Kisspeptin neuronal networks in pubertal development of domestic female ruminants

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Summary

The pubertal activation of high-frequency episodic pulses of GnRH is believed to occur as a result of a change in the balance between inhibitory and excitatory stimuli to GnRH neurons. Kisspeptin neurons have been identified as major components of the pathway that regulates GnRH neuronal activity and appear to mediate the effects of estradiol in the control of GnRH secretion. The influence of nutrition on timing the onset of puberty may also involve kisspeptin neurons. Neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons in the hypothalamus interact with kisspeptin neurons in a network that is likely to mediate the nutritional and gonadal steroid regulation of reproductive function. Pre- and postnatal programming of these neuronal networks may be a primary mechanism by which nutrition and endocrine factors time the onset of a pubertal pattern of GnRH secretion. The genetic components underlying the function of these networks have begun to be revealed with the availability of high-throughput technologies and computational tools. A complex, highly interactive network of regulatory genes appears upstream to *KISS1* and probably involves multiple cellular phenotypes. Characterization of the cellular location, temporal activation, and biological function of the various molecular components of the genetic network that regulate kisspeptin neuronal pathways will be essential for a full understanding of the role of *KISS1* in the process of pubertal maturation.

Introduction

Age at first parturition has a great impact on lifetime productivity of food-producing mammals, including ruminants. Therefore, understanding the mechanisms that regulate the onset of puberty and the establishment of regular estrous cycles in domestic ruminant species is critical for optimizing reproductive efficiency in production systems. It is well established that the onset of regular ovulatory cycles follows the maturation of the reproductive neuroendocrine axis and the establishment of a pubertal pattern of episodic release of GnRH (Foster & Jackson 2006).

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The pubertal activation of high-frequency pulsatile secretion of GnRH is believed to occur as a result of a decreased inhibition and increased excitation of GnRH neurons by afferent signals that act directly or indirectly to regulate GnRH release. Among these signals, kisspeptin has been demonstrated to be essential. In the past decade, a number of studies have supported the precept that kisspeptin is a potent stimulator of gonadotropin secretion in various species, including ruminants (Messager *et al* 2005, Kadokawa *et al* 2008). It also appears that kisspeptin neurons integrate endocrine and metabolic signals that mediate the nutritional control of reproductive neuroendocrine function (Castellano *et al* 2005). However, a more thorough understanding of the role of kisspeptin in the process of pubertal development is essential before kisspeptin, and kisspeptin receptor agonists and antagonists, can be utilized effectively in novel strategies to control the timing of the onset of puberty and improve reproductive efficiency. In this article, we present an overview of the involvement of kisspeptin neurons, and the associated neuronal network, on the control of GnRH secretion during pubertal development with emphasis in domestic female ruminants.

Neuroendocrine control of the onset of puberty

Puberty in females can be defined as the first ovulation followed by an estrous cycle of normal length for the species. Initiation of a high-frequency pattern of pulsatile release of GnRH, and consequently LH, is considered a critical neuroendocrine event for the support of final stages of follicular growth and maturation, elevated gonadal steroidogenesis and first ovulation (Foster & Jackson 2006). In ruminants, first ovulation during pubertal transition is often followed by a short luteal phase (Berardinelli *et al* 1979). Although ovulation can be induced in prepubertal females by pharmacological treatments, a return to an anovulatory state is common if not accompanied by maturation of the neuroendocrine axis (Redmond *et al* 2011a).

The presence of the gonads is a major determining factor for maintenance of infrequent episodic release of LH during the prepubertal period. Ovariectomy leads to an increase in the frequency in LH release, and estradiol replacement maintains LH pulsatility similar to that of intact, prepubertal females (Day *et al* 1984, Ebling *et al* 1990). Therefore, estradiol is the major gonadal hormone maintaining the release of LH at a pattern typical of the prepubertal period.

Role of estradiol positive and negative feedback

Heightened sensitivity of the reproductive neuroendocrine axis to estradiol negative feedback maintains the pulsatile release of LH during the infantile and juvenile periods at a low frequency. As the female matures, the sensitivity to estradiol inhibition attenuates and the frequency of release of LH increases. This developmental change has been observed in ovariectomized, estradiol-replaced heifers and lambs exhibiting increases in the frequency of LH release concurrent with the increase in LH pulsatility observed in peripubertal, intact females (Day *et al* 1984, Ebling *et al* 1990). Therefore, a reduction in estradiol negative-feedback during pubertal transition plays a critical role in the maturation of the reproductive neuroendocrine axis. Enhanced gonadotropin stimulation leads to increased circulating concentrations of estradiol, which in turn, triggers a surge in GnRH/LH release that causes ovulation. The mechanisms by which estradiol controls reproductive function include genomic and non-genomic actions involving the classical estrogen receptors ESR1 (alpha) and ESR2 (beta), the recently-characterized membrane receptor G protein-coupled estrogen receptor 1 (formerly GPR30), and the putative membrane receptors mER-Gαq and ER-X (reviewed by Sinchak &

Wagner 2012). Studies in mice indicate that the actions of estradiol in the control of release of LH appear to be mediated primarily by ESR1 (Dorling *et al* 2003). Because GnRH neurons in sheep do not appear to contain ESR1 (Lehman & Karsch 1993), estradiol regulation of episodic secretion of GnRH is likely mediated by estradiol-sensitive afferent pathways to GnRH neurons.

Nutritional and metabolic control of pulsatile GnRH release

The influence of nutrition on the reproductive development of domestic female ruminants is well established. Feed restriction during the juvenile period delays puberty (Foster & Olster 1985, Day *et al* 1986) primarily by inhibiting the pulsatile release of GnRH (I'Anson *et al* 2000). In contrast, increased rate of body weight gain and adiposity during the juvenile period advances the onset of puberty (Gasser *et al* 2006). The interactive influence of nutrition and estradiol on timing pubertal onset is also evident. Inhibition of pulsatile secretion of LH by undernutrition is enhanced in ovariectomized ewe lambs treated with estradiol (Foster & Olster 1985) and the early onset of puberty in heifers fed to gain body weight at high rates is associated with attenuation of estradiol negative feedback (Gasser *et al* 2006).

Hormones and metabolic factors have been implicated in signaling nutritional status to the central control of reproduction. Among these factors, leptin, an adipocyte-derived hormone, has generated great interest. Although early studies in mice and rats indicated that leptin was able to advance puberty, later studies in laboratory rodents and cattle demonstrated that leptin alone is insufficient to trigger puberty (Cheung *et al* 2001, Maciel *et al* 2004, Zieba *et al* 2004). Nevertheless, leptin is considered a necessary signal for normal pubertal development. The mechanism by which leptin exerts a permissive effect remains to be fully determined, but likely involves neurons located in the arcuate (ARC) nucleus (Satoh *e al* 1997) and possibly the premammillary region of the hypothalamus (Donato *et al* 2011). Neurons in these areas are proposed to relay leptin signals to the cellular network that controls GnRH neuronal function. Direct effects of leptin on GnRH neurons are unlikely because conditional deletion of functional leptin receptor gene in GnRH neurons does not impair reproduction in mice (Quennell *et al* 2009). Among the potential mediators of leptin action, neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons in the ARC are primary candidates. Kisspeptin neurons may also mediate the effects of leptin in the regulation of reproductive neuroendocrine functions.

Kisspeptin neurons as mediators of the activation of GnRH secretion during pubertal development

Kisspeptin as a GnRH secretagogue

A role for kisspeptin in the regulation of reproductive function was revealed with the report that mutations in the gene encoding kisspeptin receptor (*KISS1R*) was associated with disruption of normal pubertal development in humans and mice (de Roux *et al*. 2003, Seminara *et al*. 2003). Two populations of kisspeptin neurons are observed in the brain of mammals with neuronal cell bodies located in the preoptic (POA)/rostral periventricular (PeV) area and in the ARC nucleus (Franceschini *et al*. 2006, Smith *et al*. 2007, Ohkura *et al* 2009). These two populations are biochemically and functionally distinct (Goodman *et al*. 2007) and may have discrete roles in regulating GnRH secretion.

Kisspeptin is produced in various isoforms cleaved from a precursor peptide (Kotani *et al* 2001), but the decapeptide corresponding to the C-terminus has full biological activity.

Kisspeptin is a potent stimulator of gonadotropin release (Messager *et al* 2005, Kadokawa *et al* 2008), an effect that appears to occur through stimulation of GnRH secretion (Messager *et al* 2005, Arreguin-Arevalo *et al* 2007). Direct effects of kisspeptin in GnRH neurons is supported by the observations that *KISS1R* is present in GnRH neurons (Smith *et al* 2011), kisspeptin projections are in close proximity to GnRH neurons (Smith *et al* 2008a), and kisspeptin increases firing potentials of GnRH neurons in tissue preparations (Han *et al* 2005).

The kisspeptin stimulation of GnRH release appears critical for pubertal activation of the reproductive neuroendocrine axis. Exogenous administration of kisspeptin advances the onset of puberty in rats (Navarro *et al* 2004) and mice (Han *et al* 2005), whereas treatment with a kisspeptin antagonist delays the onset of puberty (Pineda *et al* 2010). In prepubertal ewe lambs, intermittent injections of kisspeptin stimulated the episodic release of LH, an effect that was associated with increased circulating concentrations of estradiol and ovulation/follicular luteinization (Redmond *et al* 2011a). The stimulatory effect of kisspeptin on gonadotropin secretion appears to be developmentally regulated because the kisspeptin-induced release of GnRH and LH increases with age (Castellano *et al* 2006). Developmental changes are also observed in kisspeptin innervation of GnRH neurons. As ewe lambs mature, the number of kisspeptin neuronal projections in close proximity to GnRH neurons increases (Nestor *et al* 2012). These morphological alterations may be essential for the role of kisspeptin in the pubertal activation of episodic release of GnRH. Stimulation of gonadotropin release by direct effects of kisspeptin in the adenohypophysis is also possible (Smith et al., 2008b; Suzuki *et al* 2008); however, it is unclear whether it is physiologically relevant for the control of gonadotropin release (Arreguin-Arevalo *et al* 2007, Smith *et al* 2008b).

The absolute requirement of kisspeptin for normal reproductive function has been challenged by studies demonstrating that deletion of kisspeptin cells during fetal development does not impair pubertal development and fertility in mice (Mayer & Boehm 2011). These studies have also demonstrated that when ablation of kisspeptin cells is performed postnatally, fertility is compromised. Although the ablation of kisspeptin neurons in the hypothalamus was not complete, these studies indicate the potential development of compensatory mechanisms that may overcome the absence of normal kisspeptin signaling in mice. Another possibility is that a small number of kisspeptin neurons is sufficient for sustaining GnRH secretory activity.

The functional distinction of the two kisspeptin neuronal populations is demonstrated by the heterogeneity in colocalization of various neuropeptides in kisspeptin neurons. Kisspeptin neurons located in the ARC nucleus colocalize neurokinin B and dynorphin (Goodman *et al* 2007, Wakabayashi *et al* 2010), but those located in the POA do not. Heterogeneity in kisspeptin afferent projections to brain areas and target cells is also observed between the populations of kisspeptin neurons (Yeo & Herbison 2011). Physiologically, the relevance of this functional distinction is demonstrated by the response to local administration of kisspeptin. Kisspeptin injection in both the POA and ARC stimulates the release of LH in ovariectomized, estradiol-replaced rats, and the injection of kisspeptin antagonist into the ARC inhibits the pulsatile release of LH (Li *et al* 2009). However, this inhibitory effect is not observed when the kisspeptin antagonist is injected into the POA. Estradiol regulation of *KISS1* expression is also distinct between the two populations of kisspeptin neurons (Smith *et al* 2005; Smith *et al* 2007) and the distinction appears to constitute the pathway by which estradiol controls the episodic and surge modes of GnRH secretion.

Kisspeptin neurons as mediators of estradiol control of gonadotropin secretion

Kisspeptin neurons express receptors for gonadal steroid hormones and are direct targets for estradiol. In mice (Smith *et al* 2005), rats (Takase *et al* 2009) and pigs (Tomikawa *et al*

2010), estradiol increases *KISS1* expression in the rostral periventricular region. In ewes, the number of *KISS1* mRNA-containing cells in the POA/PeV increases during the late-follicular phase (Smith *et al* 2009), a time in which circulating concentrations of estradiol are expected to be elevated. The stimulatory actions of estradiol on *KISS1* expression in the POA led to the hypothesis that estradiol positive feedback stimulation of the preovulatory surge of LH is mediated by activation of kisspeptin neurons in that region. In sheep, this notion is supported by studies indicating that kisspeptin neurons in the POA/PeV are activated (based on the presence of FOS immunoreactivity) during the preovulatory GnRH/LH surge (Hoffman *et al* 2011, Merkley *et al* 2012). However, discrepancies in the extent of the involvement of ARC kisspeptin neurons in the estradiol positive feedback exist. The studies by Merkley *et al* (2012) indicate that kisspeptin neurons in the ARC are also activated in response to estradiol positive feedback. In contrast, Hoffman *et al* (2011) did not detect FOS protein in kisspeptin neurons in the ARC at the time of the peak of the GnRH surge (26 h after estradiol implants were inserted). Methodological differences and timing of tissue collection in relation to the estradiol stimulation of the GnRH/LH surge may have been sources of inconsistency between studies. Therefore, the extent of the involvement of kisspeptin neurons in the estradiol positive feedback remains to be fully elucidated.

There is greater consistency in support of a role for kisspeptin neurons in the ARC in mediating estradiol negative feedback. At low, basal concentrations, estradiol inhibits *KISS1* expression (Smith *et al* 2007, Takase *et al* 2009). Estradiol withdrawal in ovariectomized, estradiol-replaced ewes is associated with an acute increase in the number of kisspeptin-immunoreactive cells in the ARC (Merkley *et al* 2012). In addition, saporin ablation of kisspeptin neurons in the ARC of rats markedly reduces the elevation in circulating concentrations of LH that follows ovariectomy (Mittelman-Smith *et al* 2012). Because the mediobasal hypothalamus has been demonstrated to be a major area for estradiol negative feedback regulation of gonadotropin secretion (Caraty *et al* 1998), the involvement of estrogen receptor-containing kisspeptin neurons in the ARC is a compelling pathway mediating estradiol inhibition of GnRH release.

Estradiol appears to have a major influence on the postnatal activation of kisspeptin synthesis. An increase in kisspeptin expression and immunoreactivity in the ARC is evident in rats from the early infantile period to adulthood (Takase *et al* 2009, Desroziers *et al* 2012). Such effects appear to require estradiol because kisspeptin immunoreactivity in the ARC is diminished in aromatase knockout mice (Clarkson *et al* 2009). In female sheep, kisspeptin immunoreactivity in the ARC is increased in postpubertal compared to prepubertal ewes (Nestor *et al* 2012). The activation of *KISS1* expression during pubertal development has been demonstrated in various mammalian species including sheep (Redmond *et al* 2011a). Although increased *KISS1* expression is observed in both POA/PeV and ARC populations, the timing of the activation of gene expression within each population appears distinct. In ovariectomized, estradiol-replaced ewe lambs, the number of cells containing *KISS1* mRNA in the POA/PeV increases during the juvenile period; however, this increase is not associated directly with changes in the pattern of pulsatile LH release (Redmond *et al* 2011b). In contrast, the increase in the number of *KISS1*-expressing cells in the ARC is associated with increased frequency of LH release (Fig. 1). This observation indicates that the developmental decrease in sensitivity to estradiol negative feedback, and consequent increased frequency of LH release, is associated with activation of *KISS1* expression in the ARC. This hypothesis is supported by observations in the rat (Takase *et al* 2009). However, in contrast to ewe lambs, the increase in *KISS1* expression during this period in female rats was observed in both POA/rostral PeV and ARC regions. The role of estradiol and ESR1 signaling in kisspeptin neurons was demonstrated in studies using conditional knockout of *ESR1* in kisspeptin neurons (Mayer *et al* 2010). In that study, lack of ERS1 signaling

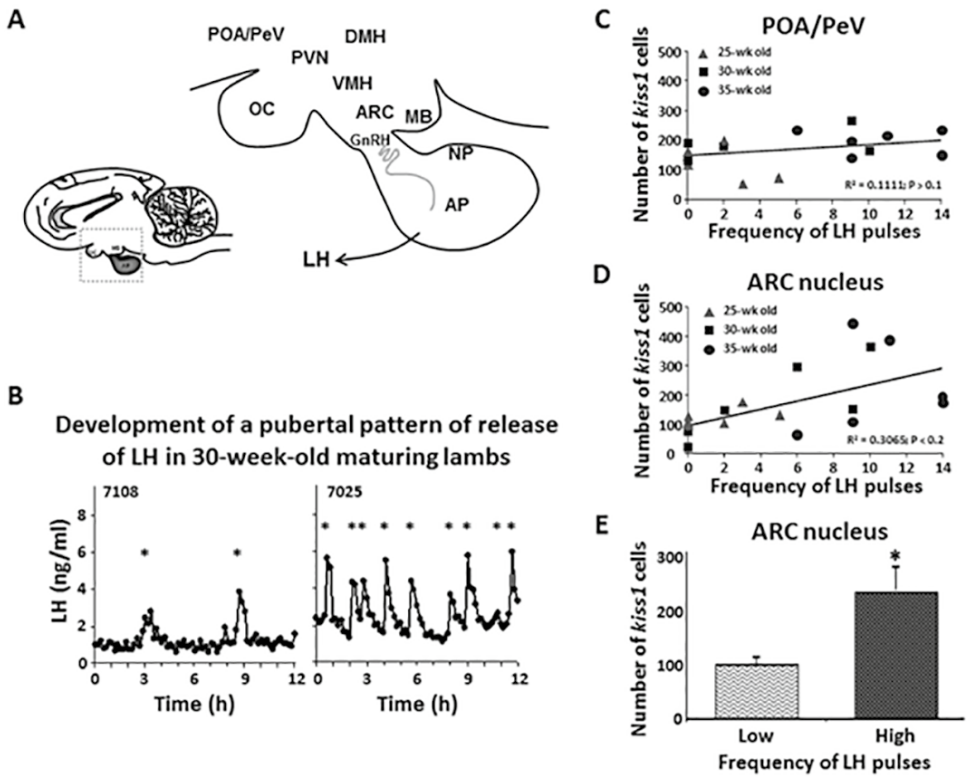

Fig. 1. Activation of *KISS1* during pubertal development of a high-frequency, pulsatile release of LH in ewe lambs. A) Drawing representing the preoptic area (POA) and hypothalamus (boxed area at the base of the brain), and the location of various hypothalamic regions and their anatomical relationship with the adenohypophisis (AP) and neurohypophysis (NP; magnified drawing). *KISS1* is expressed in the POA/periventricular area (PeV) and in the arcuate (ARC) nucleus. Gonadotropin-releasing hormone (GnRH) released in the hypothalamic-hypophyseal vasculature stimulates the release of LH from gonadotropes in the AP. B) Pattern of secretion of LH in ewe lambs at 30 weeks of age exhibiting low (left) and high (right) frequency of LH release. C, D) Regression analysis of the number of *KISS1* mRNA-containing cells in the POA/PeV (C) and ARC (D) as a function of the frequency of LH pulses detected in developing ewe lambs. The number of *KISS1* cells increase as the frequency of LH pulses increase in the ARC, but not in the POA/PeV. E) Number of *KISS1* cells in the ARC is greater in ewe lambs exhibiting a high frequency of LH release. (Modified from Redmond *et al* 2011b). DMH, dorsomedial hypothalamus; MB, mammillary body; OC, optic chiasm; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus.

in kisspeptin neurons was associated with advanced puberty in mice. This observation supports the hypothesis that a reduction in estradiol negative feedback in kisspeptin neurons is critical for timing the onset of puberty.

Integrative role of kisspeptin neurons in hypothalamic pathways mediating the metabolic control of pubertal development

Nutritional and metabolic signals have been shown to regulate kisspeptin neuronal function. Inhibition of LH release by undernutrition is associated with decreased expression of *KISS1* in

rats (Castellano *et al* 2005). Kisspeptin administration in fasted rats restores the release of LH and alleviates the delay in pubertal onset (Castellano *et al* 2005). In addition, the observation that leptin increases *KISS1* expression in lean, ovariectomized ewes (Backholer *et al* 2010) indicates that leptin's action in the regulation of reproductive neuroendocrine function may involve the kisspeptin pathway. However, the functional relevance of kisspeptin neurons as direct targets of leptin is unclear. Leptin receptor mRNA has been observed in kisspeptin neurons (Smith *et al* 2006, Backholer *et al* 2010), but kisspeptin neurons do not appear to be activated by leptin (Quennell *et al* 2011). In addition, targeted deletion of leptin receptor in kisspeptin neurons does not affect the onset of puberty or fertility in mice (Donato *et al* 2011). Therefore, intermediary pathways may integrate the metabolic regulation of kisspeptin neuronal activity.

Neuronal circuits encompassing NPY and POMC cells in the hypothalamus are considered major pathways by which nutritional signals control the reproductive neuroendocrine axis. These neurons are responsive to changes in nutritional status, an effect that involves leptin signaling. Neuropeptide Y and POMC neurons in the ARC contain leptin receptor (Iqbal *et al* 2001), and leptin inhibits the expression of *NPY* and stimulates the expression of *POMC* (Backholer *et al* 2010). In ruminants, NPY has a predominantly inhibitory effect on the release of GnRH/LH in the presence and absence of estradiol (McShane *et al* 1992, Gazal *et al* 1998, Ichimaru *et al* 2001). In contrast, the POMC-derived peptide, melanocyte-stimulating hormone alpha (αMSH), appears to stimulate GnRH release because the melanocortin receptor agonist, melanotan II, increases the release of LH in sheep (Backholer *et al* 2009). The effects of NPY and αMSH might occur, at least in part, through direct actions on GnRH neurons. In rats, projections containing NPY and POMC are in apposition to GnRH neurons (Leranth *et al* 1988, Li *et al* 1999), and in mice, both NPY and αMSH affect GnRH neuronal depolarization frequency in a manner consistent with excitatory (αMSH) and inhibitory (NPY) effects (Roa & Herbison 2012).

Neuropeptide Y and POMC neuronal projections to kisspeptin neurons may also have a functional relevance for the nutritional control of reproductive neuroendocrine function. Projections containing NPY are observed in apposition to kisspeptin neurons in sheep (Backholer *et al* 2010) and these projections may form synaptic inputs (Amstalden *et al* 2011). Projections containing POMC have also been observed in close proximity to kisspeptin neurons in sheep (Backholer *et al* 2010) and cattle (RC Cardoso, GL Williams & M Amstalden, unpublished observations). In a study using anestrous ewes primed with progesterone, melanotan II stimulated *KISS1* expression in the POA, but inhibited expression in the ARC (Backholer *et al* 2009). Projections containing kisspeptin are observed in proximity to NPY and POMC neurons (Backholer *et al* 2010), and the physiological relevance of these reciprocal projections have begun to be revealed. Intracerebroventricular injection of kisspeptin has been observed to enhance *NPY* and inhibit *POMC* expression in the ARC (Backholer *et al* 2010). In mice, kisspeptin increases excitation of POMC neurons and inhibit depolarization in NPY neurons (Fu *et al* 2010). Although central administration of kisspeptin has been observed to decrease food intake in mice (Stengel *et al* 2011), such an effect has not been observed in rats (Castellano *et al* 2005) or sheep (Clarke *et al* 2012). Therefore, a role for kisspeptin on the control of nutrient homeostasis and energy expenditure via NPY and POMC neurons, particularly during pubertal development, is still unclear.

Functional programming of the kisspeptin neuronal network for timing the onset of puberty

Hypothalamic pathways involved in the control of neuroendocrine functions are established during development; however, neuronal projections and connections among cells in the

hypothalamus continue to develop postnatally (Coupé *et al* 2010). Steroid hormones and metabolic factors have a major impact on this process. By altering early postnatal nutrition in mice via manipulations of litter size, Caron *et al* (2012) observed that kisspeptin projections in adults are impacted by undernutrion during the infantile period. This effect may involve leptin signaling. An early postnatal elevation in circulating concentrations of leptin has been proposed to be a critical event programming neuroendocrine functions in mammals (reviewed by Bouret 2010). Regulation of gene expression in the hypothalamus and differential innervation of hypothalamic areas by NPY and POMC neurons are some of the apparent effects of leptin during the early postnatal period (Coupé *et al* 2010). The postnatal increase in leptin is largely associated with maternal nutrition during pregnancy (Coupé *et al* 2010, Long *et al* 2011) and early postnatal nutrition of the offspring (Ehhardt *et al* 2003). Overall, these observations indicate that neurons within the kisspeptin network are predisposed pre- and postnatally to programming by endocrine and metabolic factors.

To better understand the role of infantile and juvenile nutrition in the programming of neuroendocrine functions controlling the onset of puberty, we have conducted studies using an animal model developed by Gasser *et al* (2006) in which age at puberty is advanced in heifers fed high-concentrate diets to promote elevated body weight gain between 4 and 8 mo of age. In a study in which heifers were fed to gain body weight at high (0.9 kg/day) or low (0.45 kg/day) rate from approximately 4 to 6.5 months of age, we observed an intricate regulation in the expression of genes in the ARC concurrent with increased circulating concentrations of leptin in high-gain heifers (Allen *et al* 2012). Genes detected to be differentially regulated in this study (Fig. 2) included those involved in the nutritional and metabolic control of feed intake (e.g., *NPY* and *POMC*). In addition, differential expression of genes involved in neuronal remodeling, axonal growth, synaptic vesicle transport and synaptic transmission is also observed and appears to be relevant for the nutritional programming of puberty. Using a similar nutritional model, we have investigated whether nutrition during the infantile and juvenile period influences kisspeptin, NPY and αMSH projections in various regions of the hypothalamus and toward GnRH neurons. In juvenile heifers, high rate of body weight gain was associated with decreased expression of *NPY* (BRC Alves, GL Williams & M Amstalden, unpublished observations) and increased expression of *POMC* in the ARC (RC Cardoso, GL Williams & M Amstalden unpublished observations). Although, no clear differences in NPY innervation of kisspeptin neurons were observed, the proportion of kisspeptin neurons in close proximity to αMSH-containing fibers was increased in heifers gaining body weight at high rates. Decreased NPY innervation of GnRH neurons, particularly those located in the MBH, was also observed (BRC Alves, GL Williams & M Amstalden, unpublished observations). In another study in which a nutritional regimen was used to promote differential body weight gain and adiposity in peripubertal ewe lambs, we observed that the proportion of GnRH neurons located in the MBH in close apposition with three or more kisspeptin varicosities was greater in ewe lambs growing at high rates (M Bedenbaugh & M Amstalden, unpublished observations). However, at that stage of development, only a small number of GnRH neurons was observed to be in close proximity to kisspeptin fibers. Nevertheless, a complex interaction among kisspeptin, NPY and POMC neurons appears to exist and the function of this neuronal network may be regulated during infantile and juvenile development. Because reciprocal projections among kisspeptin, NPY and POMC neurons are evident, changes in the neuronal activity and connectivity within cells of this neuronal network is likely to regulate the output of downstream signals including GnRH secretory activity.

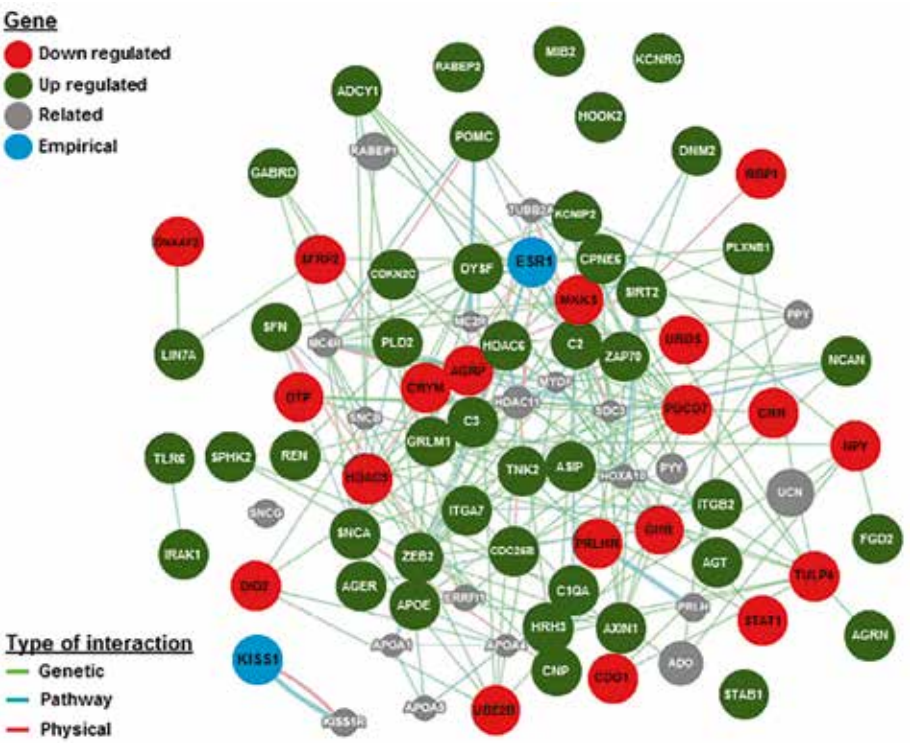

Fig. 2. Network of genes expressed in the arcuate nucleus of 6.5 month-old prepubertal heifers covering gene ontology (GO) terms for cell organization and morphogenesis, response to hormones, and cell metabolism. An increased rate of body weight gain during the juvenile period leads to increased (up-regulated, green) and decreased (down-regulated, red) expression of genes involved in control of a number of biological functions including feeding behavior, response to nutrients and hormones, cell maturation, cell-cell communication, membrane organization, and synaptic transmission. Genes illustrated in gray represent genes highly-related to those observed to be differentially expressed. Genes illustrated in blue (empirical) have been observed to be critical for pubertal development in female mammals but were not observed to be differentially-expressed in the experiment by Allen *et al* (2011). *ESR1* is highly interconnected to differentially-expressed genes, indicating a potentially central hierarchical role in the network. In contrast, interactions with *KISS1* investigated in this network are minimal indicating a potential downstream role for kisspeptin in the pubertal activation of episodic release of GnRH later in development. [Network constructed using data reported previously (Allen *et al* 2011) and the GeneMANIA algorithm (http://www.genemania.org)].

Systems biology approach for understanding the role of kisspeptin in the control of pubertal development

Computational biology has become a valuable tool to investigate the genomic control of neuroendocrine systems timing the onset of puberty. A number of genes involved in regulating intracellular signaling and intercellular communications appear to be critical for the control of GnRH neuronal activity during pubertal development (Roth *et al* 2007). The approach of investigating the genetic networks involved in the control of pubertal maturation has identified highly-interactive components that are organized in a hierarchical order (reviewed by Lomniczie *et al* 2013a). The *KISS1* gene is located downstream in this complex regulatory network (Heger

et al 2007, Mastronardi *et al* 2006) and, although *KISS1* is essential for normal reproductive function, it is regulated extensively by other genes in the hierarchical order. Thus, alterations in this genetic network are likely to influence the function of neurons within the pathways controlling GnRH neuronal activity.

A potential subordinate role for *KISS1* in the genetic network controlling pubertal development in ruminant females is supported by various physiological studies. Although administration of kisspeptin stimulates ovulation/follicle luteinization in prepubertal lambs, regular estrous cyclicity is not established until a time coincident with saline-treated lambs (Redmond *et al* 2011a). This observation indicates that kisspeptin alone does not activate upstream components of the neuroendocrine pathway essential to sustain the pubertal pattern of GnRH secretory activity. Indeed, it appears that activation of *KISS1* expression, particularly in the ARC, occurs as a later event in the maturation of the reproductive neuroendocrine axis and coincident with increased frequency of LH release (Redmond et al., 2011b). In addition, in models in which puberty is facilitated by increased rates of body weight gain during the infantile and juvenile periods, *KISS1* does not emerge as a major differentially-regulated gene during earlier pubertal development in heifers (Fortes *et al* 2010, Allen *et al* 2012, Fortes *et al* 2012). This is confirmed by the limited association of *KISS1* in the network of genes regulated by nutrition during juvenile development (Fig. 3). Nevertheless, *KISS1* appears downstream to major regulatory genes critical for pubertal maturation. These include genes such as *TTF1* (Mastronardi *et al* 2006) and *PTTG1* (Heger *et al* 2007), both of which are known to regulate *KISS1* expression activity (Mueller *et al* 2011). The RNA-binding protein encoded by *LIN28*

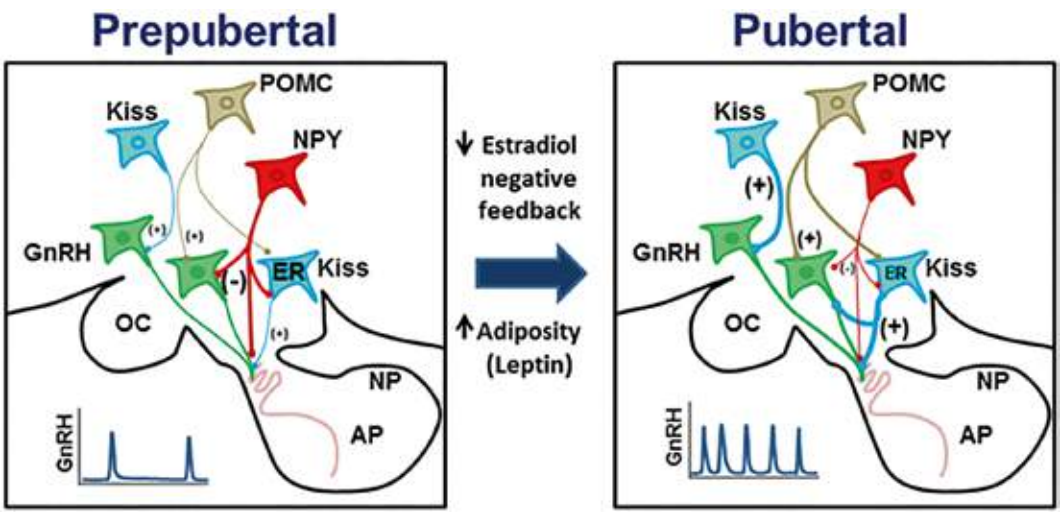

Fig. 3. Model depicting the involvement of kisspeptin neuronal networks in the pubertal activation of high-frequency GnRH release. A decrease in the sensitivity to estradiol negative feedback involves functional and structural changes in estrogen receptor (ER)-containing kisspeptin (Kiss) neurons. These changes include decreased ER signaling in kisspeptin neurons and increased kisspeptin contacts with GnRH neurons. Signals of nutritional status (e.g., leptin) act on neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons to regulate GnRH secretion. Plasticity in connections among cells in this neuronal networks and regulation of local release of transmitters and modulators facilitate the integration of endocrine and metabolic signals for the control of GnRH neuronal activity. The increase in excitatory stimuli and diminished inhibitory inputs to GnRH neurons result in increased frequency of episodic GnRH release and onset of puberty.

regulates the transcription of regulatory factors that appear upstream to *KISS1* (Lomniczi *et al* 2013a). Similarly to *KISS1*, *LIN28* has also been observed to play a role in pubertal development in humans (Ong *et al* 2009), mice (Zhu *et al* 2010) and rats (Sangiao-Alvarellos *et al* 2013). In preliminary studies in cattle, we have observed evidence for changes in the methylation pattern of *LIN28B* in heifers fed to gain body weight at high rates during the prepubertal period (BRC Alves, GL Williams & M Amstalden, unpublished observations). Moreover, epigenetic changes in transcriptional regulators that affect *KISS1* activity have been demonstrated (Lomniczi *et al* 2013b) and may contribute to the mechanisms by which kisspeptin neurons are involved in the control of pubertal development in mammals.

Conclusions

Activation of *KISS1* expression appears to be critical for the pubertal onset of high-frequency release of GnRH. Functional changes in the kisspeptin neuronal network may decode the changes in the sensitivity to estradiol negative feedback and integrate nutritional signals that time the onset of puberty. The control of a complex network of genes expressed in the hypothalamus underlies, at least in part, the molecular mechanisms controlling *KISS1* expression. Additional studies characterizing the biological roles of these genes, and their relevance within each cellular component of the kisspeptin neuronal network, are essential for improving our understanding of mechanisms involved in the pubertal activation of GnRH secretion.

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