin-immunoreactive cells in the ARC of High-gain heifers was detected, no differences in NPY innervation of kisspeptin neurons between Low- and High-gain heifers were observed. These results do not support the hypothesis that advanced activation of *KISS1* expression is involved in the mechanism mediating the nutritional facilitation of early puberty in heifers. In addition, it remains unclear whether plasticity of NPY projections toward kisspeptin neurons plays a role in this process. Nevertheless, kisspeptin is critical for pubertal maturation of the reproductive neuroendocrine axis (de Roux et al., 2003; Seminara et al., 2003; Han et al., 2005b; Shahab et al., 2005; Kauffman, 2010) and appears to mediate estradiol feedback actions (Smith et al., 2005a; Smith et al., 2005b; Franceschini et al., 2006; Smith et al., 2007). Therefore, activation of kisspeptin synthesis and release may occur at a later maturational stage beyond that investigated in the current study.

It has been well documented that *KISS1* expression increases during pubertal development in mice (Clarkson and Herbison, 2006), rats (Navarro et al., 2004a; Cao and Patisaul, 2011), monkeys (Shahab et al., 2005) and sheep (Redmond et al., 2011a). However, the mechanisms regulating changes in *KISS1* expression in the two distinct *KISS1* neuronal populations (POA/PeV and ARC) appear to differ. In ovariectomized, estradiol-replaced ewe lambs, the number of *KISS1* cells in the ARC increases concurrently with the increase in the frequency of episodic release of LH. Conversely, the number of *KISS1*-expressing cells in the POA increases with age, but unrelated to changes in episodic release of LH in maturing lambs (Redmond et al., 2011a). Because heifers fed to gain body weight at high rates during the juvenile period exhibit early onset of puberty associated with an early increase in the frequency of pulsatile LH release (Gasser et al., 2006d), we predicted that *KISS1* expression in the ARC would be increased in High-gain heifers. However, the number of *KISS1* cells in the mid ARC was reduced in High-gain compared to Low-gain heifers. Although the basis for this discrepancy is unclear, it is possible that the maturational stage of the heifers in the current study is still reflective of earlier juvenile development. Studies in female rats have indicated that *KISS1* expression in the ARC is elevated during the infantile period, decreases during the juvenile period, and then increases during the pubertal transition (Navarro et al., 2004a; Cao and Patisaul, 2011; Desroziers et al., 2012). If such a developmental pattern in *KISS1* expression occurs in heifers, the decreased *KISS1* expression observed in the ARC of High-gain heifers may reflect a developmental transition preceding the pubertal activation in *KISS1* expression. A more comprehensive

study examining the temporal pattern of *KISS1* expression in developing heifers would be necessary to clarify this possibility.

It is also possible that the decrease in *KISS1* expression in the ARC of High-gain heifers is associated with changes in estradiol negative feedback. Pubertal onset of high-frequency release of LH is believed to occur in response to a decreased sensitivity to estradiol negative feedback (Day et al., 1987). An earlier escape from estradiol negative feedback appears to occur in heifers that gain body weight at high rates during the juvenile period (Gasser et al., 2006b).The mechanisms and pathways involved in these changes are unknown, but they might involve regulation of kisspeptin neuronal function. Because estradiol inhibits *KISS1* expression in the ARC (Smith et al., 2005a; Smith et al., 2005b; Smith et al., 2007), enhanced follicular development and estradiol synthesis could explain the reduction in *KISS1* expression in the ARC in High-gain heifers used in the current studies. Although changes in follicular growth and steroidogenesis were not investigated in the current study, earlier studies by Gasser et al. (Gasser et al., 2006c) indicate that heifers fed high-concentrate diets to gain body weight at high rates exhibit increased size of the dominant follicle compared to age-matched heifers growing at lower rates. However, it is unclear whether increased follicular steroidogenic capacity is accompanied by increased follicular development because mean circulating concentrations of estradiol does not differ between heifers gaining weight at high and low rates (Gasser et al., 2006b).

The expression of *KISS1* in the POA/PeV of heifers in the current study did not differ significantly between the High-gain and Low-gain groups. Earlier studies in sheep

indicate that changes in the number of *KISS1*-expressing cells in the POA/PeV occur during juvenile development independent of the maturation of the reproductive neuroendocrine axis (Redmond et al., 2011a). These observations appear to be in contrast to those in rodent studies. In intact female rats (Takase et al., 2009) and mice (Clarkson and Herbison, 2006; Clarkson et al., 2009), increased *KISS1* expression in the anteroventral periventricular area (AVPV) has been reported to occur during pubertal maturation. Nevertheless, the limited number of *KISS1*-expressing cells in the POA/PeV detected in the current study indicates that heifers were in an early developmental stage, comparable to those reported in studies in mice (Gill et al., 2010), rats (Cao and Patisaul, 2011) and lambs (Redmond et al., 2011a). In female mice (Smith et al., 2006b) and rats (Takase et al., 2009), expression of *KISS1* in the AVPV decreases with ovariectomy. This may reflect the stimulatory effect of estradiol on *KISS1* expression in the population of kisspeptin neurons localized in this area because in mature female mice (Smith et al., 2005a) and rats (Adachi et al., 2007), estradiol stimulates *KISS1* expression in the AVPV. However, the effects of estradiol on *KISS1* expression in the POA in adult ewes are unclear, with reports of no effects (Smith et al., 2007) and stimulatory effects (Smith et al., 2008).

The changes in kisspeptin-immunoreactivity observed in the ARC of High-gain heifers were not consistent with changes in the number of *KISS1*-expressing cells in the ARC. Contrary to *KISS1* expression, there was a tendency for the number of kisspeptin neurons to be increased in the mid and caudal portions of the ARC of High-gain heifers. The basis for the discrepancy between expression and immunoreactivity is unclear. Post-

transcriptional regulation and peptide synthesis, transport and accumulation may be involved. Although post-transcriptional regulation of kisspeptin synthesis has not been investigated extensively, lack of consistency between changes in the mRNA encoding kisspeptin and kisspeptin immunoreactivity has been reported previously in the developing mice (Gill et al., 2010). Nevertheless, the number of kisspeptin neurons in the ARC was greater in intact, postpubertal compared to intact, prepubertal ewe lambs (Nestor et al., 2012). Whether the tendency for an increased number of kisspeptin neurons in the ARC indicates a more advanced developmental stage in High-gain heifers remains to be determined.

Although *KISS1* expression in the POA/PeV was detected in both High-gain and Low-gain heifers, the number of kisspeptin immunoreactive cells in these areas was highly variable and insufficient to perform comparative analysis. A limited detection of kisspeptin neuronal soma in the POA/PeV was also reported in studies using ewe lambs (Nestor et al., 2012) and may represent a state of exposure to low estradiol characteristic of the prepubertal state in the heifer. This developmental change is evident in studies reporting that the number of kisspeptin-immunoreactive cells in the POA increases with reproductive maturity in female rats (Takase et al., 2009).

Undernutrition inhibits *KISS1* expression in rats (Castellano et al., 2005; Kalamatianos et al., 2008; Castellano et al., 2011), mice (Luque et al., 2007) and sheep (Backholer et al., 2010b). In contrast, increased body weight gain during the prepubertal period induced by reduced litter size (Castellano et al., 2011) or feeding a high-fat diet (Li et al., 2012), conditions in which pubertal development is advanced in rats, increases

KISS1 expression in the POA and ARC. These observations indicate that *KISS1* expression in the rat is regulated by nutritional status. The effects of nutrition on kisspeptin neuronal function may be mediated by leptin, a metabolic hormone shown to increase *KISS1* expression in undernourished rats (Castellano et al., 2006) and sheep (Backholer et al., 2010b). Although in the current study High-gain heifers exhibited increased average daily gain and increased concentrations of leptin (please, see Chapter III), no differences in the *KISS1* expression in the POA was observed, and expression in the ARC was decreased compared to Low-gain heifers. Because a lack of effect of under- and overnutrition on the expression of *KISS1* has also been observed in mice and rats (Luque et al., 2007; Smith and Spencer, 2012), it is possible that distinct experimental conditions may influence the ability to clearly detect changes in *KISS1* expression in response to nutritional regimens. In addition, although a small proportion of kisspeptin neurons in the ARC contain leptin receptor (Smith et al., 2006a; Backholer et al., 2010b), leptin signaling through kisspeptin neurons does not appear to be important for reproductive function (Donato Jr et al., 2011; True et al., 2011b). Nevertheless, as discussed earlier, potential developmental changes that appear to exist in the expression of *KISS1* may have obscured the effects of increased growth rate on *KISS1* expression in heifers.

Neuropeptide Y is believed to mediate the nutritional regulation of reproduction (McShane et al., 1992a; Kalra and Kalra, 1996). In cows, NPY inhibits the episodic release of GnRH (Gazal et al., 1998). Neuropeptide Y projects to GnRH neurons and appears to exert the inhibitory effects on the secretion of GnRH by direct actions on

GnRH neurons (Li et al., 1999; Klenke et al., 2010). Because NPY fibers have been observed in close proximity to kisspeptin neurons in sheep (Backholer et al., 2010b) and appear to form synaptic inputs onto kisspeptin neurons (Amstalden et al., 2011), we hypothesized that changes in NPY projections toward kisspeptin neurons would occur in heifers fed to gain body weight at high rates during the juvenile period. The proportion of GnRH neurons highly-innervated by NPY fibers is decreased in High-gain heifers (please, see Chapter III). Such an alteration is conducive for early activation of a pubertal pattern of GnRH secretion because decreased inhibition of GnRH activity may be associated with the pubertal development of high-frequency release of LH observed several weeks before the first ovulation in heifers (Day et al., 1984). However, in the current study, there was no evidence for plasticity of NPY projections toward kisspeptin neurons. Therefore, the results do not support the hypothesis that decreased NPY innervation of kisspeptin neurons is involved in the mechanism of nutritional programming of accelerated puberty in heifers.

The current study did demonstrate that a high nutritional plane adopted during early development in heifers leads to reduced *KISS1* expression in the ARC during the juvenile period. Although contrary to the hypothesis that high growth rate would increase *KISS1* expression, this alteration may indicate differences in the development of the kisspeptin system between High-gain and Low-gain heifers. The tendency for an increased number of kisspeptin-immunoreactive neurons in the ARC indicates that differences in post-transcriptional processes and peptide accumulation in the neuronal

soma also occur during juvenile development in the heifers. However, plasticity of NPY projections toward kisspeptin neurons does not appear to play a role in this regulation.

CHAPTER V

PATTERN OF DNA METHYLATION IN THE ARCUATE NUCLEUS IN PREPUBERTAL HEIFERS GROWING AT HIGH AND LOW RATES

Overview

A relatively high rate of body weight gain during the juvenile period appears to program molecular events within the arcuate nucleus (ARC) leading to an advanced onset of puberty. We hypothesized that the nutritional programming involves an altered pattern of DNA methylation in genomic regions within the ARC. In the current study, DNA methylation was assessed in genomic DNA obtained from the ARC of juvenile heifers fed to gain body weight at high (1Kg/day; High Gain, n = 4) and low (0.5 Kg/day; Low Gain, n = 4) rates from 4.5 to 8.5 mo of age. The methylcytosine enrichment assay coupled with a custom oligonucleotide array was used to determine the methylation status of target DNA sequences. Data were analyzed using three approaches: 1) the Sliding *t*-test, in which differences between dietary groups were accessed by the $\log_2$ ratio of immediate adjacent sequences; 2) the Three-point Average, in which the $\log_2$ ratio values of three neighboring sequences were averaged for each experimental unit (heifer) independently and the average was compared between dietary groups using the *t*-test; and 3) the Categorical Comparison, in which the Fisher's exact test was used to test whether the proportion of heifers identified as exhibiting differential rmethylation of a given sequence differed between dietary groups. Differential

methylation of genomic regions identified were observed in genes in which activity has been reported to be involved in the modulation of growth and metabolism (*GHR, IGF2*, *IGF1R*, *LEPR, PEG3*) and timing of the onset of puberty (*LIN28B* and *HMGA2*). Hence, relative increases in the rate of body weight gain during the juvenile period promotes altered methylation pattern of genomic DNA obtained from the ARC and these changes that may be critical for programing the age at puberty in heifers.

Introduction

Nutrition plays a major role in timing the onset of puberty in humans, laboratory animals and livestock (for review see Schillo et al., 1992; Roa et al., 2010; Cheng et al., 2012). Studies in cattle have demonstrated that a high percentage of heifers fed high-concentrate diets during the juvenile period exhibit precocious puberty (defined as puberty before 300 days of age; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d). This indicates that timing of reproductive maturation in heifers can be programmed by nutritional regimens applied during the juvenile period. The limiting physiological event for the onset of puberty is the increase in the frequency of pulsatile gonadotropin releasing hormone (GnRH) release which stimulates the secretion of luteinizing hormone (LH) (Foster et al., 1985; Ojeda and Terasawa, 2002). It is believed that the acceleration of puberty induced by accelerated body weight gain during the juvenile period comprises changes in cellular functions within the hypothalamus that facilitate earlier establishment of high-frequency episodic release of

LH (Gasser et al., 2006d). However, the cellular mechanisms and pathways involved in this process remain unclear.

Previous studies have shown that nutritional status during the perinatal and early developmental periods influences cellular processes in the hypothalamus that include changes in neuronal functions involved in the control GnRH release and pubertal development (Plagemann et al., 2009; Coupe et al., 2010; Stevens et al., 2010; Castellano et al., 2011). In a recent experiment investigating the transcriptome of the hypothalamic arcuate nucleus (ARC), it was observed that the expression of a number of regulatory genes is altered in heifers fed to gain body weight at relatively high rates between 4 and 6 mo of age (Allen et al., 2012). Functional analysis of the genes altered by this nutritional regimen indicated that biological processes involving responses to hormones and metabolites, synaptic transmission, synaptic vesicle transport, regulation of cell morphogenesis, feeding behavior and developmental maturation were affected (Allen et al., 2012). Moreover, altered pattern of neuronal inputs onto GnRH neurons (see Chapters III and IV of this dissertation) and neurotransmitter release (Cardoso et al., 2012) appear to also occur in heifers fed to gain body weight at higher rates during the juvenile period.

Recent reports have proposed that the mechanisms involved in the nutritional regulation of gene expression in the ARC include modifications to the epigenome (Plagemann et al., 2009; Stevens et al., 2010; Begum et al., 2012). These epigenetic alterations may play a role in timing the onset of puberty (Lomniczi et al., 2013). The addition of methyl groups to cytosine-guanine dinucleotides (CpGs) is one of the

epigenetic modifications that promote fine control of gene expression and may persistently affect the transcriptional profile of genes (Goldberg et al., 2007). Therefore, we hypothesized that an increased rate of body weight gain in heifers during the juvenile period leads to alterations in the pattern of methylation of genes in the ARC nucleus, and these alterations facilitate earlier pubertal maturation. To test this hypothesis, we used a nutritional model well characterized for accelerating puberty in heifers (Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d; Cardoso et al., 2012).

Material and methods

The study described herein used tissue collected from experiments described earlier in this dissertation (Chapter III). Animal-related procedures used for that experiment were approved by the Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System. Briefly, heifers were weaned at approximately 3.5 mo of age and fed individually to gain body weight at high (High Gain, 1Kg/d) or low (Low Gain, 0.5 Kg/d) rate for approximately 4 mo. This nutritional regimen resulted in heifers in the High-gain group exhibiting greater body weight and circulating concentrations of leptin at slaughter at 8.5 mo of age. Heifers (n=4/group) were selected based on similarity of the genetic background (¾ *Bos taurus* – Angus and ¼ *Bos indicus* – Brahman or Nelore) and body weight at slaughter. Heifers were determined to be prepubertal by confirming the absence of functional luteal tissue

(concentrations of progesterone in circulation less than 1 ng/ml in samples obtained twice a week during the four weeks before the slaughter and *post-mortem* evaluation of the ovaries). At slaughter, the brain of each heifer was dissected and a block of tissue containing the septum, POA, and hypothalamus was collected and snap frozen in liquid nitrogen vapor. Frozen tissue blocks were cut in coronal sections of 20 µm using a cryostat, mounted on SuperFrost Plus slides and stored at -80° C until used for the current study.

Genomic DNA isolation and methylated DNA enrichment assay

Genomic DNA was isolated from tissue obtained from bilateral scrapes of an area of approximately 1 mm in diameter delimited within the mid ARC nucleus. Scrapes were performed on 12 tissue sections (200 µm apart) for each heifer and followed procedures similar to those described earlier (Allen et al., 2012). The subdivisions of the ARC nucleus were determined according to anatomical references (Lehman et al., 1993; Allen et al., 2012). Tissue scrapes from each heifer were pooled in a tube containing 500 µl NTES buffer (50mM NaCL, 500mM Tris, 12.5mM EDTA, 0.5% SDS) added to 40 µg of proteinase K and incubated overnight at 55°C. Genomic DNA was obtained by phenol-chloroform extraction followed by ethanol precipitation. The quantity and purity of DNA obtained was determined by spectrophotometry (NanoDrop, Thermo Scientific NanoDrop Technologies, Wilmington, DE). The DNA was stored at -20° C until further processing.

The methylated DNA enrichment assay was performed to capture fragments of methylated DNA (methylated-enriched DNA) using the MethylMiner™ kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. In this assay, fragments of DNA containing methylated cytosine residues were captured by methyl-CpG-binding proteins (MBD2) attached to magnetic beads. The methylated DNA was eluted from the MBDs with a 2 M NaCl solution and purified by ethanol precipitation.

Optimization and validation of the methylated DNA enrichment assay and whole-genome amplification of bovine hypothalamic DNA

To develop, optimize and validate the use of the methylated DNA enrichment assay for the bovine hypothalamic DNA, a series of experiments was performed using genomic DNA isolated from bovine hypothalamus and white-blood cells. Genomic DNA was fragmented by enzymatic digestion using *Alu1* endonuclease (New England BioLabs Inc., Beverly, MA) at 37° C overnight. The enzyme was then inactivated by incubation at 65° C for 20 min. To determine the size of the products obtained by endonuclease digestion, DNA fragments were resolved by agarose gel electrophoresis (1.5% agarose gel and ethidium bromide staining). The methylated DNA enrichment assay was performed using 500 ng of fragmented DNA.

Because the total quantity of methylated-enriched DNA isolated from hypothalamic explants was limited, a procedure to amplify the DNA recovered from the methylated DNA enrichment assay was used. Ten µg of input and methylated-enriched DNA were amplified by two rounds of whole genome amplification (WGA) using the

GenomePlex® amplification method (Sigma-Aldrich, St. Louis, MO) and following procedures reported previously (Dindot et al., 2009). The amplified DNA was purified using the PureLink® PCR Purification Kit (Invitrogen, Carlsbad, CA).

Amplification of *OCT4* (*POUF1*) and *GAPDH* genes was used to determine the efficiency of the methylated DNA enrichment assay and WGA procedures. *OCT4* is a highly-methylated gene, while *GAPDH* is an un-methylated gene (Stevens et al., 2010). Primers used for PCR amplification were designed using Primer3 software (http:// www.primer3.com) and are listed in **Table 5.1**.

Table 5.1 | Sequences of primers used for PCR

Gene	Name	Ref. Seq.	Primer Sequence	Amplicon Length
OCT4	Octamer-Binding Transcription Factor 4	AC_000180.1	F 5'- CCTACTGTGCGCCGCAGGTT - 3' R 5'- AGGCCCCCTCGGAGTTGCTC - 3'	111 bp
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	AC_000162.1	F 5'- GCCTTCGAGGGCCTCCTCCA - 3' R 5'- GGTCCTCACCGCATTGCGCT - 3'	130 bp

Amplifications of *OCT4* and *GAPDH* sequences were performed by PCR in the input (total DNA after fragmentation), unbound and methylated-enriched fractions. Reactions were performed in 25 µl reaction mixtures containing 25 ng of DNA, GoTaq reaction buffer, 1 mM of MgCl, 0.2 mM of each deoxynucleotide triphosphate, 4 pmol of each forward and reverse primers, 0.5 M betaine, and 0.625 units of GoTaq DNA polymerase (Promega, Madison, WI). Thirty-five cycles of 95°C for 30 s, 53°C for 30 s

and 72°C for 60 s were employed. The amplicons were resolved by agarose gel electrophoresis (1.5% agarose gel and ethidium bromide staining).

Pattern of methylation of target DNA in the ARC nucleus of juvenile heifers

Genomic DNA extracted from the ARC nucleus of the heifers was isolated and fragmented by enzymatic digestion using *Alu1* endonuclease as described earlier. Fragmented DNA was processed through the MethylMiner™ procedure (Invitrogen, Carlsbad, CA) to obtain methylated-enriched DNA. The DNA concentrations of the input and methylated-enriched fractions were accessed using a Qubit fluorometer and the Quant-iT dsDNA BR procedure (Invitrogen, Carlsbad, CA). Input and methylated-enriched DNA were amplified using the GenomePlex® amplification kits (WGA2 and WGA3; Sigma-Aldrich, St. Louis, MO). Amplified DNA was then purified using PureLink® PCR Purification Kit (Invitrogen, Carlsbad, CA) as described earlier in the optimization and validation of procedures section. Equal amounts of input and methylated-enriched DNA within individual hypothalamic genomic DNA samples were used for WGA for consistent comparison between input and methylated-enriched fractions. The total amount of DNA used among samples obtained from High-gain and Low-gain heifers ranged from 6 to 15 μg, depending on the output of the second round of the WGA procedure. Amplification of *OCT4* by PCR in methylated-enriched fractions was used to confirm successful WGA in DNA obtained from hypothalamic samples.

Oligonucleotide array

A 60 000-oligonucleotide array (8 X 60K format) was generated using the Agilent E-array software database (Agilent Technologies, Santa Clara, CA). The Baylor 4.0/bosTau4 bovine genome assembly was used to generate the array. A total of 10 249 oligonucleotides (probes) with average size of 52 nucleotides were designed to cover targeted genomic sequences associated with 97 genes (and associated 2K base pair sequence upstream and downstream regions) and two wider genomic intervals which correspond to the *DLIK1-DIO3* (Dindot et al., 2004; Rocha et al., 2008) and *IGF2-H19* (Dindot et al., 2004; Robbins et al., 2012) imprinted clusters. Genes observed to be responsive to nutritional inputs in heifers (Allen et al., 2012) and predicted to be involved in the control of pubertal development in mammals (Daftary and Gore, 2005; Ong et al., 2009; Fortes et al., 2010; Hawken et al., 2012; Mueller et al., 2012) were included in the array. The list of genes and target genomic intervals selected for probe design is presented in **Table 5.2**.

Table 5.2 | Genes and target intervals used for designing probes included in array

Interval	Gene	Region for probes	Interval	Gene	Region for probes
1	*RBP1*	chr1:131864504-131901453	51	*FAM83D*	chr13:68309059-68342033
2	*ITGB2*	chr1:146750368-146789523	52	*MYC*	chr14:12146577-12161133
3	*STAM2*	chr2:46133987-46191568	53	*ARRB1*	chr15:54041525-54124996
4	*ZEB2*	chr2:54397583-54497541	54	*REN*	chr16:928980-948995
5	*STAT1*	chr2:83378381-83420778	55	*KISS1*	chr16:950542-963542
6	*IGFBP2*	chr2:108805787-108844273	56	*RGS2*	chr16:11063845-11077152
7	*LIN 28A*	chr2:130964723-130984591	57	*GABRD*	chr16:47980943-48001509
8	*C1QA*	chr2:134667736-134680624	58	*MIB2*	chr16:48275281-48297580
9	*INSR*	chr3:15409199-15434858	59	*AGRN*	chr16:48673613-48721521
10	*LEPR*	chr3:85611671-85697000	60	*NPY2R*	chr17:2160011-2174222
11	*TRAF3IP1*	chr3:125292340-125361965	61	*WSB2*	chr17:60080562-60104970
12	*HDAC9*	chr4:28306681-28517815	62	*GAS2L1*	chr17:72033183-72048647
13	*NPY*	chr4:74063972-74081098	63	*FHOD1*	chr18:33868654-33894874
14	*LIN7A*	chr5:12345874-12511415	64	*TPPP3*	chr18:34015652-34030563
15	*RAPGEF3*	chr5:35709370-35740929	65	*LCAT*	chr18:34438828-34452072
16	*HMGA2*	chr5:51726181- 51902985	66	*SIRT2*	chr18:48202012-48231849
17	*LET7-I*	chr5:55133987-55134032	67	*APOE*	chr18:52418183-52430869
18	*ITGA7*	chr5:62108747-62142899	68	*SPHK2*	chr18:55080530-55095398
19	*KRAS*	chr5:91185713- 91186139	69	*PEG3*	chr18:64346467-64379975
20	*NPY1R*	chr6:2410744-2428446	70	*SEPT4*	chr19:8684399-8707902
21	*SNCA*	chr6:36499250-36656064	71	*PLD2*	chr19:26912471-26935177
22	*TLR6*	chr6:60350277-60394131	72	*MPRIP*	chr19:35985956-36062542
23	*NCAN*	chr7:3939006-3979379	73	*IGFBP1*	chr19:38704348-38754256
24	*ISYNA1*	chr7:4658839-4672035	74	*TNS4*	chr19:41956261-41985523
25	*HOOK2*	chr7:11013019-11033531	75	*CNP*	chr19:43485122-43501006
26	*DNM2*	chr7:13654605-13752930	76	*STAT3*	chr19:43746450-43830335
27	*PNPLA6*	chr7:14845367-14879170	77	*SOCS3*	chr19:55412550-55423249
28	*KISS1R*	chr7:42471597-42485886	78	*GHR*	chr20:33891757-34211083
29	*UBE2B*	chr7:45123930-45146548	79	*OSMR*	chr20:37742962-37819945
30	*NR3C1*	chr7:53954676-54083159	80	*IGF1R*	chr21:6564323-6874285
31	*LET7-A*	chr8:89745989-89746049	81	*IDLK1-DIO3**	chr21:65743366-65898529
32	*CDK5RAP2*	chr8:115159765-115352986	82	*OXTR*	chr22:18280690-18300357
33	*LIN28B*	chr9:47239907-47265263	83	*STAB1*	chr22:49468255-49503581
34	*PRPS1*	chr9:98628704-98640743	84	*CISH*	chr22:50651185-50666604
35	*IGF2R*	chr9:100071966-100074442	85	*PLXNB1*	chr22:52608104-52621354
36	*CDO1*	chr10:4164206-4185727	86	*FGD2*	chr23:11251679-11287945
37	*CPNE6*	chr10:21424582-21439641	87	*OCT4.*	chr23:27905664-27906341
38	*GERM1*	chr10:29524388-29546874	88	*PPPIR18*	chr23:28241971-28260609
39	*DNAAF2*	chr10:42732912-42749601	89	*MC4R*	chr24:61724588-61736383
40	*DIO2*	chr10:94211358-94230305	90	*AXIN1*	chr25:761006-805117
41	*ZAP70*	chr11:3326029-3375822	91	*RAB11FIP3*	chr25:852856-921019
42	*DYSF*	chr11:13463191-13734907	92	*CRYM*	chr25:20228817-20262375
43	*POMC*	chr11:76241975-76279453	93	*RABEP2*	chr25:27725758-27748007
44	*GPR21*	chr11:97352139-97364322	94	*KCNIP2*	chr26:22849588-22862882
45	*KCNRG*	chr12:18844412-18859880	95	*PRLHR*	chr26:39261151-39273428
46	*BFSP1*	chr13:37814036-37862393	96	*NPY4R*	chr28:41782436-41798211
47	*CDC25B*	chr13:51949757-51969354	97	*AGT*	chr28:45968498-45987336
48	*OXT*	chr13:52667972-52678870	98	*IGF2-H19**	chr29:49860608-51627535
49	*HRH3*	chr13:55717151-55730353	99	*HDAC6*	chrx:55583140-55620215
50	*SNTA1*	chr13:63742371-63778617			

**imprinted regions*

DNA labeling and hybridization

Input and methylated-enriched DNA (3 µg each) were labeled with Alexa Fluor® 555 and Alexa Fluor® 647, respectively, using the BioPrime® Plus Array CGH Genomic Labeling Module (Invitrogen, Carlsbad, CA) and following the manufacturer's instructions. Concentrations of DNA and fluorophore intensities were determined using a NanoDrop (Thermo Scientific NanoDrop Technologies, Wilmington, DE). Input and methylated-enriched labeled DNA were co-hybridized to the array (2 µg each), using the Oligo aCGH/ChIP-on-chip Hybridization Kit (Agilent Technologies, Santa Clara, CA) at 65° C for 23 h following procedures described previously (Dindot et al., 2009). The array was scanned using the G2505C Microarray Scanner (Agilent Technologies, Santa Clara, CA). Signal intensities were quantified using the Agilent Feature Extraction Software (version 10.7, Agilent Technologies, Santa Clara, CA). A resolution of 5µm and extended dynamic range (XDR) of 0.05 were applied to obtain the signal intensities.

Data analyses

The Chip Interactive Analysis application of the Agilent Genomic Workbench software (version 7.0, Agilent Technologies, Santa Clara, CA) was used to analyze signal intensity data obtained from the array. Signal intensities were normalized according the Agilent Feature Extraction output, which applies the Lowess (Locally Weighted Polynomial Regression) method. This approach normalizes the channel within each array using a nonlinear polynomial fit to the data and corrects for dye-related artifacts (Cleveland and Devlin, 1988). The ratios of the $\log_2$ (enriched/input) of the

normalized intensities were determined. The p-value of the difference in signal intensities between enriched and input fractions was calculated based on the Agilent Universal Error modeling (Zahurak et al., 2007). Differences in intensities between enriched and input fractions were used to assign a methylation status to target DNA sequences. A given sequence was determined hypermethylated when the $\log_2$ ratio (enriched/input) of normalized intensities was greater than 0.3 and the p-value of the difference was less than 0.05. If these criteria were not met, the methylation status of target DNA sequences was determined equal between input and methylated-enriched fractions and the $\log_2$ ratio of the normalized intensity was set to zero for further analysis. This process was repeated for each probes in all eight arrays (one array per heifer used in the experiment).

In order to determine differences in the methylation profiles between dietary groups (High Gain and Low Gain), data were analyzed using three approaches. For the first approach (Sliding *t*-test), differences between dietary groups were accessed taking into account the $\log_2$ ratio values of immediate adjacent sequences. The $\log_2$ ratios within group were averaged and three consecutive averaged values were compared between High-gain and Low-gain groups using the Sliding *t*-test as described previously (Dindot et al., 2009). This approach generates a 3X3-cell comparison. For the second approach (Three-point Average), the $\log_2$ ratio values of three neighboring sequences were averaged for each experimental unit (heifer) independently and the average was compared between dietary groups (High Gain and Low Gain) using the *t*-test. This approach generates a 4X4-cell comparison. For these two approaches, significant

differences in methylation status were accepted when the p-value for the difference was <0.05. After the initial analysis using the Sliding *t*-test and Three-point Average methods, sequences that were detected as differently methylated were ranked according to the magnitude of the difference between the log_2 ratio of the High-gain and Low-gain groups. The results from these two analyses were then intersected to determine the extent of the concordance between the two approaches.

For the third approach (Categorical Comparison), sequences identified with log_2 ratio >0.3 and $p<0.05$ for each heifer were assigned the value 1 (differently methylated). Sequences that did not meet these criteria were assigned the value 0 (unmethylated or equal methylation between groups). The Fisher's exact test was then used to test whether the proportion of heifers in each group exhibiting differential methylation of a given target sequence (value 1) differed between the High-gain and Low-gain groups. For this approach, a $p<0.07$ was accepted as statistically-significant difference. This significance level indicates that at least 3 of 4 heifers within a group differed in their methylation status for a target sequence.

Results

Optimization and validation of the methylated DNA enrichment assay and whole-genome amplification of bovine hypothalamic DNA

Amplicons of the PCR reaction for *GAPDH* were readily detected in reactions using input and unbound DNA, but not in the methylated-enriched DNA (**Figure 5.1A**).

In contrast, amplification of *OCT4* was detected in the input and enriched DNA, but not in the unbound DNA (**Figure 5.1B**). These results indicate the effectiveness of MBD proteins used in the methylated DNA enrichment assay to capture methylated sequences of *OCT4* from the input sample. However, unmethylated sequences, such as those associated with *GAPDH*, are not captured and undetectable in the methylated-enriched sample. Therefore, the methylated DNA enrichment assay method was accepted to perform enrichment of methylated DNA isolated from scrapes of hypothalamic tissue.

Because the amount of DNA obtained in the methylated-enriched fraction of scrapes of the ARC nucleus was below the minimum required for labeling procedures, WGA was performed to produce an adequate amount of DNA for array hybridization. The efficiency of the WGA procedures was investigated by detection of the highly-methylated *OCT4*. A sequence corresponding to a region expected to be methylated in *OCT4* was readily detected in round one (**Figure 5.1C**) and round two (**Figure 5.1D**) WGA reactions using methylated-enriched DNA.

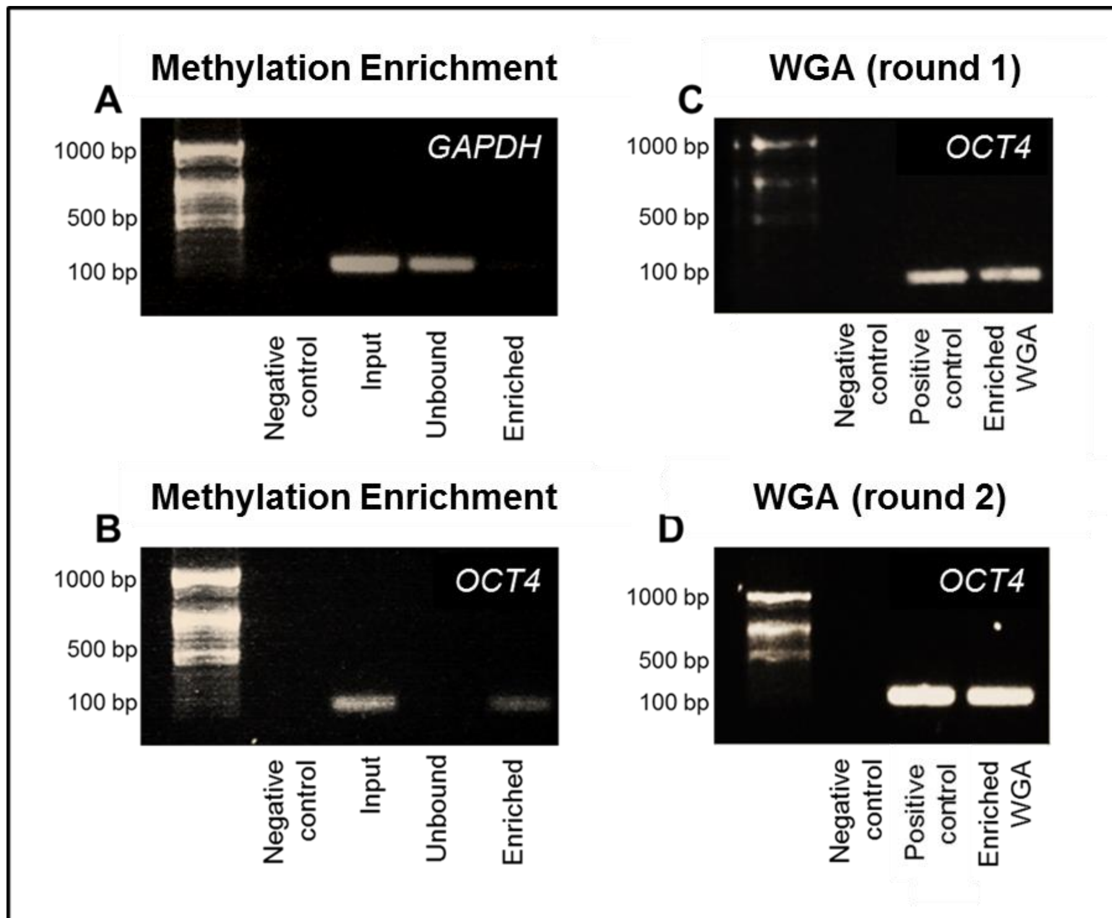


Figure 5.1 | PCR amplification of *GAPDH* and *OCT4* from bovine hypothalamic DNA. A. Detection of PCR amplification of GAPDH in enzymatically-digested DNA obtained from bovine hypothalamus (input) and in DNA not isolated in the methylated DNA enrichment assay (unbound). No amplicon was detected in PCR reactions performed using the methylated-enriched DNA (enriched). **B**. Detection of PCR amplification of *OCT4* in enzymatically-digested DNA obtained from bovine hypothalamus (input) and in DNA eluted from the methylated DNA enrichment assay (enriched). Amplicons were undetectable in PCR reactions performed using the unbound DNA. **C, D**. Detection of *OCT4* amplicons in input and methylated-enriched DNA samples subjected to round 1(**C**) and round 2 (**D**) of whole genomic amplification(WGA). Positive control (**C, D**) represents PCR amplification of undigested DNA. Negative control represents reactions in which DNA template was omitted.

Pattern of methylation of target DNA in the ARC nucleus of juvenile heifers

Methylation enrichment was detected in a number of target-DNA sequences investigated, and included sequences known to be hypermethylated in the bovine genome. Hypermethylation of the *DLK1-DIO3* cluster was detected in three of the four heifers in each dietary group (**Figure 5.2**) in a region located upstream to *MEG3*, a gene previously observed to be imprinted in cattle (Dindot et al., 2004). Hypermethylation was also observed in the *IGF2-H19* cluster. However, although all heifers in both groups exhibited hypermethylation that spanned this genomic region, there was variation in the specific subregions in which hypermethylation was detected (**Figure 5.2**). The *IGF2-H19* cluster is also known to be imprinted in the bovine genome by methylation of various CpG islands (Dindot et al., 2004). Therefore, the observation that these regions are hypermethylated in heifers of both groups indicates successful recovery of methylated DNA fragments isolated from the ARC nucleus.

Region	Sequence	Low Gain Heifer				High Gain Heifer			
		1	2	3	4	5	6	7	8
DLK1-DIO3	chr21:65773223-65773283								
IGF2-H19	chr29:50035564-50035609								
	chr29:50036451-50036498								
	chr29:50037142-50037202								
	chr29:51403742-51403787								
	chr29:51404983-51405043								

Figure 5.2 | Distribution of methylated-enriched DNA intervals in genomic regions encompassing *DLK1-DIO3* and *IGF2-H19* among individual Low-gain and High-gain heifers. The shaded cells represent genome intervals detected as methylated in each heifer analyzed in the Low-gain (n=4) and High-gain (n=4) dietary groups.

The analysis of differential methylation between Low- and High-gain heifers resulted in detection of 130, 304 and 69 differentially-methylated regions based on the methods of Sliding *t*-test, Three-point Average and Categorical Comparison, respectively (**Table 5.3**). The ranks of the 10 greatest differences between the $\log_2$ ratio of the average values of High-gain and Low-gain groups are presented in **Table 5.4** (Sliding *t*-test method) and **Table 5.5** (Three-point Average method), and include the genomic regions spanned by the three consecutive probes included in the analysis considered by these two methods.

Table 5.3 | Number of regions targeted by individual probes that were differently methylated in High-gain heifers

	Hypomethylated	Hypermethylated	Total
Sliding *t-test*	17 (13%)	113 (87%)	130
Three-point Average	114 (38%)	190 (64%)	304
Categorical Comparison	44 (64%)	25 (36%)	69

Numbers in parenthesis indicate the percentage of the total number of probes found differently methylated in each method.

Table 5.4 | Greatest differences in $\log_2$ ratio between High-gain and Low-gain groups determined by the Sliding *t*-test method

Gene	Position	Region	Log_2 Ratio	Gene	Position	Region	Log_2 Ratio
DYSF	intragenic	chr11: 13728399-13728444	-0.611	***IGF1R***	intragenic	chr21: 6734412-6734835	1.426
SNCA	intragenic	chr6: 36540342-36541031	-0.557	***GPR21***	intragenic	chr11: 97358267-97358771	0.889
HMGA2	intragenic	chr5: 51885717-51886310	-0.477			chr11: 97358460-97358938	0.860
		chr5: 51887306-51887826	-0.327	***CDK5RAP2***	upstream	chr8: 115164062-115165161	0.886
HOOK2	upstream	chr7: 11014834-11016214	-0.336	***OSMR***	intragenic	chr20: 37756367-37756837	0.861
IGF1R	intragenic	chr21: 6668338-6671788	-0.318		downstream	chr20: 37818274-37819042	0.685
ARRB1	intragenic	chr15: 54088355-54089584	-0.312	***GHR***	intragenic	chr20: 33984719-33985154	0.800
MPRIP	intragenic	chr19: 35992837-35993999	-0.286	***SNCA***	intragenic	chr6: 36511914-36512404	0.739
KCNRG	intragenic	chr12: 18850865-18851511	-0.258	***PEG3***	upstream	chr18: 64352071-64352532	0.729
ZAP70	intragenic	chr11: 3364748-3368697	-0.243	***FAM83D***	downstream	chr13: 68337835-68340456	0.704

ARRB1: arrestin, beta 1; CDK5RAP2: CDK5 regulatory subunit associated protein 2; DYSF: Dysferlin; FAM83D: family with sequence similarity 83, member D; GHR: Growth hormone receptor; GPR21: G-coupled protein receptor 21; HMGA2: High mobility group protein HMGI-C; HOOK2: hook microtubule-tethering protein 2; IGF1R: insulin-like growth factor 1 receptor; KCNRG: Potassium channel regulator; OSMR: oncostatin M receptor; PEG3: paternally expressed 3; SNCA: Alpha-synuclein; ZAP70: Zeta-chain-associated protein kinase 70.

Table 5.5 | Greatest differences in $\log_2$ ratio between High-gain and Low-gain groups determined by the Three-point Average method

Gene	Position	Region	Log_2 Ratio
RAB11FIP3	intragenic	chr25: 892148-893894	-1.479
		chr25: 893339-894636	-1.479
		chr25: 890759-893399	-1.209
		chr25: 882739-884152	-0.786
IGF1R	intragenic	chr21: 6811841-6813286	-1.241
		chr21: 6811547-6812215	-1.178
		chr21: 6811547-6812215	-1.148
NCAN	intragenic	chr7: 3971080-3972430	-0.642
HMGA2	intragenic	chr5: 51875822-51876790	-0.629
		chr5: 51876736-51877401	-0.629

Gene	Position	Region	Log_2 Ratio
IGF1R	intragenic	chr21: 6734412-6734835	1.426
		chr21: 6734586-6735636	0.901
CDK5RAP2	intragenic	chr8: 115164062-115165161	0.886
TRAF3IP1	intragenic	chr3: 1253322985-125334083	0.881
GHR	intragenic	chr20: 34201105-34202666	0.844
		chr20: 34201412-34203385	0.844
KRAS	downstream	chr5: 91216333-91216809	0.752
		chr5: 91216566-91217477	0.752
LIN7A	intragenic	chr5: 12364271-12365295	0.752
		chr5: 12363639-12364331	0.752

CDK5RAP2: CDK5 regulatory subunit associated protein 2; GHR: Growth hormone receptor; HMGA2: High mobility group protein HMGI-C; IGF1R: insulin-like growth factor 1 receptor; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; LIN7A: protein lin-7 homolog A; NCAN: Neurocan; RAB11FIP3: RAB11 family interacting protein 3 (class II); TRAF3IP1: TNF receptor-associated factor 3 interacting protein 1.

The results of the analyses performed using the Sliding *t-test* and Three-point Average methods were intersected to determine the degree of concordance. Thirty-six genomic regions were observed to be differently methylated in these two methods. These regions and associated genes/genomic intervals are presented in **Tables 5.6, 5.7** and **5.8.**

These regions were contained within 22 genes (or corresponding upstream or downstream regions). Among those genes, four were observed to be hypomethylated (**Table 5.6**) and 17 were observed to be hypermethylated in High-gain heifers (**Table 5.7**). In addition, the *IGF1R* genomic region exhibited a subregion that was hypomethylated and subregions that were hypermethylated in High-gain heifers (**Table 5.8**).

Table 5.6 | Genes and spanned regions detected to be hypomethylated in High-gain heifers concurrently by the Sliding *t-test* and Three-point Average methods

Gene	Description	Position	Region
INSR	Insulin receptor	downstream	chr3:15431413-15431975
DYSF	Dysferlin	intragenic	chr11:13728186-13728651
ARRB1	Arrestin, beta 1	intragenic	chr15:54088355-54089584
MPRIP	Myosin phosphatase Rho interacting protein	intragenic	chr19:35992837-35993999

Table 5.7 | Genes and spanned regions detected to be hypermethylated in High-gain heifers concurrently by the Sliding *t-test* and Three-point Average methods

Gene	Description	Position	Region
KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1	upstream	chr29:50110938-50111858 chr29:50268088-50269251 chr29:50397757-50402049 chr29:50541791-50543406 chr29:50610664-50612151
LEPR	Leptin receptor	upstream	chr3:85614443-85616158
		intragenic	chr3:85626884-85640135 chr3:85663096-85693582
LIN28B	Lin28 homolog B	intragenic	chr9:47133692-47134101 chr9:47181416-47181944 chr9:47233280-47233672
ZEB2	Zinc finger E-box binding homeobox 2	intragenic	chr2:54410057-54410637 chr2:54470411-54470924
HDAC9	Histone deacetilase 9	intragenic	chr4:28312734-28313252 chr4:28408694-28410034
GHR	Growth hormone receptor	intragenic	chr20:34021218-3402297 chr20:34069113-34069610
STAT1	Signal transducer and activator of transcription 1	intragenic	chr2:83386692-83388413
LIN7A	Lin-7 homolog A	intragenic	chr5:12499546-12500151
HMGA2	High mobility group protein HMGI-C	intragenic	chr5:51799612-51801028
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	intragenic	chr5:91211541-91212211
CDK5RAP2	CDK5 regulatory subunit associated protein 2	intragenic	chr8:115164062-115165161
CDO1	Cysteine dioxygenase, type I	downstream	chr10:4181971-4183808
FAM83D	Family with sequence similarity 83, member D	intragenic	chr13:68326273-68326751
MYC	V-myc avian myelocytomatosis viral oncogene homolog	downstream	chr14:12159168-12159788
RFC5	Replication factor C (activator 1) 5	intragenic	chr17:60100746-60101614
PEG3	Paternally expressed 3	upstream	chr18:64352071-64352532
CISH	Cytokine inducible SH2-containing protein	downstream	chr22:50661526-50663062

Table 5.8 | Genes and spanned regions exhibiting both hypo- and hypermethylated sequences in High-gain heifers concurrently by the Sliding *t-test* and Three-point Average methods

Gene	Description	Position	Region	Methylation Status
IGF1R	Insulin-like growth factor 1 receptor	intragenic	chr21:6571296-6572012	Hypermethylated
			chr21:6668338-6671778	Hypomethylated
			chr21:6734412-6734835	Hypermethylated
			chr21:6808975-6809509	Hypermethylated

The 69 genomic regions observed to be differentially methylated by the Categorical Comparison method are presented in **Tables 5.9, 5.10 and 5.11.** Those regions were contained within 30 genes (or corresponding upstream or downstream regions). Among those genes, 17 were observed to be hypomethylated in High-gain heifers (**Table 5.9**) and six were observed to be hypermethylated in High-gain heifers (**Table 5.10**). Seven exhibited subregions that were hypomethylated and subregions that were hypermethylated in High-gain heifers (**Table 5.11**). The majority of the differently methylated regions detected in the Categorical Comparison method were contained within regions detected by the Sliding *t*-test, the Three-point Average or both. These regions are indicated in **Tables 5.9, 5.10 and 5.11.**

Table 5.9 | Genes and spanned regions observed to be hypomethylated in High-gain heifers by the Categorical Comparison method

Gene	Description	Position	Region
DYSF	Dysferlin	intragenic	chr11:13502125-13502179 * chr11:13517084-13517135 * chr11:13544732-13544777 * chr11:13728186-13728231 ***
RAB11FIP3	RAB11 family interacting protein 3 (class II)	intragenic	chr25:884107-884152 * chr25:893339-893399 *
RBP1	Retinol binding protein 1	intragenic	chr1:131893504-131893551 *
STAM2	Signal transducing adapter molecule 2	intragenic	chr2:46155596-46155656 *
IGFBP2	Insulin-like growth factor binding protein 2	intragenic	chr2:108812004-108812062
C1QA	Complement component 1, q subcomponent, A chain	intragenic	chr2:134675170-134675215 *
DNM2	Dynamin 2	intragenic	chr7:13731826-13731871 *
SNTA1	Syntrophin, alpha 1	intragenic	chr13:63750230-63750275 *
ARRB1	Arrestin, beta 1	intragenic	chr15:54049208-54049253
GAS2L1	Growth arrest-specific 2 like 1	upstream	chr17:72034736-72034781 *
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1	intragenic	chr19:38749621-38749666 *
STAT3	Signal transducer and activator of transcription 3	intragenic	chr19:43789152-43789212 *
DLK-1	Delta-like 1 homolog	intragenic	chr21:65722081-65722133
AXIN1	Axin 1	intragenic	chr25:797682-797727
RABP2	Rabaptin, RAB GTPase binding effector protein 2	intragenic	chr25:27741072-27741117 *
KCNIP2	Kv channel-interacting protein 2	intragenic	chr26:22857601-22857646 *
AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	intragenic	chr28:45981789-45981834 *

** Region contained within an interval observed to be differently methylated by the Three-point average method.*
**** Region contained within an interval observed to be differently methylated by the Sliding t-test and Three-point average methods.*

Table 5.10 | Genes and spanned regions observed to be hypermethylated in High-gain heifers by the Categorical Comparison method

Gene	Description	Position	Region
GHR	Growth hormone receptor	intragenic	chr20:34021218-34021276 *** chr20:34059500-34059552 * chr20:34202607-34202666 *
CDK5RAP2	CDK5 regulatory subunit associated protein 2	intragenic	chr8:115164930-115164990 *** chr8:115165103-115165161 ***
SNCA	Synuclein, alpha	intragenic	chr6:36512184-36512244 ** chr6:36592778-36592838 *
HDAC9	Histone deacetilase 9	intragenic	chr4:28404677-28404727 *
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	intragenic	chr5:91213838-91213898 *
POLE2	Polymerase (DNA directed), epsilon 2	intragenic	chr10:42748900-42748960 *

** Region contained within an interval observed to be differently methylated by the Three-point average method.*
*** Region contained within an interval observed to be differently methylated by the Sliding t-test method.*
**** Region contained within an interval observed to be differently methylated by the Sliding t-test and Three-point average methods.*

Table 5.11 | Genes and spanned regions exhibiting both hypo- and hypermethylated sequences in High-gain heifers by the Categorical Comparison method

Gene	Description	Position	Region	Methylation Status
KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1	upstream	chr29:50022026-50022086 *	Hypermethylated
		upstream	chr29:50172098-50172153 *	Hypomethylated
		upstream	chr29:50324708-50324753 *	Hypomethylated
		upstream	chr29:50429383-50429436 *	Hypermethylated
		upstream	chr29:50492301-50492361 *	Hypermethylated
		upstream	chr29:50497219-50497265 *	Hypomethylated
		intragenic	chr29:50645089-50645134	Hypomethylated
		intragenic	chr29:50648896-50648941 *	Hypomethylated
		intragenic	chr29:50782117-50782167 *	Hypomethylated
		intragenic	chr29:50784657-50784704 *	Hypomethylated
		intragenic	chr29:50807293-50807338 *	Hypomethylated
		intragenic	chr29:50896277-50896323 *	Hypomethylated
IGF2	Insulin-like growth factor 2	upstream	chr29:51220344-51220389 *	Hypomethylated
		downstream	chr29:51291236-51291291 *	Hypomethylated
		downstream	chr29:51301146-51301191 *	Hypomethylated
		downstream	chr29:51340334-51340379 *	Hypomethylated
		downstream	chr29:51561153-51561207 *	Hypermethylated
		downstream	chr29:51617696-51617743 *	Hypomethylated
HMGA2	High mobility group protein HMGI-C	intragenic	chr5:51775073-51775133 *	Hypermethylated
		intragenic	chr5:51778469-51778529 *	Hypermethylated
		intragenic	chr5:51805099-51805159	Hypomethylated
		intragenic	chr5:51826185-51826237 *	Hypermethylated
		intragenic	chr5:51844308-51844360 *	Hypermethylated
		intragenic	chr5:51856152-51856212 *	Hypermethylated
		intragenic	chr5:51876736-51876790 *	Hypomethylated
		downstream	chr5:51896841-51896901 *	Hypermethylated
LIN28B	Lin-28 homolog B	upstream	chr9:47109809-47109869	Hypomethylated
		intragenic	chr9:47155622-47155682 *	Hypermethylated
		intragenic	chr9:47169739-47169794 *	Hypermethylated
		downstream	chr9:47244040-47244085 *	Hypomethylated
		downstream	chr9:47246621-47246666 *	Hypomethylated
NCAM	Neurocan	intragenic	chr7:3945457-3945505 *	Hypomethylated
		intragenic	chr7:3947593-3947638 *	Hypermethylated
		intragenic	chr7:3971599-3971644 *	Hypomethylated
TRAF3IP1	TNF receptor-associated factor 3 interacting protein 1	intragenic	chr3:125334023-125334083 *	Hypomethylated
		intragenic	chr3:125356147-125356196	Hypermethylated
IGF1R	Insulin-like growth factor 1 receptor	intragenic	chr21:6733198-6733258	Hypomethylated
		intragenic	chr21:6836296-6836356	Hypermethylated

** Region contained within an interval observed to be differently methylated by the Three-point average method.*

Within the 50.6 K to 51.6 K region of the imprinted gene cluster of chromosome 29 (which contains the *IGF2*/*H19* and *KCNQ1* controlling regions), 12 sequences were detected to exhibit differential methylation in High-gain heifers according to the Categorical Comparison method. These regions included six intervals located within CpG islands. The genomic location of the probes assigned to those regions and CpG islands are depicted in **Figure 5.3**.

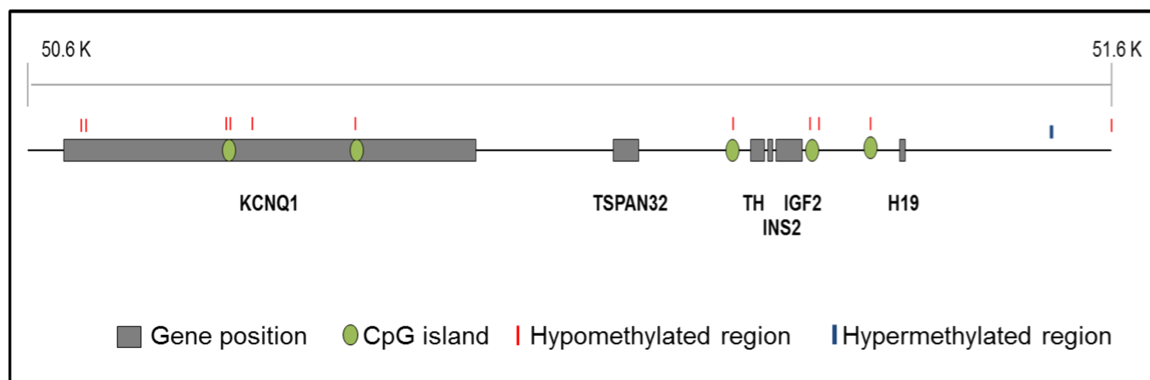


Figure 5.3 | Genomic location of sequences within the imprinted gene cluster in the bovine chromosome 29. Eleven regions exhibited hypomethylation and one region exhibited hypermethylation in High-gain heifers. *KCNQ1*: Potassium voltage-gated channel, KQT-like subfamily, member 1; *TSPAN32*: tetraspanin 32; *TH*: tyrosine hydroxylase, *INS2*: insulin 2; *IGF2*: Insulin-like growth factor 2.

Discussion

In this study, we used a customized oligonucleotide array to investigate differential methylation in genomic DNA regions containing candidate genes that appear to be involved in the nutritional programming of puberty. Using an animal model in which puberty is advanced by feeding heifers to gain body weight at a relatively

increased rate (Garcia et al., 2003; Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d), we observed a distinct pattern of methylation of genomic DNA obtained from the ARC nucleus. The selection of candidate DNA regions to be investigated in this study was based on previous studies investigating the transcriptome of the ARC in heifers exposed to a similar nutritional model (Allen et al., 2012) and other published literature (Ong et al., 2009; Fortes et al., 2010; Hawken et al., 2012; Mueller et al., 2012). Because the ARC nucleus of the hypothalamus is a major metabolic-sensing area in the brain (Cone et al., 2001), and epigenetic modifications are tissue-specific (Dindot et al., 2009), genomic DNA isolated specifically from this hypothalamic nucleus was used in the current study. Using the methylated DNA enrichment assay, we identified a number of differentially methylated genes involved in the physiological and cellular control of metabolic functions.

The methylated DNA enrichment assay method has been employed successfully in various experimental models to analyze methylation profiles of genomic DNA. This assay efficiently retrieves methylated-enriched fractions of DNA and produces results comparable to those obtained by bisulfite sequencing (Weber et al., 2005; Rauch et al., 2006; Dindot et al., 2009), although it doesn't provide the resolution of bisulfite sequencing. One advantage of the methylated DNA enrichment assay is the possibility of investigating methylation status in a large number of sequences if coupled with oligonucleotide array analysis. Therefore, it allows interrogation of intra- and inter-genic regions, including promoters and associated CpG islands (Weber et al., 2005; Rauch et al., 2006; Dindot et al., 2009). In the current study, differential methylation was

observed more frequently in intragenic regions. Furthermore, differential methylation patterns, including hypermethylated and hypomethylated regions in High-gain heifers within a given gene (e.g., *IGF2*, *NCAM*, *LIN28B*, *HMGA2* and *IGF1R*), were also observed. The differential pattern of methylation within genes may represent an additional level of control of gene expression. For instance, while methylation in the promoter is often associated with repression of gene expression (Rauch et al., 2009), methylation in regions downstream to gene sequences has been proposed to regulate polyadenylation of mRNA, suppress transcription of antisense RNA and regulate premature transcriptional termination (Rauch et al., 2009). In addition, methylated intragenic regions may regulate insulator activity (Wallace and Felsenfeld, 2007), microRNA expression (Han et al., 2007) and other complex pathways for regulation of gene transcription (Mitsuya et al., 1999).

The use of the Sliding *t*-test and the Three-point Average methods enhances the detection of highly-methylated regions of DNA because they take into consideration a group of adjacent sequences that have a tendency to exhibit similar methylation status (Weber et al., 2005). In addition, these methods can detect not only the presence of methylation, but also the magnitude of methylation within regions because the amount of methylated DNA retrieved by methylated DNA enrichment assay is proportional to the number of methylations present in the sequence (Rauch et al., 2009). Therefore, greater differences in signal intensity ($\log_2$ ratio) indicate greater differential methylation in target genomic regions between dietary groups. This approach identified the region associated with the Paternally Expressed 3 gene (*PEG3*) as a hypermethylated region in

High-gain heifers. The *PEG3* gene has been demonstrated to be imprinted (Huang and Kim, 2009) and expected to be methylated in both dietary groups. In support of this concept, the proportion of heifers exhibiting methylation of *PEG3* did not differ between the two groups when determined by the Categorical Comparison method. However, the $\log_2$ ratio of the intensities differed, indicating that the genomic region associated with *PEG3* is hypermethylated in heifers gaining body weight at accelerated rates during the juvenile period.

In contrast, the Categorical Comparison approach evaluates each sequence independently. This approach is useful to detect differential methylation that may occur in regions with low-intensity signals. However, the caveat of the categorical method is that it misses differential methylation in regions that are methylated regardless of the experimental group. Nevertheless, the use of parametrical (Sliding *t*-test and Three-point Average) and non-parametrical (Categorical Comparison) methods are expected to be complementary and enhance our ability to detect relevant differentially-methylated regions that would be otherwise overlooked. Notably, most of the intervals detected as differently methylated by the Categorical Comparison methods were contained within the intervals detected as differently methylated by one or both of the parametrical tests. These include genes related to regulation of growth and early development, such as *GHR, IGF1R, HMGA2* and *LIN28B*. However, differentially-methylated regions detected only by the non-parametrical test were observed and include genes that appeared to be unmethylated in High-gain heifers (*IGFBP2*, *ARRB1*, *DLK1* and *AXIN1*) or have differential site of methylation compared to Low-gain heifers (*IGF1R*).

The alterations in the pattern of methylation observed in the current study indicate that nutrition during the juvenile period influences the epigenome of the ARC nucleus. Because increased rates of body weight gain during the juvenile period advances the onset of puberty in heifers (Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d) and alters the pattern of gene expression in the ARC (Allen et al., 2012), epigenetic control of gene expression may be a mechanism mediating the nutritional programming of puberty. Notably, genes encoding components of the pathways involved in the regulation of growth (*GHR, IGF1R* and IGFBPs), energy metabolism (*PEG3* and *LEPR*) early development (*IGF2*), and control of pubertal onset (*HMGA2* and *LIN28B*) appear to be particularly involved.

The growth-promoting effect of growth hormone (GH) is mediated largely by GH receptor (GHR) activation of insulin-like growth factor 1 (IGF-1) synthesis and secretion in the liver (Sjögren et al., 1999). In addition, to act in peripheral tissues (Tiong and Herington, 1991), circulating IGF-1 is transported across the blood-brain barrier (Reinhardt and Bondy, 1994; Pulford and Ishii, 2001) to act as an endocrine signal to regulate hypothalamic functions (Hiney et al., 1991; Fernandez-Galaz et al., 1999; Daftary and Gore, 2003). Synthesis of IGF-1 is also observed in the brain (Hojvat et al., 1982; Murphy et al., 1987; Daftary and Gore, 2003), where IGF-1 appears to act in a paracrine/autocrine manner to control cellular function (Daftary and Gore, 2005). The high-affinity receptor for IGF-1, IGFR1, is expressed in the central nervous system (Evercooren et al., 1991). Studies have indicated that both *IGF1* and *IGF1R* mRNA (Hiney et al., 1991; Daftary and Gore, 2004), as well as the protein encoded by these

messengers (Pons et al., 1991; Hiney et al., 1996; Daftary and Gore, 2003), increase in the hypothalamus during postnatal development. Insulin-like growth factor 1 and IGFR1 have been demonstrated to influence synaptic plasticity (Fernandez-Galaz et al., 1999). Moreover, bioavailability of IGF-1 and binding of IGF-1 to its receptor are modulated by IGF binding proteins (IGFBPs; Firth and Baxter, 2002), which are produced both in the liver and in the central nervous system (Funston et al., 1995; Pfaffl et al., 2002). Therefore, regulation of expression of the various components of the growth axis may, directly or indirectly, influence hypothalamic function. The observation that nutrition during the juvenile period influences the methylation status of genes reported to be differentially-expressed in the ARC nucleus in response to dietary regimens (Allen et al., 2012) supports the hypothesis for epigenetic mechanisms play a role in controlling growth at the hypothalamic level.

In heifers, immunization against GH-releasing hormone, which decreases circulating concentrations of GH and IGF-1, delays puberty (Simpson et al., 1991a; Schoppee et al., 1996). Whether this effect is mediated by peripheral or central actions of GH and IGF-1 is unclear. The *GHR* gene is expressed in NPY neurons in the ARC nucleus (Chan et al., 1996; Kamegai et al., 1996) and GH increases *NPY* expression and NPY neuronal activity in the ARC nucleus (Kamegai et al., 1994; Minami et al., 1998). In heifers fed to gain body weight at higher rates, *NPY* expression in the ARC is reduced concurrently with a reduction in *GHR* expression (Allen et al., 2012). Therefore, if enhanced DNA methylation of the *GHR* gene, as observed in the current study, leads to repression of *GHR* expression in High-gain heifers, diminished GHR-mediated

stimulation may contribute to reduced abundance of *NPY* mRNA in the ARC nucleus of juvenile heifers gaining body weight at increased rates (Allen et al., 2012; see Chapter III of this dissertation). In addition to regulating feed intake and energy expenditure (Woods et al., 1998), NPY also has a role in regulating reproductive functions. The secretion of GnRH in cows is inhibited by NPY (Gazal et al., 1998), and female rats chronically treated with NPY exhibit delayed puberty (Catzeflis et al., 1993). In this context, epigenetic modifications that regulate GH and IGF-1 actions in the ARC nucleus may lead to a reduction in *NPY* expression in High-gain heifers. Diminished inhibition of GnRH secretion by NPY is conducive to early pubertal development as observed in heifers fed to gain body weight gain at increased rates during the juvenile period (Gasser et al., 2006a; Gasser et al., 2006b; Gasser et al., 2006c; Gasser et al., 2006d).

The leptin receptor (*LEPR*) and *PEG3* genes were observed to be hypermethylated in High-gain heifers in this study. Leptin, a hormone secreted primarily by adipocytes, is believed to play a major role in timing the onset of puberty in heifers (Garcia et al., 2003; Zieba et al., 2005). Leptin signals nutritional status to the central nervous system via direct modulation of NPY neurons (Hakansson et al., 1998). Therefore, altered methylation of *LEPR* may influence its expression in High-gain heifers. However, no differential expression of *LEPR* was observed in the studies by Allen et al. (2012); therefore, the relevance of the differential methylation of *LEPR* observed in this study is unclear. Similarly, *PEG3* was also observed to be hypermethylated in High-gain heifers. This gene encodes a zinc finger protein that has

been involved in leptin resistance and regulation of expression of *NPY* (Curley et al., 2005). Interestingly, mutations in the *PEG3* gene have been associated with a delay in the onset of puberty in mice (Curley et al., 2004). Therefore, altered methylation of *LEPR* and *PEG3* appears to reinforce potential mechanisms that regulate neuronal activity in the ARC.

Another functionally-relevant group of genes that was observed to be differently methylated in this study include the *LIN28B* and *HMGA2*. Genome-wide association studies identified genetic variants of *LIN28B* influencing age at menarche in girls (He et al., 2009; Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009; Ong et al., 2011). In goats, polymorphism in *LIN28B* was also associated with puberty (Cao et al., 2013). In mice, overexpression of *LIN28A* (a homologue of *LIN28B*) leads to delayed puberty (Zhu et al., 2010). In rats and monkeys, the hypothalamic expression of *LIN28B* decreases from neonatal to juvenile development and is minimal during the pubertal period (Sangiao-Alvarellos et al., 2013). In the current study, intragenic sequences in *LIN28B* were observed to be hypermethylated whereas downstream and upstream related sequences were observed to be hypomethylated in High-gain heifers. Although it is uncertain whether this differential pattern of methylation influences *LIN28B* expression, hypomethylation of a putative transcription factor binding site within *LIN28B* has been implicated in upregulation of *LIN28B* expression (Viswanathan et al., 2009). In contrast, methylation of the promoter region of the *LIN28* has been associated with inhibition of expression (Dansranjavin et al., 2009). Hence, an altered pattern of methylation within

LIN28B and in regulatory regions may represent a mechanism by which rate of body weight gain during the juvenile period influences the age at puberty.

The product of the *LIN28B* gene is a DNA binding protein that inhibits transcription of the micro RNA let-7 (Viswanathan et al., 2008). Let-7 is involved in mRNA degradation and translation silencing (Bagga et al., 2005; Pillai et al., 2005), and in progression of cellular differentiation (Johnson et al., 2007). Let-7 also regulates the expression of *IGF2BP1, KRAS* and *HMGA2* (Polesskaya et al., 2007; Boyerinas et al., 2008; Sangiao-Alvarellos et al., 2013; Shyh-Chang and Daley, 2013). The *IGF2BP1* gene encodes an RNA binding protein which represses the translation of *IGF2* (Nielsen et al., 1999). The oncogene *KRAS* has been observed to play a role in regulating the cellular metabolism of glucose and glutamine (Gaglio et al., 2011). High mobility group AT-hook 2 (HMGA2) is a chromatin-associated protein that modulates gene transcription by altering chromatin structure (Reeves, 2001). Expression of *HMGA2* is upregulated in fetal neural stem cells and declines with cell differentiation (Zhou et al., 1995; Anand and Chada, 2000; Nishino et al., 2008). In addition, translation of *HMGA2* mRNA is inhibited by let-7 (Lee and Dutta, 2007). Studies using mouse models indicate that the Lin28/Let-7/Hmga2 pathway is critical for the control of glucose metabolism (Zhu et al., 2011). Our observation that nutritional status influences the pattern of methylation of *LIN28B* and *HMGA2* in the ARC nucleus supports the involvement of these molecules in the control of neuroendocrine functions at the hypothalamic level. Moreover, studies investigating associations between single nucleotide polymorphisms

and age at puberty in cattle identified *HMGA2* as part of a network of transcription factors that appear to regulate age at puberty (Fortes et al., 2011).

Differential methylation was also observed in genomic regions contained in the imprinted gene cluster of the distal end of bovine chromosome 29 (orthologous region in chromosome 7 of mice and chromosome 11 of humans). These regions contain imprinted genes demonstrated to be critical for the regulation of growth and development of the conceptus (Reik and Walter, 2001). Disruption of normal methylation pattern in this region is related to excessive fetal growth in humans (Beckwith-Wiedemann syndrome; Gicquel et al., 2003) and the large offspring syndrome in cattle (Hori et al., 2010; Robbins et al., 2012). The pattern of DNA methylation regulates the imprinted status and the expression of genes in this region. In cattle (Robbins et al., 2012), humans (Vu et al., 2000; Mancini-DiNardo et al., 2003) and mice (Thorvaldsen et al., 1998; Mancini-DiNardo et al., 2006), two differently methylated regions (imprinting control regions) are independent regulatory sites for imprinting-driven expression of the genes contained in this cluster. One of the imprinting control region located within the 10th intron of the *KCQN1* gene (Robbins et al., 2012) was observed to be less methylated in High-gain heifers. Methylation of *KCQN1* occurs in the maternal chromosome and unmethylated paternal allele regulates silencing of neighboring genes in the paternal chromosome (Fitzpatrick et al., 2002). Loss of methylation in this imprinted control region in the maternal allele is associated with Beckwith-Wiedemann syndrome in humans (Lee et al., 1999; Weksberg et al., 2003). Therefore, decreased methylation of this region in the ARC nucleus appears to be

associated with the central control of nutrient utilization and may be involved in the mechanisms by which elevated body weight gain during the juvenile period facilitates early onset of puberty in heifers.

A second imprinting control region occurs in the coding region for the *IGF2* and *H19* genes (Thorvaldsen et al., 1998). Methylation of this region leads to expression of *IGF2* and silencing of *H19*, while no methylation leads to the reverse pattern of expression (Bell and Felsenfeld, 2000;Hark et al., 2000). In bovine fetal brains, monoallelicaly (Dindot et al., 2004) or biallelicaly (Curchoe et al., 2005) expression of *IGF2* has been described. In mature cattle, *IGF2* is observed to be expressed monoallelicaly (Curchoe et al., 2005). In this current study, we observed a region upstream to the imprinting control region in the *IGF2* that was hypomethylated in High-gain heifers. In the mouse, this region has been characterized to function as an enhancer for the expression of *IGF2* (Jones et al., 2001). Deletion of this region reduces expression of *IGF2* in the brain and enhances adiposity (Jones et al., 2001). The reduced methylation in this region in High-gain heifers would be expected to lead to increased expression of *IGF2*. Because IGF-2 is involved in the control of cellular metabolism via its action through IFG1R and insulin receptor (INSR; Nakae et al., 2001),the nutritional influence on methylation pattern of *IGF2, IGF1R* and *INSR* observed in the current study may be an integral regulatory mechanism by which metabolic signals program the hypothalamic control of energy expenditure.

Collectively, the results of the current study support the hypothesis that rate of body weight gain during the juvenile period alters the methylation pattern of genomic

DNA obtained from cells of the ARC nucleus. The genes that were observed to be differentially-methylated are known to be involved in the physiological and cellular control of growth and metabolism. In addition, they are also involved in the regulation of reproductive function. Therefore, DNA methylation may be an additional mechanism by which expression of genes within the ARC nucleus is regulated for the fine control of growth and development in maturing heifers.

CHAPTER VI

SUMMARY AND CONCLUSION

Accelerated body weight gain during the juvenile period advances the onset of puberty in heifers, an effect that appears to involve earlier escape from estradiol negative feedback control of gonadotropin secretion. Onset of puberty is characterized by an enhanced frequency of release of GnRH, likely resulting from alterations in neuronal inputs that control GnRH secretion. In the current studies, it was hypothesized that high rates of body weight gain between 4 and 8 mo of age in heifers would lead to functional changes in the hypothalamus that are conducive to diminished inhibition and increased stimulation of GnRH secretion. The studies reported herein support this hypothesis by demonstrating that expression of *NPY* in the arcuate nucleus and NPY innervation of GnRH neurons is diminished in heifers that gained weight at high rates during the juvenile period. Because NPY is a major neurohormone mediating the metabolic control of reproductive function and inhibits secretion of GnRH in cattle, the results of the current studies indicate that decreased NPY inhibitory tone favors early onset of puberty in heifers. In addition, decreased NPY innervation of GnRH neurons was observed, particularly in GnRH neurons located in the mediobasal hypothalamus, a GnRH neuronal population whose activation that has been involved in pulsatile release of LH.

The neuropeptide kisspeptin is considered a critical signal for pubertal development in mammalian species. Kisspeptin neurons are sensitive to estradiol and metabolic signals. Undernutrition and estradiol inhibits *KISS1* expression in the ARC.

However, contrary to our hypothesis, an accelerated rate of body weight gain in young heifers was associated with down-regulation of *KISS1* in the mid ARC. This inhibition of *KISS1* expression in High-gain heifers is speculated to occur in response to changes in the sensitivity to estradiol inhibition that has been characterized in heifers undergoing pubertal transition. Although decreased NPY innervation of GnRH neurons in High-gain heifers was observed in these studies, no differences in NPY innervation of kisspeptin neurons were detected between groups. Therefore, it is still unclear whether kisspeptin neurons have a major role in mediating the nutritional programming of accelerated puberty in heifers.

Epigenetic alterations are major regulatory mechanisms of cellular functions. Epigenetic mechanisms involved in the control of gene expression include methylation of specific DNA sequences in the genome. In the current studies, the methylation profile of target genomic sequences of DNA isolated from the ARC nucleus was investigated. Differential methylation of genes associated with modulation of growth and metabolism (*GHR, IGF2*, *IGF1R, LEPR*) and age at puberty (*PEG3*, *LIN28B* and *HMGA2*) was observed to occur between heifers gaining weight at high and low rates during the juvenile period. These observations support the hypothesis that alteration in methylation of genomic DNA in the ARC is an additional regulatory mechanism important for the regulation in the expression of genes that are relevant for the nutritional control of pubertal development in heifers.

In conclusion, an accelerated rate of body weight gain during the juvenile period promotes functional alterations in the hypothalamus that are conducive for facilitating

the early onset of puberty in heifers. These alterations affect neuronal pathways and regulatory cellular molecules that are involved directly or indirectly in the control of GnRH release.

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