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# FROM PRECOCIOUS PUBERTY TO INFERTILITY: METABOLIC CONTROL OF THE REPRODUCTIVE FUNCTION

Topic Editors
Meenakshi Alreja, Carol F. Elias
and Jennifer W. Hill

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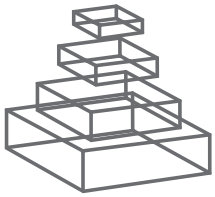

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# FROM PRECOCIOUS PUBERTY TO INFERTILITY: METABOLIC CONTROL OF THE REPRODUCTIVE FUNCTION

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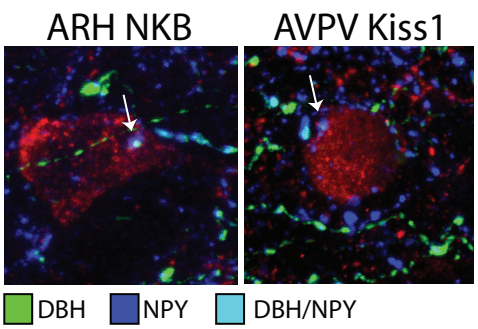


Close appositions of brainstem catecholamine fibers on arcuate nucleus (ARH) NKB and AVPV Kiss1cells. Figure taken from True C, Grove KL and Smith MS (2011) Beyond leptin: emerging candidates for the integration of metabolic and reproductive function during negative energy balance. Front. Endocrin. 2:53. doi: 10.3389/fendo.2011.00053

The existence of a fundamental link between nutrition and reproduction is well established. It is known for decades that a critical amount of stored energy is required for sexual maturation and maintenance of fertility. This concept is based on the idea that when survival is threatened by scarcity of food or increased energy demands, male and female of most species divert energy away from reproduction. This includes sexual maturation, the production of reproductive hormones and gametes, and the maintenance of pregnancy and lactation. If excessive leanness occurs in young women, puberty is often delayed. On the other hand, excess stored energy also negatively impacts fertility. Elevated adiposity aggravates polycystic ovarian syndrome, ovulatory dysfunctions and may induce hypothalamic hypogonadism in women. In obese men, fertility is usually decreased likely due to altered activity of the hypothalamus-pituitary axis and defective steroidogenesis in testis. Recently, studies have documented the link between the advance of obesity and the increasing rates of "precocious puberty". In 1997, an epidemiologic study reported that 6.7% of American girls had clinical evidence of puberty at age 7 years, and 14.7% at age 8 years. That study described the youngest ever reported population age

at puberty onset of 9.96 ± 1.82 years. In September of 2010, an alarming study showed that this phenomenon has been aggravated in the last decade. The authors found an increment of 5-8% in the number of girls with clinical evidence of puberty at age 7 and 8 years, compared to the 1997 study. They also reported a high correlation of early puberty onset and childhood obesity. These observations suggest the existence of a previously unrecognized deleterious effect of the increasing rates of childhood obesity: "the precocious puberty", which will bring profound social and health implications for the next generations. Earlier menarche in girls is associated with increased risk of adult obesity, type 2 Diabetes and breast cancer. Thus, changing levels of key metabolic cues is an essential signal for the onset of puberty and maintenance of the tone of the reproductive system. As the activity of gonadotropin releasing hormone (GnRH) neurons is essential for the development and maturity of the reproductive axis, efforts have been made to identify the factors that directly regulate the activity of GnRH neurons. For example, sensory signals and metabolic cues (e.g. glucose) may reach the reproductive control sites of the central nervous system via sensory inputs conveyed by the vagus nerve or by direct action in the caudal brainstem. In addition, circulating metabolic factors such as leptin, insulin and ghrelin also inform the brain about the individual nutritional state. The goal of this Research Topic is to assemble multidisciplinary specialists to provide up-to-date reviews on the recent advances and achievements in the field.

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# From precocious puberty to infertility: metabolic control of the reproductive function

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Reproduction is calorically expensive. The energy demands of mate seeking, gamete production, pregnancy, and lactation require increased food consumption and appropriate regulation of energy expenditure. Therefore, control of the reproductive function by the brain must be responsive to the metabolic state of the animal. Conversely, when survival is threatened by insufficient fuels or increased energy demands, males and females of most species divert energy away from reproduction by reducing copulatory motivation and behavior, halting ovulation, terminating pregnancies, or ceasing lactation. In addition, when prepubertal animals, including humans, are exposed to energy deprivation, the onset of puberty is delayed or even blocked, until a favorable energy balance is achieved. These mechanisms serve to optimize reproductive success in environments where energy availability fluctuates. Recent studies suggest that excess body fat can trigger early onset of puberty, especially in females. In males, on the other hand, the prevalence of delayed puberty is about fivefold higher than in females, indicating sexual dimorphism in the sensitivity of the reproductive axis to metabolic cues. Understanding the interaction between energy balance and fertility has critical implications for the treatment reproductive deficits caused by metabolic dysfunction.

Given the involvement of the hypothalamus in the management of food intake, energy use, reproductive behavior, and the hormonal control of gametogenesis and ovulation, ongoing studies are focused on the interplay between the hypothalamic circuits driving these functions. Gonadotropin releasing hormone (GnRH) neurons are specialized neurons often described as the "master regulators" of the hypothalamus-pituitary-gonads (HPG) axis. The intermittent release of GnRH controls pituitary release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) and, by extension, function of the gonads. Surprisingly, few GnRH neurons are sufficient to initiate puberty in males and females and to maintain fertility in the male. However, more are required for females to generate LH surges and ovulate. These additional GnRH neurons may modulate GnRH pulsatility in response to environmental, nutrition, stress, or other cues conveying adverse situations. However, GnRH neurons on their own seem to sense few metabolic cues. Instead, neighboring neurons and glia may perceive circulating factors, such as leptin, insulin, and ghrelin that serve as signals of the nutritional state of the individual. If these cells are not able to sense metabolic cues, for example in states of insulin or leptin resistance, the repercussions may include both imbalanced metabolic homeostasis and reproductive dysfunction. Indeed, leptin-deficient patients become hyperphagic, massively obese, and infertile. This eBook has assembled multidisciplinary specialists to provide up-to-date information on recent advances in understanding the complex physiologic interaction between metabolism and reproduction.

In the initial article, True et al. (2011) discuss the role of the adipocyte hormone leptin as a key metabolic signal and predominant focus of interest in the field. In their review, it is emphasized that although leptin may be an important permissive signal for reproductive function as indicated by many years of research, factors other than leptin must critically contribute to negative energy balance-induced reproductive inhibition. Schneider et al. (2012) call attention to the "metabolic hypothesis," which predicts that sensory systems monitor the availability of oxidizable metabolic fuels and allow behavioral responses to optimize reproductive success. Following these provocative introductory articles, three reviews discuss the role of specific groups of neurons in this physiologic regulation. Bianco (2012) highlights the Kisspeptin system as the converging target of environmental, metabolic, and hormonal signals, and proposes a potential correlation between the existence of a sexual dimorphism of pubertal disorders in children of different ethnicities and the sexually dimorphic expression of kisspeptin neurons. Supported by recent genetic studies, Xu et al. (2012) focused their review on two sets of hypothalamic neurons: the pro-opiomelanocortin (POMC) neurons in the arcuate nucleus and the steroidogenic factor-1 (SF1) neurons in the ventromedial hypothalamic nucleus. Their discussion calls attention to exciting new findings showing that disruption of metabolic signals (e.g., leptin and insulin) or reproductive signals (e.g., estradiol) in these neurons leads to impaired regulation of both energy homeostasis and fertility. Donato and Elias (2011) discuss the role of the ventral premammillary nucleus as integrator of environmental, metabolic, and reproductive cues, and its emergence as a critical previously unrecognized hypothalamic site linking metabolism and reproduction. Acosta-Martínez (2012) proposes a role for phosphatidylinositol-3-kinase (PI3K) signaling pathway as potential integrator of a number of peripheral metabolic cues, including insulin and leptin, in the metabolic control of the reproductive function. Tolson and Chappell (2012) offer an

insightful discussion on pubertal timing, outlining a potential role of endogenous timing mechanisms including cellular circadian clocks in pubertal initiation. They propose that these clocks may be altered by metabolic factors leading to reproductive deficits. In a provocative review, Clasadonte et al. (2011) discuss the action of non-neuronal components in GnRH regulation. They suggest that synaptically associated astrocytes and perijunctional tanycytes are integral modulatory elements of GnRH neuronal function at the cell soma/dendrite and terminal levels. Finally, two important articles call the attention to differences in the metabolic modulation of the reproductive physiology in different species. Klingerman et al. (2011) highlight the metabolic influence on sexual behavior, and food intake or food hoarding in hamsters, and suggest a role for neuropeptide Y (NPY) and gonadotropin inhibiting hormone (GnIH) expressing cells in these processes. Amstalden et al. (2011) emphasize observations made in ruminant species in a very welcome comparative perspective. Clearly, research examining the metabolic control of reproduction is advancing at a rapid pace. The articles in this eBook highlight some of the most critical and intriguing areas for future study.

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# Beyond leptin: emerging candidates for the integration of metabolic and reproductive function during negative energy balance

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Reproductive status is tightly coupled to metabolic state in females, and ovarian cycling in mammals is halted when energy output exceeds energy input, a metabolic condition known as negative energy balance. This inhibition of reproductive function during negative energy balance occurs due to suppression of gonadotropin-releasing hormone (GnRH) release in the hypothalamus. The GnRH secretagogue kisspeptin is also inhibited during negative energy balance, indicating that inhibition of reproductive neuroendocrine circuits may occur upstream of GnRH itself. Understanding the metabolic signals responsible for the inhibition of reproductive pathways has been a compelling research focus for many years. A predominant theory in the field is that the status of energy balance is conveyed to reproductive neuroendocrine circuits via the adipocyte hormone leptin. Leptin is stimulatory for GnRH release and lower levels of leptin during negative energy balance are believed to result in decreased stimulatory drive for GnRH cells. However, recent evidence found that restoring leptin to physiological levels did not restore GnRH function in three different models of negative energy balance. This suggests that although leptin may be an important permissive signal for reproductive function as indicated by many years of research, factors other than leptin must critically contribute to negative energy balance-induced reproductive inhibition. This review will focus on emerging candidates for the integration of metabolic status and reproductive function during negative energy balance.

**Keywords: leptin, GnRH, Kisspeptin, GnIH**

## INTRODUCTION

Metabolic status is a known regulator of reproductive function, with both over- and under-nutrition resulting in reproductive dysfunction. In female mammals, this is frequently observed as a disruption of reproductive cycling leading to anovulation. Cyclic reproductive function is controlled by the hypothalamic-pituitary–gonadal axis, in which gonadotropin-releasing hormone (GnRH) is released from the hypothalamus, causing the release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary followed by estrogen and progesterone release from the ovary. Disruptions at any level in this pathway can result in acyclicity. Despite years of intense study in this field, many key questions remain unanswered in our understanding of how changes in metabolic status result in disruption of the hypothalamic-pituitary–gonadal axis.

One well-studied candidate for the integration of metabolic and reproductive function is the adipocyte hormone leptin (for reviews see Clarke and Henry, 1999; Cunningham et al., 1999; Bluher and Mantzoros, 2007; Tena-Sempere, 2007; Hill et al., 2008). Circulating leptin levels directly correlate to adipose stores and are highly sensitive to changes in metabolic status, making it an ideal candidate to signal changes in energy balance to central and peripheral systems (Maffei et al., 1995; Blache et al., 2000). Within the hypothalamic-pituitary–gonadal axis, there is evidence that leptin acts to stimulate GnRH release through an intermediate cell population, rather than through direct actions on GnRH neurons (Yu et al., 1997; Watanobe, 2002; Quennell et al., 2009). The candidates for this intermediate cell population include arcuate nucleus kisspeptin cells (discussed below) and glutamate cells in the ventral premammillary nucleus. The latter population has only been described recently for its role in leptin's reproductive regulation, based on the high number of cells expressing leptin receptors in this area and direct projections from these cells to GnRH neurons (Leshan et al., 2009; Louis et al., 2011; Patterson et al., 2011). In addition to the morphological evidence, *in vivo* studies found that lesions to the ventral premammillary nucleus prevented leptin-induced-LH stimulation (Donato et al., 2009, 2011). Given these results, glutamate cells in the ventral premammillary nucleus are clearly exciting new candidates in understanding leptin's role in reproduction.

A large portion of research has focused on the regulatory influence of leptin for the initiation of puberty. There is evidence of a developmental increase in leptin levels between postnatal day 20 and 40 in the rat, the latter date corresponding to vaginal opening and followed soon after by the first estrus cycle (Gruaz et al., 1998). Similar results have been found in humans, suggesting leptin may be critical to stimulate normal pubertal development (Mantzoros et al., 1997). There is an abundance of data to support

this hypothesis, most notably the evidence that leptin deficient ob/ob mice do not undergo puberty and are infertile, a phenotype rescued with exogenous leptin treatment (Ingalls et al., 1950; Swerdloff et al., 1976; Barash et al., 1996; Chehab et al., 1996). Zucker Fatty Rats lacking a functional leptin receptor also have delayed pubertal development and reduced LH levels (Zucker and Zucker, 1961; Saiduddin et al., 1973; Phillips et al., 1996; Todd et al., 2003). These transgenic studies recapitulate findings in humans in which genetic mutations in the leptin signaling system have been reported to result in both dramatically delayed and absent pubertal development (Clement et al., 1998; Strobel et al., 1998). In addition, overexpression of leptin, or exogenous treatment of wild-type mice results in early onset of puberty in rodents (Ahima et al., 1997; Chehab et al., 1997). Given this evidence it is clear that leptin plays a critical role in signaling sufficient metabolic energy stores required for the initiation of GnRH release and puberty in rodents and humans. Notably, puberty in the non-human primate is not preceded by a rise in circulating leptin, suggesting other signals are responsible for the initiation of puberty in this species (Plant and Durrant, 1997).

## NEGATIVE ENERGY BALANCE-INDUCED ACYCLICITY AND HYPOLEPTINEMIA

To understand the role of leptin for the integration of energy balance and reproductive function, investigators have relied in part on animal models of negative energy balance, where energy output exceeds energy input. Negative energy balance-induced reproductive acyclicity is a highly conserved phenomenon, present in all female mammals investigated to date. It is well understood that the halting of ovarian cycling in this case likely occurs through inhibition of GnRH release from the hypothalamus, since exogenous GnRH rescues cyclic reproductive function (Bronson, 1986; Bergendahl et al., 1991; Cameron and Nosbisch, 1991; Kile et al., 1991; Aloi et al., 1997). Given the proposed stimulatory role of leptin in GnRH release, the prevailing hypothesis in the field is that reproductive dysfunction during negative energy balance occurs due to hypoleptinemia and thus a decrease in stimulatory drive for GnRH release. This hypothesis is supported by multiple studies demonstrating that exogenous leptin treatment during fasting models of negative energy balance stimulates GnRH release as measured by circulating LH levels (Ahima et al., 1996; Nagatani et al., 1998, 2000). However, these studies used pharmacological doses of leptin that resulted in levels at least 50-fold higher than normal circulating levels (Ahima et al., 1996). Even with pharmacological doses of leptin replacement, Ahima et al. (1996) found only partial restoration of LH levels, suggesting a continued inhibitory source for GnRH release was still present. This latter finding indicates that while hypoleptinemia may play a role in suppression of GnRH during negative energy balance in rodents, other players are also likely involved.

Recent research suggests that metabolic signaling beyond leptin may also be critical for the reversal of GnRH inhibition upon exit from negative energy balance back to a normal metabolic state. Research from our lab examined the effects of restoring leptin to physiological levels during lactation, a naturally occurring condition of negative energy balance in which the energy requirement for milk production exceeds energy intake. Restoring leptin during mid-lactation had no effect to restore LH levels (Xu et al., 2009b). However, it could be argued that lactation is a complicated physiological model, with other known sources of GnRH inhibition (Brogan et al., 1999); therefore, it is possible that leptin's effects were masked by continued inhibitory inputs specific to lactation (Li et al., 1999c). To test this hypothesis a long-term caloric restriction (CR) model was developed to mimic the duration and intensity of lactation-induced negative energy balance, but once again restoration of leptin to physiological levels did not normalize mean LH levels (True et al., 2011b). This data was in direct contrast to previous results; however, these earlier studies used a short-term fasting model of negative energy balance and also employed much higher pharmacological levels of leptin replacement (Ahima et al., 1996; Nagatani et al., 1998). To determine whether these discrepancies were due to the different models of negative energy balance or to the dose of leptin, leptin was replaced in a 48 hour fasting model to both physiological and pharmacological levels. Despite partial normalization of LH levels with the pharmacological dose of leptin, consistent with previous results (Ahima et al., 1996; Nagatani et al., 1998), restoration of physiological levels of leptin had no effect on fasting-suppressed LH levels (True et al., 2011b). Additional research arguing against a critical role for leptin in the restoration of LH upon exit from negative energy balance comes from the lean ewe model. Szymanski et al. (2007) demonstrated that when lean acyclic ewes are refed, LH levels rise quickly, and importantly this increase occurs prior to any increase in circulating leptin levels. Together, these studies confirm earlier indications that while hypoleptinemia may play a permissive role in negative energy balance-induced reproductive dysfunction it does not appear to be critical for GnRH inhibition (Smith et al., 2010b). It should be noted that physiological levels of leptin seem more effective at preventing LH inhibition in humans, pointing to important species differences in this field (Welt et al., 2004). In the rodent many other candidates involved in the integration of metabolic and reproductive function exist, and there are many reviews on the role of appetitive hormones and hypothalamic peptides in this process (Kalra and Kalra, 1996; Schioth and Watanobe, 2002; Smith and Grove, 2002; Fernandez-Fernandez et al., 2006; Garcia et al., 2007; Hill et al., 2008; Tena-Sempere, 2008; Smith et al., 2010b). The goal of this review is to highlight more recent and emerging candidates involved in GnRH regulation that also have ties to metabolic regulation, and discuss their potential roles during negative energy balance.

## INHIBITION OF KISSPEPTIN AS A CENTRAL MECHANISM OF GnRH INHIBITION

It would be remiss to discuss regulation of GnRH release without mention of the neuropeptide kisspeptin (Kiss1). Kiss1 was found to be the endogenous ligand of an orphan receptor, g-protein coupled receptor 54 (GPR54), and is an extremely potent stimulator of GnRH release (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Navarro et al., 2004; Thompson et al., 2004; Dumalska et al., 2008; Popa et al., 2008). Mutations in either the *Kiss1* or *GPR54* gene results in hypogonadotropic hypogonadism in humans, indicating Kiss1 signaling is critical for reproductive function (de Roux et al., 2003; Seminara et al., 2003). Two major populations of

Kiss1 neurons have been identified in several species, including the rodent, one in the arcuate nucleus and another in the anteroventral periventricular nucleus (AVPV). These two populations are thought to be involved in negative and positive steroid feedback of GnRH release, respectively (Smith et al., 2005a,b, 2006b; Dungan et al., 2006; Clarkson et al., 2008; Popa et al., 2008; Clarkson and Herbison, 2009). Interestingly, these two populations of Kiss1 neurons have different projections, with AVPV Kiss1 cells contacting GnRH cell bodies in the preoptic area and arcuate nucleus Kiss1 cells sending fibers to the median eminence, where they are in close contact with GnRH fibers (True et al., 2011a). Kiss1 appears to be regulated by metabolic conditions suggesting it may also be important for negative energy balance-induced GnRH suppression. In lactating and CR models Kiss1 levels are suppressed in both the arcuate nucleus and AVPV (Yamada et al., 2007; Xu et al., 2009b; True et al., 2011b). While studies reporting the effect of fasting on Kiss1 levels have been inconsistent, there are reports of inhibition in both nuclei in this model as well (Castellano et al., 2005; Luque et al., 2007; Forbes et al., 2009; Backholer et al., 2010). These results suggest that inhibition of GnRH release during negative energy balance may occur upstream at Kiss1 populations, which in turn results in decreased stimulatory drive for GnRH (Hill et al., 2008; Roa et al., 2008; Castellano et al., 2009, 2010).

Surprisingly, very little is known about afferent signals that regulate AVPV and arcuate nucleus Kiss1 cells. Numerous studies have found evidence for a stimulatory role of leptin for Kiss1 expression (Castellano et al., 2006, 2010; Luque et al., 2007; Backholer et al., 2010). Double-label *in situ* hybridization found leptin receptor expression colocalized with arcuate nucleus Kiss1 cells in the mouse and sheep (Smith et al., 2006a; Backholer et al., 2010), and leptin treatment resulted in rapid depolarizations of arcuate nucleus Kiss1 cells in guinea pigs, suggesting a direct regulatory relationship (Qiu et al., 2011). However, studies using the leptin receptor–green fluorescent protein (GFP) transgenic mice show virtually no colocalization of GFP with Kiss1-immunoreactivity (Louis et al., 2011). Further work in mice and rats has demonstrated a lack of pSTAT3, a signal transducer and activator of transcription stimulated by leptin receptor signaling, in Kiss1 cells after treatment with high levels of leptin (Louis et al., 2011; Quennell et al., 2011; True et al., 2011b); however, it remains possible that leptin signaling in Kiss1 cells may be through cascades not involved in gene transcription and pSTAT3 activation, as suggested by rapid electrophysiological responses (Qiu et al., 2011). Importantly, restoring leptin to physiological levels does not restore arcuate nucleus Kiss1 levels in either lactation or CR, suggesting hypoleptinemia may not be a required signal for arcuate nucleus Kiss1 inhibition during negative energy balance (Xu et al., 2009b; True et al., 2011b). Given the important role of Kiss1 in GnRH regulation, and its implicated involvement in negative energy balance, understanding the regulatory afferent inputs for Kiss1 cells will be critical to our understanding of the integration of energy balance and reproduction.

## REGULATION OF NEUROKININ B AND DYNORPHIN AS A CENTRAL MECHANISM OF GnRH INHIBITION

Arcuate nucleus Kiss1 cells also express the tachykinin neuropeptide, neurokinin B (NKB; Goodman et al., 2007; True et al., 2011a). Similar to Kiss1, NKB also appears critical for development of reproductive function since mutations in the genes encoding NKB and the NKB receptor NK3, also result in hypogonadotropic hypogonadism in humans (Topaloglu et al., 2009). NKB levels are inhibited by estradiol in several species and there is strong evidence that NKB plays a role in negative steroid feedback (Rance and Young, 1991; Danzer et al., 1999; Goubillon et al., 2000; Pillon et al., 2003; Rance, 2009; Navarro et al., 2011). For many years results were inconsistent as to whether NKB was stimulatory or inhibitory for GnRH release (Sahu and Kalra, 1992; Sandoval-Guzman and Rance, 2004; Corander et al., 2010), but more recent evidence has supported a stimulatory role, consistent with reproductive dysfunction in humans with mutations in the NKB system (Billings et al., 2010; Ramaswamy et al., 2010; Wakabayashi et al., 2010; Navarro et al., 2011). There is evidence in the rat that NKB may directly stimulate GnRH release through fiber contacts in the external zone of the median eminence (Krajewski et al., 2005). In addition to direct regulation of GnRH cells, there is also evidence that NKB may indirectly regulate GnRH release through stimulating-autoregulatory actions on arcuate nucleus Kiss1/NKB cells (Navarro et al., 2009, 2011; Wakabayashi et al., 2010).

Our laboratory has investigated whether NKB may play a role in negative energy balance-induced inhibition of GnRH release. Real-time PCR analysis has demonstrated that arcuate nucleus NKB mRNA expression is decreased during lactation, and is also inhibited during severe 50% CR (Xu et al., 2009b; True et al., 2011b). This is in contrast to a lack of inhibition during more moderate 40% CR and a 48-h fast (True et al., 2011b). These findings suggest that arcuate nucleus NKB inhibition may be involved in more severe conditions of negative energy balance to shut off cyclic reproductive function. Similar to Kiss1, little is known about the upstream regulatory input for arcuate nucleus NKB expression, although work from our group found no evidence that leptin regulates NKB expression during negative energy balance (Xu et al., 2009b; True et al., 2011b). It is of interest to note that arcuate nucleus Kiss1/NKB cells also express dynorphin, yet another neuropeptide involved in reproductive regulation (Burke et al., 2006; Goodman et al., 2007). Similar to NKB, dynorphin receptors are also found on arcuate nucleus Kiss1/NKB/dynorphin (KNDy) cells suggesting a potential autoregulatory action (Navarro et al., 2009). However, dynorphin is thought to be inhibitory for GnRH release and unlike NKB, dynorphin is not differentially regulated during negative energy balance (Schulz et al., 1981; Kinoshita et al., 1982; Leadem and Kalra, 1985; Xu et al., 2009b; True et al., 2011b). A similar example of juxtaposed coexpressing neuropeptides exists in the hypothalamic feeding cells containing α-melanocyte-stimulating hormone and β-endorphin, which are stimulatory and inhibitory for food intake, respectively (Imura et al., 1985; Tsujii and Bray, 1989; Kim et al., 2000). It remains unclear how coexpression of these two counteracting neuropeptides may be coordinated to control food intake in the hypothalamus (Hughes et al., 1988). In the case of KNDy neurons it is tempting to speculate that differential expression of NKB and dynorphin may tightly regulate the release of Kiss1 into the median eminence. Furthermore, precisely timed Kiss1 release may be physiologically significant for the regulation of basal pulsatile GnRH release.

## GONADOTROPIN-INHIBITORY HORMONE

While Kiss1 and NKB are likely important stimulatory signals for GnRH and gonadotropin release, research over the past decade has uncovered a similarly important inhibitory signal aptly named gonadotropin-inhibitory hormone (GnIH). GnIH was first isolated from avian brains and characterized by its ability to inhibit gonadotropin release from pituitary explants (Tsutsui et al., 2000). Over time the mammalian homolog termed RF-amide related protein 3 (RFRP3) was characterized (Hinuma et al., 2000; Yano et al., 2003; Kriegsfeld et al., 2006; Johnson et al., 2007; Ubuka et al., 2009). Like its avian counterpart, RFRP3 has also been localized to the median eminence and shown to inhibit gonadotropin release from the pituitary (Clarke et al., 2008). In addition to pituitary actions, there is also evidence that GnIH/RFRP3 can inhibit GnRH cell firing (Ducret et al., 2009; Wu et al., 2009b), suggesting there may also be hypothalamic actions of GnIH/RFRP3. This finding is supported by immunohistochemical data demonstrating GnIH/RFRP3 fibers in close contact with GnRH cell bodies in birds, rats, sheep, and non-human primates (Johnson et al., 2007; Smith et al., 2008, 2010a; Ubuka et al., 2008). In fact, results demonstrating a lack of hypophysiotropic effects of RFRP3 in the rat have lead to the hypothesis that RFRP3 action may be solely hypothalamic in this species (Anderson et al., 2009; Rizwan et al., 2009). More research is needed to understand the site of action of RFRP3 in the rat given contradictory evidence regarding (1) the presence of RFRP3 fibers in the external zone of the median eminence (Johnson et al., 2007; Rizwan et al., 2009; Bentley et al., 2010) and (2) *in vivo* actions of intracerebral ventricular RFRP3 administration on LH release (Johnson et al., 2007; Murakami et al., 2008; Anderson et al., 2009; Rizwan et al., 2009).

Given the inhibitory action of GnIH/RFRP3 on gonadotropin release, many studies have investigated the role of this peptide during conditions of GnRH suppression (Smith and Clarke, 2010; Clarke, 2011). In both birds and sheep GnIH/RFRP3 appears to be higher in the non-breeding season suggesting a potential role in seasonal regulation of reproductive function (Bentley et al., 2003; Smith et al., 2008; Clarke and Smith, 2010). GnIH/RFRP3 has also been shown to inhibit sexual behavior in birds and rats (Bentley et al., 2006; Johnson et al., 2007). Although GnIH/RFRP3 has been implicated in inhibited reproductive function, the role of this peptide in governing cyclic fluctuations of gonadotropins during ovarian cycling in mammals is still not well understood. Work in the rat found exogenous RFRP3 reduced c-Fos activity in GnRH and AVPV cells at the time of an induced-LH surge, but RFRP3 had no affect on basal pulsatile GnRH release (Anderson et al., 2009). In the hamster Fos activation is decreased in RFRP3 cells at the time of the LH surge (Gibson et al., 2008). Differential regulation of RFRP3 during the ovarian cycle was found in hamsters and monkeys, but in opposite directions. While RFRP3 was high during diestrus in the hamster (Gibson et al., 2008), non-human primates showed elevated levels immediately prior to the GnRH/LH surge (Smith et al., 2010a). The latter finding was surprising since high levels of RFRP3 would be expected to suppress GnRH/LH levels; therefore, it is possible RFRP3's role in ovarian cyclicity is not always strongly inhibitory.

Studies focusing on the reproductive aspects of the GnIH/RFRP3 system have also noted a potential role for GnIH/RFRP3 in appetite regulation. Exogenous administration of GnIH in chicks was found to potently stimulate food intake, potentially through an opioid receptor system (Tachibana et al., 2005, 2008). RFRP3 was also found to stimulate food intake in rats, resulting in a doubling of food consumption during the photophase (Johnson et al., 2007; Murakami et al., 2008), consistent with the well established role of RF-amide related proteins and appetite regulation (Dockray, 2004). The evidence for a regulatory role of GnIH/RFRP3 in reproduction and appetite suggests this neuropeptide may be important for integration of energy balance and reproductive function (Smith and Clarke, 2010; Clarke, 2011). The characteristics of inhibitory effects on reproductive function and stimulatory effects on food intake makes it tempting to hypothesize that RFRP3 may be upregulated during negative energy balance. If RFRP3 is elevated during negative energy balance it could work to both conserve energy output through inhibition of reproductive cycling and increase energy input through food intake. Future research will undoubtedly be aimed at answering this question. Additional future directions for GnIH research may be electrophysiological and anatomical studies to determine if RFRP3 has any direct influence on Kiss1 release, since fiber distribution analysis suggests RFRP3 terminals are in the region of both the AVPV and arcuate nucleus Kiss1 populations (Rizwan et al., 2009). Additionally, investigations using GnIH antagonists could determine whether blocking a potential negative energy balance-induced rise in GnIH/RFRP3 prevents inhibition of GnRH release.

## ALARIN

Galanin-like peptide (GALP) has been previously linked to the integration of energy balance and reproduction (Krasnow et al., 2003; Kageyama et al., 2005; Lawrence and Fraley, 2010). Recent evidence suggests a splice variant of the GALP gene, termed alarin (Santic et al., 2006), may have a similar regulatory function. Alarin appears orexigenic in rodents since intracerebral ventricular injections of alarin result in a fivefold acute increase in food intake and increased body weight gain after chronic administration (Boughton et al., 2010; Van Der Kolk et al., 2010). Alarin has also been implicated in reproductive regulation due to its expression in sexually important nuclei such as the preoptic area and other hypothalamic regions including the arcuate nucleus (Eberhard et al., 2011). Furthermore, work in male rodents has found evidence that alarin may be stimulatory for LH release, potentially in a GnRH-dependent manner (Boughton et al., 2010; Van Der Kolk et al., 2010). Further research is needed to understand the role of alarin in regulation of reproductive function in females, since past studies have focused only on males. Future studies in females will also be important in determining whether steroid hormone environment affects the direction of alarin's regulatory action on LH release as it does for many other orexigenic neuropeptides (Crowley and Kalra, 1987; Pu et al., 1998).

The apparent stimulatory action of alarin on LH release observed in males is in contrast to the inhibitory effects found with most other orexigenic neuropeptides, such as NPY, MCH, and orexin (Kalra and Kalra, 1996; Pu et al., 1998; Tamura et al., 1999; Murray et al., 2000; Chiocchio et al., 2001; Kohsaka et al.,

2001; Small et al., 2003; Wu et al., 2009a). There is significant evidence that these well-studied orexigenic neuropeptides regulate GnRH release through direct projections to GnRH cell bodies (Li et al., 1999b; Iqbal et al., 2001; Campbell et al., 2003; Small et al., 2003; Williamson-Hughes et al., 2005). Therefore, direct inhibitory effects on GnRH cells would be greatly enhanced during states of negative energy balance when their activities are upregulated (Xu et al., 2009a; Rondini et al., 2010; Smith et al., 2010b). However, it is unclear how alarin may influence GnRH regulation. One study found alarin-like immunoreactivity in the preoptic area (Eberhard et al., 2011), but it has not been examined whether this immunoreactivity is in close proximity to GnRH cell bodies. It is also unclear how alarin might be differentially regulated during negative energy balance, since orexigenic drive should be high while LH stimulation would be expected to be low. Future research may elucidate how alarin contributes to the integration of energy balance and reproductive function, and what if any role this neuropeptide has in negative energy balance-induced acyclicity.

## BRAINSTEM GLUCOSE-SENSING POPULATIONS

Certain neuronal populations are capable of sensing metabolic status through changes in circulating glucose levels. This characteristic offers an attractive and relatively simple mechanism through which metabolic status may be acutely sensed in the brain and potentially relayed to reproductive circuits. One such glucose-sensing population exists in the ventrolateral medulla of the brainstem (for review see Ritter et al., 2006). Glucose-deprivation achieved with administration of 2-deoxy-D-glucose, a glucose molecule unable to undergo glycolysis, results in c-Fos activation in the catecholaminergic A1/C1 subregion of the ventrolateral medulla (Ritter et al., 1998). To determine if the A1/C1 catecholamine neurons contributed to the reproductive inhibition associated with glucoprivation this population was ablated by injection of a conjugated dopamine-beta hydroxylase saporin toxin complex (I'Anson et al., 2003). Interestingly, when A1/C1 catecholamine neurons were destroyed this prevented 2-deoxy-D-glucose-induced reproductive acyclicity, strongly implicating a role for this population in the integration of energy balance and reproductive function. Microdialysis during 2-deoxy-D-glucose administration revealed increases in noradrenaline release in the hypothalamus, and intervention to block this noradrenaline rise prevented inhibition of LH (Nagatani et al., 1996). In addition, it appears that noradrenaline's role in LH suppression is not specific to the 2-deoxy-D-glucose model of negative energy balance, given similar results were observed in fasting animals (Cagampang et al., 1992; Maeda et al., 1994). These studies suggest that increases in noradrenaline, potentially from the A1/C1 subregion, during negative energy balance may be critical for inhibition of LH release. This inhibitory role for catecholamines, and noradrenaline specifically, on LH release is consistent with previous literature (Maeda et al., 1994; Tsukamura et al., 1994; Nagatani et al., 1996), and electrophysiological recordings as well as anatomical results suggest this inhibitory action may be exerted directly upon GnRH cells (Todman et al., 2005; Campbell and Herbison, 2007; Han and Herbison, 2008). However, noradrenaline is also stimulatory for LH release, and these contradictory regulatory influences are thought to be dependent on steroid hormone levels, with noradrenaline stimulatory for LH in the presence of high levels of estradiol and inhibitory in the presence of low levels of estradiol (Gallo and Drouva, 1979; Leung et al., 1982; Meyer and Goodman, 1985; Havern et al., 1991; Cagampang et al., 1992; Robinson and Kendrick, 1992; Herbison, 1997), as would be associated with negative energy balance.

In the lactation model of negative energy balance the A1 noradrenergic region was also found to have c-Fos activation in response to pup suckling (Li et al., 1999c). Pup suckling is strongly implicated in LH inhibition during lactation (Brogan et al., 1999; Smith and Grove, 2002; Smith et al., 2010b), once again supporting an inhibitory role for noradrenaline during negative energy balance. Noradrenergic neurons of A1 activated by the suckling stimulus were also found to project to the arcuate nucleus (Li et al., 1999a). Contradictory evidence for A1 noradrenaline projections to GnRH cells exists, suggesting it is possible that an intermediary population may be involved in mediating noradrenaline's effects on GnRH (Wright and Jennes, 1993; Simonian et al., 1999; Campbell and Herbison, 2007). Given the projection to the arcuate nucleus, we sought to determine whether noradrenaline might regulate the upstream arcuate nucleus Kiss1/NKB population. Immunohistochemistry presented here demonstrates that arcuate nucleus Kiss1/NKB and AVPV Kiss1 neurons appear to have close appositions from DBH/NPY positive fibers (**Figure 1**). This finding indicates Kiss1 populations may receive regulatory inputs from brainstem catecholamine neurons, since this is the only known area where catecholamines and NPY are colocalized in the same neurons (Everitt et al., 1984; Bai et al., 1985). It remains to be confirmed whether brainstem catecholamine fibers contacting Kiss1 cells are from the ventrolateral medulla A1 region and future tract-tracing studies will be aimed at defining the source of this catecholaminergic input.

## CONCLUSION

It is clear that although leptin is undoubtedly involved in the regulation of reproductive and metabolic status, leptin's role in negative energy balance is likely permissive and not causative for

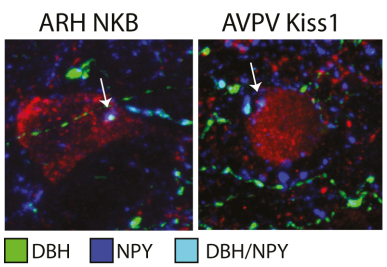


**FIGURE 1 | Close appositions of brainstem catecholamine fibers on arcuate nucleus (ARH) NKB and AVPV Kiss1cells.** Immunohistochemistry of colocalized NPY (AB1583, Millipore; 1:5000; blue) and dopamine-beta hydroxylase (MAB308, Millipore; 1:5000; green) fibers represents projections from brainstem catecholamine populations that coexpress NPY (for detailed methods see True et al., 2011a). Colocalized NPY/DBH fibers (light blue) were found in close contact with ARH NKB cells (3/61, a gift from Dr. Phillipe Ciofi; 1:4000; red, left photomicrograph) and AVPV Kiss1 cells (AB9754, Millipore; 1:1000; red, right photomicrograph).

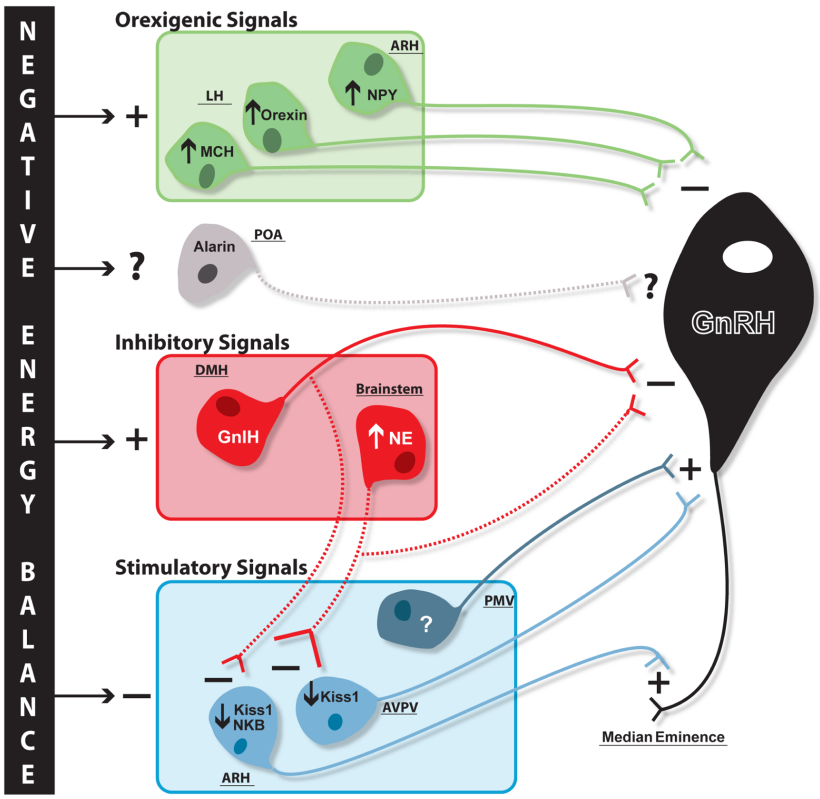


**FIGURE 2 | Proposed schematic of negative energy balance-induced changes in reproductive and metabolic circuits contributing to GnRH inhibition.** Negative energy balance results in differential regulation of systems both stimulatory and inhibitory for GnRH release. Orexigenic neuropeptides (green) melanin-concentrating hormone (MCH) and orexin in the lateral hypothalamus (LH) and neuropeptide Y (NPY) in the arcuate nucleus (ARH) are all stimulated (plus sign) with negative energy balance and inhibit (minus sign) GnRH (black) release through direct regulation at cell bodies. It is unknown whether alarin is differentially regulated during negative energy balance, but it is proposed these neurons may also project to GnRH cell bodies (hypothesized regulatory influence and projections represented with dashed lines). Negative energy balance stimulates additional cell populations that are inhibitory for GnRH release (red), namely the dorsomedial hypothalamus (DMH) GnIH and brainstem noradrenaline (NE) populations. While GnIH, and potentially brainstem NE, cells project to GnRH cell bodies, it is hypothesized these cells may also inhibit upstream stimulatory Kiss1 populations. ARH kisspeptin/neurokinin B (Kiss1/NKB) and anteroventral periventricular nucleus (AVPV) Kiss1 populations (blue), which stimulate GnRH terminals and cell bodies, respectively, are inhibited during negative energy balance. Negative energy balance is also proposed to inhibit an unknown stimulatory cell population in the ventral premammillary nucleus (PMV) which has direct projections to GnRH cell bodies.

the severe inhibition of GnRH release. The finding that restoration of leptin to normal physiological levels does not restore GnRH release in multiple models of negative energy balance supports this conclusion. Given this evidence, the search continues for signals that may be critical for integrating reproductive and metabolic function. One obvious candidate not previously discussed here is the orexigenic gut hormone ghrelin, which has already been extensively reviewed for its potential role in the integration of energy balance and reproduction (Fernandez-Fernandez et al., 2006; Garcia et al., 2007; Tena-Sempere, 2008). Similar to leptin, ghrelin is a predominantly peripheral-derived hormone capable of sensing changes in metabolic state, but unlike leptin ghrelin is orexigenic and elevated during negative energy balance. Ghrelin has also been found to be inhibitory for GnRH release in several species (Tschop et al., 2000; Wren et al., 2000; Ariyasu et al., 2001; Furuta et al., 2001; Fernandez-Fernandez et al., 2004, 2005a,b; Vulliemoz et al., 2004; Iqbal et al., 2006). In looking beyond leptin, future research investigating the circuits and modes through which ghrelin regulates GnRH release may prove critical for our understanding of negative energy balance-induced reproductive dysfunction. One potential intermediary for ghrelin's actions on GnRH release may be Kiss1 (Forbes et al., 2009). Kiss1 has been strongly implicated in the integration of energy balance and reproduction given arcuate nucleus Kiss1/NKB and AVPV Kiss1 expression levels are low in various states of negative energy balance. Significantly, arcuate nucleus Kiss1/NKB and AVPV Kiss1 levels were also not restored with physiological leptin treatment during negative energy balance, and methods aimed at preventing negative energy balance-induced hyperghrelinemia may elucidate whether this rise in ghrelin is critical for Kiss1 inhibition. Given the importance of the Kiss1 system for negative energy balance acyclicity, the search for other metabolic signals influencing this neuronal population is a leading research question in this field.

Gonadotropin-inhibitory hormone/RFRP3, alarin, and glucose-sensing ventrolateral medulla neurons are just a few of the

emerging candidates that have been linked to reproductive and metabolic regulation and as such may play a role in the integration of these systems. These three candidates are diverse in both their mechanism of metabolic sensing and their site of action in reproductive neuroendocrine circuits (**Figure 2**). GnIH/RFRP3 and noradrenergic ventrolateral medulla cells are both inhibitory signals for GnRH release that are increased during negative energy balance. While noradrenergic A1 cells are likely activated by low glucose levels, it is unclear how GnIH/RFRP3 might be upregulated during negative energy balance. However, once activated GnIH/RFRP3 cells likely contribute to increased orexigenic drive during negative energy balance, and it will be of interest to understand how GnIH may integrate with other well known metabolic/food intake systems of the hypothalamus, such as NPY, MCH, and orexin, to contribute to this orexigenic drive. Although GnIH/RFRP3 and the A1 populations may both be inhibitory for GnRH release, there is evidence that they may differ in their site of action within the hypothalamic-pituitary–gonadal axis. There is strong evidence that GnIH/RFRP3 acts directly at GnRH cells. New data presented here suggests there is potential brainstem noradrenergic input to arcuate nucleus Kiss1/NKB and AVPV Kiss1 cells, and future research will be aimed at determining whether the source of these inputs is in fact the noradrenergic A1 population. GnIH/RFRP3 fibers are also found in the arcuate nucleus indicating that this population may regulate Kiss1/NKB as well, though more detailed histological research will be needed to test this hypothesis. Unlike GnIH and the A1 noradrenergic population, there is little research on the role of alarin for reproductive regulation during negative energy balance given how recently this neuropeptide was discovered. However, the limited amount of research available on alarin suggests it may play a role in both food intake and reproductive regulation, much like its sister-gene product, GALP. Research examining the effects of alarin on reproductive parameters in females will be a crucial next step in determining what role this neuropeptide might play in the larger neuroendocrine pathway, including the regulatory direction of alarin's effect on LH release, as well as its potential site of action.

As more and more candidates involved in both reproductive and metabolic regulatory systems emerge, it is becoming clear that there are likely multiple signals and mechanisms working in concert to tightly couple the regulation of these two critical physiological processes. More specifically, negative energy balance results in wide spread changes in the hypothalamus, including increases in numerous orexigenic neuropeptides known to regulate GnRH release, increases in signals inhibitory for reproduction, and decreases in signals excitatory for reproduction (**Figure 2**). With such a myriad of changes in these two regulatory systems, it is unlikely any one factor is solely responsible for the subsequent inhibition of GnRH release. Similarly, there may be many metabolic signals that are required to produce sustained GnRH inhibition, for example acute decreases in glucose levels, intermediary increases in the gut orexigenic hormone ghrelin, and long-term decreases in circulating leptin. The most beneficial work of the future will likely be those studies that attempt to understand how multiple signals work in concert to control GnRH release during negative energy balance.

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# Sense and nonsense in metabolic control of reproduction

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An exciting synergistic interaction occurs among researchers working at the interface of reproductive biology and energy homeostasis. Reproductive biologists benefit from the theories, experimental designs, and methodologies used by experts on energy homeostasis while they bring context and meaning to the study of energy homeostasis. There is a growing recognition that identification of candidate genes for obesity is little more than meaningless reductionism unless those genes and their expression are placed in a developmental, environmental, and evolutionary context. Reproductive biology provides this context because metabolic energy is the most important factor that controls reproductive success and gonadal hormones affect energy intake, storage, and expenditure. Reproductive hormone secretion changes during development, and reproductive success is key to evolutionary adaptation, the process that most likely molded the mechanisms that control energy balance. It is likely that by viewing energy intake, storage, and expenditure in the context of reproductive success, we will gain insight into human obesity, eating disorders, diabetes, and other pathologies related to fuel homeostasis. This review emphasizes the metabolic hypothesis: a sensory system monitors the availability of oxidizable metabolic fuels and orchestrates behavioral motivation to optimize reproductive success in environments where energy availability fluctuates or is unpredictable.

**Keywords: appetitive behavior, hoarding, metabolic hypothesis, motivation, nutritional infertility, sex behavior, vaginal scent marking**

Research at the interface of energy balance and reproduction is gaining momentum as investigators in many different fields recognize the relevance of this topic to basic biology and clinical practice. According to a recent PubMed search (using the search keywords *energy balance and reproduction)* only 135 journal articles were published in the decade between 1980 and 1990, whereas 609 articles were published in the last decade. Eighty of the 135 articles published between 1980 and 1990 were concerned with lactation in dairy animals, whereas the 600 or more published in the last decade covered a broad range of topics including the many orexigenic and anorectic peptides that influence reproduction in a wide variety of organisms, including invertebrates and vertebrates, human and non-human primates, and males and females (**Table 1**). The recent momentum reflects an exciting synergy that arises from melding reproductive biology with neuroendocrinology of ingestive behavior. Reproductive biologists benefit from the theories, experimental designs, and methodologies used by experts in ingestive behavior and energy homeostasis. Reproductive biology and physiological ecology bring context and meaning to the study of ingestive behavior and energy metabolism. This current special issue of *Frontiers in Translational Endocrinology* illustrates that metabolic control of reproduction is now, on its own, an established field of basic biological research with the most exciting discoveries just around the corner. New investigators are beginning to recognize that by viewing energy metabolism in the context of reproductive success, we open a window into human obesity, eating disorders, diabetes, and other pathologies related to fuel homeostasis.

With so many new researchers entering the field, it might be useful to provide sign posts to the most fruitful avenues of research, as well as to potential hazards, wrong turns, and dead ends. In particular, new investigators need to know which hypotheses and assumptions are supported by a preponderance of evidence and which are largely unsupported. Unfortunately, some of the most often repeated ideas in this field happen to be as untestable as they are seductive. Future research will be centered on molecular mechanisms at the level of the gene, but will be meaningless without attention to the developmental, epigenetic, environmental, and evolutionary context.

This review will focus on the five ideas that are likely to facilitate progress in research at the interface of reproduction and energy homeostasis.

(1) We will emphasize experimental designs that incorporate *varying degrees of metabolic challenge and behavioral options relevant to the natural habitats in which those behaviors evolved*, i.e., habitats in which food is not available *ad libitum* and the experimental subjects have options to choose between reproductive and ingestive behaviors.
(2) We will illustrate the importance of measuring behavioral *motivation* by quantifying *appetitive* sex and ingestive behaviors, i.e., not only eating and copulation, but also behaviors that bring individuals in contact with food or potential mating partners, such as food hoarding and courtship.
(3) We will emphasize the *metabolic hypothesis*, the idea that neuroendocrine systems function to maintain fuel (not body fat or food intake) homeostasis.

**Table 1 | Orexigenic and anorectic peptides that influence reproduction.**

| Central peptides | Ingestive behavior effect | Reproductive effects |
|---|---|---|
| Agouti-related protein (AgRP) | Increases food intake and hoarding (Rossi et al., 1998; Day and Bartness, 2004) | Inhibits LH in the presence of E (Schioth et al., 2001), stimulates LH in males (Stanley et al., 1999) |
| α-melanocyte stimulating hormone (α-MSH), MTII | Decreases food intake and food hoarding (Shimizu et al., 1989; Keen-Rhinehart and Bartness, 2007) | Stimulates LH secretion (Alde and Celis, 1980) |
| Bombesin-like peptides | Decrease food intake (Gibbs and Smith, 1988) | Stimulate LH secretion (Babu and Vijayan, 1983) |
| β-endorphin | Increases food intake (McKay et al., 1981) | Inhibits LH secretion and sexual libido (Sirinathsinghji et al., 1983) |
| Cocaine and amphetamine-regulated transcript (CART) | Decreases food intake (Kristensen et al., 1998) | Stimulates GnRH secretion (Lebrethon et al., 2000; Parent et al., 2000) |
| Cholecystokinin (CCK) | Decreases food intake and food hoarding (Bailey and Dela-Fera, 1995; Teubner and Bartness, 2010) | Simulates LH secretion (Kimura et al., 1983; Perera et al., 1993) |
| Corticotropin releasing hormone (CRH) | Decreases food intake (Levine et al., 1983; Heinrichs and Richard, 1999) and food hoarding in rats (Cabanac and Richard, 1995) | Inhibits LH secretion and lordosis (Olster and Ferin, 1987) and sex behavior (Jones et al., 2002) |
| Dopamine (DA) | Decreases food intake (Heffner et al., 1977), increases food hoarding (Kelley and Stinus, 1985; Borker and Mascarenhas, 1991), and reward (Wise, 2004) | Stimulates sexual arousal, motivation and reward (Meisel and Mullins, 2006) |
| Galanin | Increases food intake (Kyrkouli et al., 1986; Krasnow et al., 2003) | Inhibits LH secretion (Sahu et al., 1987) |
| Galanin-like peptides | Increases food intake (Krasnow et al., 2003; Van Der Kolk et al., 2010) | Inhibits LH secretion (Gundlach, 2002; Krasnow et al., 2003; Van Der Kolk et al., 2010) |
| Glucagon-like peptide (GLP-I) | Decreases food intake (Turton et al., 1996) | Stimulates LH secretion (Beak et al., 1998) |
| Gonadotropin releasing hormone (GnRH I or II) | Decreases food intake (Kauffman and Rissman, 2004b) | Stimulates LH secretion and sex behavior (Moss and McCann, 1975; Clarke and Cummins, 1982; Temple et al., 2003) |
| Kisspeptin | Decreases food intake (Stengel et al., 2011) | Stimulates GnRH and LH secretion (Gottsch et al., 2004; Irwig et al., 2004) |
| Melanin concentrating hormone (MCH) | Increases food intake (Presse et al., 1996) | Inhibits LH secretion (Tsukamura et al., 2000a) |
| Motilin (peripheral) delete | Increases food intake in fasted rats (Garthwaite, 1985) | Inhibits LH secretion (Tsukamura et al., 2000b) |
| Neuropeptide Y (NPY) | Increases food intake (Stanley and Leibowitz, 1984) and food hoarding (Dailey and Bartness, 2009) | Inhibits LH in the absence of, stimulates LH in the presence of estradiol (Crowley et al., 1985; Sahu et al., 1987), inhibits sex behavior (Ammar et al., 2000), |
| Orexin/hypocretin | Increases food intake (Sakurai et al., 1998) | Inhibits LH in the absence of, stimulates in the presence of estradiol (Pu et al., 1998) |
| Oxytocin | Decreases food intake (Olson et al., 1991) | Stimulates sex behavior (Whitman and Albers, 1995) |
| RFamide-related peptide-3 = Gonadotropin inhibiting hormone | Increases food intake (Tachibana et al., 2005; Johnson et al., 2007) | Inhibits GnRH and LH secretion and sex behavior (Bentley et al., 2006; Kriegsfeld et al., 2006; Smith et al., 2008) |
| Secretin (move to VIP) | Decreases food intake (Cheng et al., 2011) | Stimulates LH secretion (Babu and Vijayan, 1983) |
| Serotonin (5HT) | Decreases food intake (Blundell, 1977) | Stimulates LH in the presence of estradiol (Coen and MacKinnon, 1979) Inhibits LH secretion in the absence of estradiol (Coen et al., 1980; Koh et al., 1984) |
| Thyroptropin releasing hormone | Decreases food intake (Vijayan and McCann, 1977) | Stimulates LH secretion in pituitary *in vitro* not *in vivo* (Fujihara and Shiino, 1983), and indirectly by effects on thyroid hormones (Barrett et al., 2007) |
| Urocortin | Decreases food intake (Spina et al., 1996) | Stimulates LH secretion in ewes (Holmberg et al., 2001), inhibits LH secretion (potentially; Li et al., 2005; Nemoto et al., 2010), directly inhibits Leydig cell function (Rivier, 2008) |
| Vasopressin | Decreases food intake (Meyer et al., 1989) | Inhibits LH secretion (Heisler et al., 1994) |

(4) We will urge investigators to focus specifically on mechanisms that *promote opportunistic overeating or hoarding and fuel storage in anticipation* of the high energetic demands of reproduction.
(5) All of the above concepts will be discussed within the context of a *distributed neural network* with multiple, redundant function. We will argue that metabolic control of reproduction must include the hindbrain, midbrain and forebrain including the ventral premammillary nucleus. This is in sharp contrast to the typical focus on the arcuate nucleus of the hypothalamus (Arc).

The central unifying hypothesis is that neuroendocrine systems that control energy homeostasis optimize reproductive success in environments where energy fluctuates or is unpredictable. The testable predictions that emerge from this hypothesis are fundamentally different from those that follow from the idea that neuroendocrine systems exist to maintain body weight or adiposity at some particular level.

## AN EVOLUTIONARY CONTEXT AND THE RELEVANCE OF A SEMI-NATURAL ENVIRONMENT

Metabolic control of reproduction is obscured in the laboratory when females are housed in isolation, in small cages, where locomotion is restricted, temperature is controlled, and where food availability is unlimited (Bronson, 1998). Under these conditions, there is more than enough energy available for all of the cellular and systemic processes including reproduction. Likewise, women in modern, westernized, industrial societies live surrounded by "foraging" opportunities that require very little energy expenditure. It is under these conditions of unlimited food availability that misconceptions developed about hormone effects on behavior. Two such misconceptions, for example, are that female sexual motivation in primates is emancipated from the effects of hormones, and that the main function of "satiety" peptides is to maintain stability in body weight. These naïve notions are shattered when animals are housed in semi-natural environments that mimic important aspects of their natural habitats that include a limited food supply, high energy demands, and the ability to choose between sex and ingestive behavior. When animals are studied under environmental conditions that approximate those in which the traits evolved, presumed "satiety" peptides increase sexual motivation and presumed "orexigenic" peptides promote vigilant foraging and food hoarding and opportunistic overeating. These effects are masked under conditions of *ad libitum* food intake and low energetic demand. Under these conditions, sex drive is already elevated, obscuring increases that might otherwise be induced by the release of anorectic peptides. Laboratory animals are perhaps in some ways more like modern humans in westernized societies in that, in both cases, the link between hormones, behavior, and environmental energy availability is obscured. An important function of these peptides, to orchestrate the appetites for food and sex, is revealed by studying laboratory animals under the energetically challenging conditions in which they evolved. As will be described in this review, laboratory rodents can be housed in a semi-natural burrow system that 1) requires that individuals expend energy on locomotion in order to gain access to food, and 2) provides opportunities to encounter potential mating partners during foraging expeditions. Under these conditions, the effects of hormones on motivation are revealed in sharp relief. Furthermore, fluctuation, rather than stability in ingestive behavior is the norm.

In contrast to the typical laboratory situation, food supplies in the natural habitats of most wild animals are not unlimited. Rather, in nature, food availability often fluctuates seasonally, with a nadir in the winter, dry, or rainy season depending on the geographic area. In addition, food availability varies unpredictably due to myriad factors including population density, inter and intraspecies competition, famine, drought, storms, hurricane, tornadoes, floods, fires, global climate change, and diseases that destroy edible plants, crops, prey animals, and livestock (Bronson, 1989, 1998). Given the importance of environmental energy availability on reproductive function in members of every mammalian order, it is reasonable to hypothesize the mechanisms that control energy intake, storage, and expenditure serve to optimize reproductive success in environments where energy fluctuates (Bronson, 1989; Wade and Schneider, 1992; Schneider, 2006; Schneider et al., 2007). One prediction from this hypothesis is that the effects of ovarian hormones on ingestive and sex behavior will vary with the degree of energetic challenge, that is, the balance of energy supply and expenditure. This idea is inspired by the book, *Mammalian Reproduction*, which provides evidence for metabolic control of reproduction in females from representative species of every mammalian order and for the conclusion that energy availability is the most important factor that controls reproduction in female mammals (Bronson, 1989).

There are three important features to this perspective.

First, females anticipate the energetic demands of reproduction by eating more than their immediate energetic needs and storing the extra energy as body fat or as a food cache. All ingested macronutrients derived from food, including carbohydrates and fats, can be stored as triglycerides in adipocytes, so that later, these triglycerides can be hydrolyzed to release glycerol and free fatty acids for oxidation. During pregnancy and lactation, these fuels are mobilized for the growing conceptus and to produce milk for the offspring. In rats, progesterone (elevated during the luteal phase of the ovulatory cycle and pregnancy), in the presence of estradiol, promotes increases in food intake, and body fat storage, at least in the early phases of pregnancy (Wade, 1975; Trujillo et al., 2011). Models of mechanisms that affect ingestive behavior and body weight must incorporate the ability to anticipate future energy shortages.

Second, females anticipate opportunities for fertile matings by virtue of the fact that the same hormones that induce ovulation also stimulate sexual appetite while reducing the appetite for food. Neuroendocrine mechanisms stimulate sexual (rather than ingestive) motivation during the times of highest fertility, when mate searching, courtship, and copulatory activities are most likely to pay off in terms of genetic contributions to the next generation. The sequence of hormones necessary for ovulation is permissive for copulatory behavior and these same neuroendocrine mechanisms inhibit foraging, hoarding, and eating during the most fertile part of the cycle (Zucker, 1969, 1972; Wade and Zucker, 1970; Zucker et al., 1972; Wade and Gray, 1979; Wade and Schneider, 1992; Asarian and Geary, 2006; Klingerman et al., 2010;

Michopoulos and Wilson, 2011). Thus, models of ingestive behavior and physiology must include the choice between food and sex, fluctuations in ovarian hormones, and sex differences in ingestive and sex behavior responses to ovarian steroids.

Third, females have mechanisms that delay reproductive processes during energetic challenges and re-initiate reproductive activities when the energetic conditions improve. In species from rodents to primates, sexual motivation, sexual performance, puberty, birth intervals, hypothalamic gonadotropin releasing hormone (GnRH) secretion, luteinizing hormone (LH) secretion, and ovarian steroid synthesis and secretion are inhibited by energetic challenges (McClure, 1962; Kennedy and Mitra, 1963; Morin, 1975; Ronnekleiv et al., 1978; Bronson and Marsteller, 1985; Cameron et al., 1985; Foster and Olster, 1985; Armstrong and Britt, 1987; Bronson, 1987, 1988; Lively and Piacsek, 1988; Sprangers and Piacsek, 1988; Schneider and Wade, 1989; de Ridder et al., 1990; Thomas et al., 1990; Cameron, 1996; Shahab et al., 1997, 2006; Ellison, 2001; Temple et al., 2002; Terry et al., 2005). When energetic challenges are so severe that they induce anestrous or inhibit the menstrual cycle, the primary locus of effect is the GnRH pulse generator, a diffusely located cell group in the medial basal hypothalamus. In support of this idea, pulsatile LH secretion, follicle development, and ovulation can be reinstated by treatment with pulses of GnRH at species-specific frequency and amplitude in food-deprived or -restricted rats, sheep, pigs, cows, monkeys, and women (Nillius et al., 1975; Foster and Olster, 1985; Bronson, 1986; Day et al., 1986; Armstrong and Britt, 1987; Cameron and Nosbisch, 1991; Kile et al., 1991; Manning and Bronson, 1991). Severe metabolic challenges can have effects at many levels (the gonad, pituitary, or hypothalamic GnRH generator, or the neural substrates that control sex behavior). Metabolic control of the GnRH pulse generator is the most widely studied. What about less severe metabolic challenges?

Fourth, and most recently, mild energetic challenges can have significant effects on reproductive and ingestive behavior long before there are effects on the mechanisms that govern steroid synthesis and secretion (Schneider, 2004; Schneider et al., 2007; Klingerman et al., 2010). Furthermore, in order to observe the effects of mild energetic challenges on the reproductive system, it is necessary to house animals in semi-natural environments in which energy expenditure is high relative to energy supply and both food and mates are available simultaneously (Schneider et al., 2007; Klingerman et al., 2010).

Attention to appetitive and consummatory aspects of behavior might shed light on so-called "feeding" hormones and neuropeptides which tend to stimulate ingestive behavior and inhibit aspects of the reproductive system, e.g., ghrelin, neuropeptide Y (NPY), and RF amide-peptide-3 (RFRP-3), also known as gonadotropin inhibiting hormone (GnIH), which tend to stimulate ingestive behavior and inhibit various aspects of the reproductive system (**Table 1**) (Clark et al., 1985; Guy et al., 1988; Kalra et al., 1988; Wren et al., 2000; Furuta et al., 2001; Johnson et al., 2007; Kriegsfeld et al., 2010; Shah and Nyby, 2010). It might also illuminate the functional significance of leptin, α-melanocortin stimulating hormone (α-MSH), kisspeptins, glucagon-like peptide, cholecystokinin, and GnRH, which tend to decrease ingestive behavior and stimulate reproductive behavior and physiology (**Table 1**) (Gonzalez et al., 1993; Wade et al., 1997; Schneider et al., 1998; Ammar et al., 2000; Cragnolini et al., 2000; Kauffman and Rissman, 2004a; Castellano et al., 2005, 2010; Kauffman et al., 2005; Fernandez-Fernandez et al., 2006; Crown et al., 2007; Millington, 2007). Given that many of these hormone–behavior systems were molded by natural selection in response to environmental energy availability, and that natural selection works via differential reproductive success, progress can be facilitated by attention to the influence of these hormone–behavior systems on reproductive success.

The hypothesis that putative anorectic and orexigenic peptides function to optimize reproductive success in environments where energy fluctuates or is unpredictable is relevant even for our own species. A look at modern, non-contracepting, non-industrialized societies shows that seasonal fluctuations and unpredictable scarcity in food availability have profound, measurable effects on reproductive success (Ellison, 2001). The link between reproduction and environmental energy is obvious in the !Kung, non-contracepting bush people who live in the Kalahari Desert where rainfall and hence food availability fluctuates dramatically within a year. Among the !Kung, the fluctuating food supply is coupled with a continuous need to expend energy because the workload is high throughout the year. !Kung women, for example, engage in miles of walking, carrying water, gathering firewood, harvesting, and cooking food, all while toting infants and toddlers. The !Kung show a dramatic loss of body weight during the "hungry season" and a dramatic drop in birth rate 9 months following the hungry season, suggesting that they only rarely ovulate during the this time of low food production (van der Walt et al., 1978). Given that evolutionary change occurs via differential reproductive success, plus the clear effect of seasonal fluctuation in food availability on reproductive success in extant populations of humans, it is likely that fluctuations in food availability molded ingestive and reproductive traits in our own species during human history.

Furthermore, the effects of environmental energy availability on human reproductive function are not limited to rural, indigenous, tribal peoples. Starvation and food insecurity has impact on reproductive function today in many, if not all societies. In fact, most societies have members who expend more energy than they can acquire, and nutritional amenorrhea occurs in these subpopulations, either because they experience starvation in relation to poverty or because they voluntarily engage in exercise and limit their food intake (Loucks, 2003a,b; Loucks and Thuma, 2003; Rosetta and Mascie-Taylor, 2009). For example, a high incidence of delayed menarche, adult amenorrhea, decreased birth rates, and high infant mortality result from low food availability in non-contracepting populations in India and Bangladesh (Gopalon and Naidu, 1972; Chen et al., 1974). Birth intervals are longer and birth rates plummet along with low energy balance (energy intake and storage minus expenditure) in women from extant, diverse, subsistence gardening/farming cultures, including the Lese of the Congo's Ituri Forest, the Tamang in the foothills of the Himalaya of central Nepal, and women who live in mountain valleys in rural Poland (Ellison, 2001). Not only are energetic effects on fertility present in the economically challenged people in every society, but these effects are seen in all strata of every society during famine (Chakravarty et al., 1982). Thus, examples of the link

between energy availability and reproductive success in our own species come from extant, modern populations as well as populations descended from subsistence agricultural societies. Thus, when we hypothesize that the mechanisms that control energy balance serve to optimize reproductive success in environments where energy fluctuates, this likely applies to our ancestors and to modern human beings.

The idea that the energy balancing system optimizes reproductive success is, in some ways, similar to the so-called "thrifty gene" hypothesis (Neel, 1962, 1999), with important differences. According to Neel, fluctuations in energy availability during the early evolution of *Homo sapiens* have favored genotypes that code for metabolic efficiency, the ability to overeat and store excess energy in adipose tissue that would increase the chances of survival during famine (Neel, 1962, 1999). The idea is often invoked to explain the so-called obesity epidemic. Individuals predisposed toward body fat storage had selective advantage in Paleolithic times, whereas in the presence of modern food abundance, the same individuals become obese. Various reiterations of Neel's ideas tend to be incomplete, and thus, in this review we emphasize three specific modifications. First, ingestive behavior, body weight, and body fat content are *polygenic*; many genes contribute to metabolic efficiency, body fat storage, and hunger, not just one "thrifty" gene. Second, survival during famine was not the sole function of the energy balancing system. The critical function was to modulate reproductive output according to environmental energy availability. Finally, overeating and obesity are not the inevitable outcome of one "thrifty" gene in an energy rich environment, but rather, the *interaction* of many genes with reproductive hormones, epigenetic factors, and environmental energy availability.

## APPETITIVE AND CONSUMMATORY BEHAVIORS

Reproductive behavior is far more complex than the simple act of copulation, ingestive behavior is more than the act of eating food, and these complexities are important. The hormonal effects on reproduction are not limited to the hypothalamic–pituitary–gonadal (HPG) system, but extend to the brain mechanisms that control the hunger for food and sex. Survival and reproduction involves appetitive behaviors that bring animals in contact with food or mating partners (Sherrington, 1906; Craig, 1917). Appetitive sex behaviors, however, occur separated in time from lordosis and reflect sexual motivation but not necessarily the ability to perform the sex act (Sherrington, 1906; Craig, 1917; Lorenz, 1950; Johnston, 1974, 1977; Lisk et al., 1983; Everitt, 1990). These appetitive behaviors might include mate searching and assessment, competition, courtship, and the ability to attract a mate. Similarly, ingestive behavior is more than the amount of calories ingested or meal size, it is a multifaceted array of interrelated behaviors and physiological traits that include foraging, food hoarding, food defense, and diet preference.

The history of behavioral endocrinology contains hints that we have missed something important in our narrow focus on food intake and copulation. Primate research based on laboratory studies led to erroneous conclusions about hormonal effects on primate sex behavior. These conclusions were overturned when researchers such as Kim Wallen began studying primates in semi-natural environments wherein females were able to exercise volition in their sexual interactions. Contrary to the commonly held idea that female primates are emancipated from the effects of hormones on behavior, appetitive sex behaviors, such as male-female grooming and proximity to mating partners, are correlated with peri-ovulatory increases in circulating estradiol when primates are studied in a semi-natural habitat where they experience social risks and can exercise volition with regard to sex behavior. These effects on sex behavior in a semi-natural environment have been traced to circulating levels of estradiol (Wallen, 2000, 2001). Similar misconceptions about hormonal effects on human behavior result from the narrow focus on copulation of the pair, rather than on underlying sexual motivation of the individuals in question. The idea that women are somehow emancipated from the effects of ovarian hormones on sex behavior is supported only by the difficulty in showing repeatable, statistically significant correlations between menstrual fluctuations in ovarian hormones and the incidence of sexual intercourse in married women from modern, industrialized societies (with unlimited food intake and low energetic demands). In contrast, when the motivation of individual women is assessed, there are statistically significant associations between these measures of appetitive behavior and phases of the menstrual cycle in a growing number of studies (Stanislaw and Rice, 1988; Meuwissen and Over, 1992; Gangestad et al., 2002; Gangestad and Thornhill, 2008; Durante and Li, 2009). These examples from sexual behavior in primates all suggest that it is important to create a relevant context in the study of behavioral endocrinology.

A similar problem occurs in the study of human ingestive behavior. Researchers in industry and academia alike commonly operate under the assumption that women are emancipated from effects of their ovarian hormones on ingestive behaviors. The effect of phases of the menstrual cycle on food intake in women, i.e., a periovulatory nadir in food intake, is subtle and is statistically significant in most, but not all studies (Fessler, 2003). The peri-ovulatory increase in locomotor activity is even more elusive in women (Fessler, 2003).

What would we find if sexual and ingestive *motivation* (not just food intake and the incidence of sexual intercourse) were measured in females with limited food availability and high energetic demands? Inspired by these questions, we have initiated a new line of research that manipulates energy availability and examines the effects of ovarian steroids on behavioral motivation (Klingerman et al., 2010, 2011a), and a recent example of this work is illustrated in the article by Klingerman et al. (2011b) in this issue.

The idea that any of these particular chemical messengers evolved to optimize reproductive success in environments where energy availability fluctuates is testable (i.e., it constitutes a hypotheses in which there is a realistic outcome that will refute the hypothesis). This hypothesis leads to the following testable predictions:

(a) The effects of the chemical messenger in question will vary when energy availability is manipulated. The effects of so-called orexigenic and anorectic peptides will be amplified in testing environments that mimic the habitats in which these neuroendocrine systems evolved, including the choice between food and sex, and the need to expend energy in order

to acquire energy. Specifically, the greater the energetic challenges, the more putative orexigenic peptides inhibit sexual motivation, and directly or indirectly promote vigilant foraging, hoarding, and eating. The choice between food and sex is a prerequisite for observation of the phenomenon.

(b) The "orchestration of motivation" implies effect on the choice between food and sex, because in natural environments animals do not live and forage in the absence of conspecifics and do not engage in sex in a separate enclosed space precluded from eating and foraging. Thus, sexual motivation, i.e., the appetitive aspects of behavioral choice, will be more sensitive to energetic challenges than the consummatory aspects of behavior. Food hoarding and the preference for spending time with males vs. food will be significantly affected by food restriction prior to significant changes in follicle development and estradiol secretion.

(c) Periovulatory increases in estradiol disinhibit mechanisms that control sexual motivation and behavior, thereby switching behavioral priorities from ingestive to sexual.

These hypotheses have been examined in female Syrian hamsters, rodents in which motivation (appetitive behavior) and ability (consummatory behavior) are easily measured, with respect to both ingestive and sex behavior. Sexual performance of the lordosis posture is a reflex triggered by male conspecific odors and tactile stimulation. These sensory cues are integrated in neural structures only when those neural structures are stimulated by periovulatory levels of estradiol and progesterone, which bind to their receptors, estradiol receptor-alpha (ER-α), and progestin receptor (PR). Lordosis reflects an unknown combination of both motivation and ability and consistently occurs on day 4 of the 4-day estrous cycle, known as proestrous in rats (Ciaccio and Lisk, 1971; Lisk et al., 1972; Steel, 1981). Motivation, in contrast to performance, can be measured in this species by counting the number of vaginal scent marks, or by measurement of the preference for sex vs. food. Vaginal scent marking increases gradually over days 1–3 of the Syrian hamster estrous cycle (known as diestrous 1 and 2 in rats) as circulating estradiol (but not progesterone) is rising. In addition, female hamsters decrease levels of agonist behavior toward males, and spend progressively more time in closer proximity to males as circulating levels of estradiol increase (Johnston, 1974, 1975). Hamster appetitive sex behaviors increase linearly with increasing levels of estradiol alone and are inhibited by progesterone (Ciaccio et al., 1979; Steel, 1981).

Hamsters are prodigious hoarders in the wild and in the laboratory. Metabolic challenges, such as food deprivation increase the appetitive ingestive behavior, food hoarding, but not the consummatory measure, food intake (Silverman and Zucker, 1976; Rowland, 1982). In their natural habitat, hamsters emerge from underground burrows for only 90 min per day and spend virtually every minute of this time hoarding food (Gattermann et al., 2008). These ecological observations suggest that the choice between hoarding and courtship has important consequences for reproductive success and evolution by natural selection. Thus, female hamsters were acclimated to a burrow system that included vertical tubes in a *t*-configuration leading in one direction to food and in the opposite direction to an adult male hamster. For 8 days prior to testing, they were either fed *ad libitum* or food-restricted to 75% of their *ad libitum* intake. At the onset of the dark period of the light–dark cycle, they were tested every day of the estrous cycle for their preference for males vs. food, food hoarding, and food intake (Klingerman et al., 2010).

According to our first hypothesis, we predicted that the effects of the anorectic hormone estradiol would differ according to the availability of metabolic energy, and that appetitive behaviors would be more sensitive than consummatory behaviors to energetic challenges. We first tested this by measuring ingestive and sexual motivation over the estrous cycle under two different energetic conditions: limited and unlimited food availability. In two other experiments we manipulated the available energy by increasing the demand for energy expenditure. We used two conditions in which hamsters must increase their energy expenditure: housing at cold ambient temperature (5°C for at least 7 days before testing), and access to voluntary exercise (the hamsters were housed with running wheels). In all types of energetically challenged females, whether food-limited, cold-housed, or exercising), we predicted that there would be clear fluctuations in appetitive behaviors over the estrous cycle, with food preference at its nadir and sexual motivation at its peak on estrous cycle days 3 and 4. If our first hypothesis were correct, only energetically challenged females would fluctuate over the cycle in food hoarding and the preference for males vs. food, whereas females with unlimited energy availability would not fluctuate to the same degree. Our hypothesis would be refuted if females with unlimited energy availability did not differ from those with limited intake or increased energetic demands. If our second hypothesis were correct, energetically challenged females would differ from energy unchallenged females in appetitive (food hoarding and preference for spending time with males), but not consummatory behavior (food intake and the incidence of lordosis).

As hypothesized, only the food-limited, cold-housed, and wheel-running females varied significantly over the estrous cycle in appetitive sex behaviors. Those with unlimited energy availability showed consistently high preference for sex throughout the estrous cycle. For example, food-limited, cold-housed, and wheel-running females showed significant fluctuations in male preference [(time spent with males – time spent with food) divided by total time; **Figure 1**; Klingerman et al., 2010]. Females with unlimited food, housed at room temperature without access to running wheels varied little if any over the estrous cycle in male preference (i.e., they preferred to visit the males more than 75% of the time of the time; **Figure 1**). Similarly, the food-restricted and cold-housed females varied significantly over the estrous cycle in food hoarding (**Figure 2**). Food-limited and cold-housed females showed low levels of food hoarding and high levels of male preference on the night of high circulating concentrations of estradiol and ovulation, and high levels of hoarding and low levels of male preference on all other estrous cycle day (**Figure 2**), and yet the same females showed little fluctuation in food intake over the estrous cycle (**Figure 3**). Females with unlimited food supply, housed at warm temperatures and without access to running wheels showed little variation in food hoarding over the estrous cycle, and none of the groups showed dramatic changes in food intake. With regard to sex behavior, the response to energy deficit was similar, whether the deficit

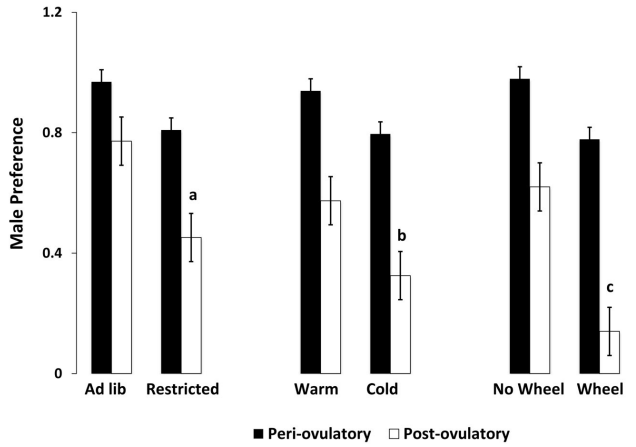


**FIGURE 1 | Mean and SEM of male preference, calculated as the (time spent with the male minus the time spent with food) divided by the total time.** Male preference is shown for females on either the periovulatory day (when circulating estradiol is high, black bars) or the postovulatory day when (circulating estradiol is low, white bars), in three experiments. Experiment 1 compared food-limited to food-unlimited females at 22˚C without access to wheels. Experiment 2 compared cold-housed (5˚C) to warm housed (22˚C) in females with unlimited food availability. Experiment 3 compared females housed with running wheels to those without running wheels at 22˚C with levels of food equal to those fed *ad libitum* without wheels. a = significantly different from *ad libitum*-fed at $P < 0.01$, b = significantly different from warm housed at $P < 0.01$, c = significantly different from no-wheel group at $P < 0.001$. Food-limited and unlimited groups adapted from Klingerman et al. (2010).

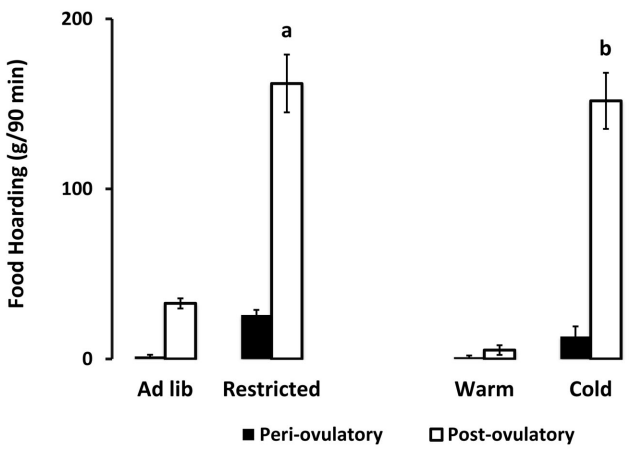


**FIGURE 2 | Mean and SEM of food hoarding shown for females on either the periovulatory day (when circulating estradiol is high, black bars) or the postovulatory day when (circulating estradiol is low, white bars), in two experiments.** Experiment 1 compared food-limited to food-unlimited females at 22˚C. Experiment 2 compared cold-housed (5˚C) to warm housed (22˚C) in females with unlimited food availability. a = significantly different from *ad libitum*-fed at $P < 0.001$, b = significantly different from warm housed at $P < 0.001$. Food-limited and unlimited groups adapted from Klingerman et al. (2010).

was due to limited food availability, cold ambient temperature, or wheel running. Food hoarding, however, was increased by limited

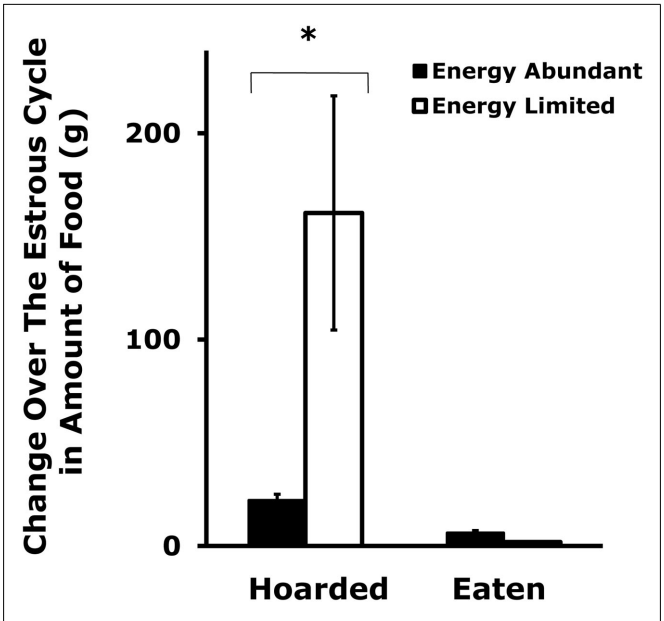


**FIGURE 3 | Mean and SE of the change in food hoarding that is seen over the estrous cycle in food-limited female Syrian hamsters compared to those with unlimited food.** * = significantly different from females with unlimited food. After Klingerman et al. (2010).

food availability and cold temperatures, but not by increases in voluntary wheel running. Females with wheels decreased their preference for males and yet hoarded very little food, perhaps providing a window into brain areas that partition energy for either sexual motivation or hunger for food.

These experiments suggest that changes in energy intake and expenditure change the responsiveness or sensitivity to ovarian steroids. A follow-up experiment showed the same effects of limited energy availability when ovariectomized (Ovx) females were compared to females treated with the hormonal regimen typically used to induce lordosis in this species (estradiol 48 h and progesterone 6 h prior to testing). Four days of food restriction increased food hoarding and decreased male preference in Ovx + vehicle females relative to Ovx + estradiol and progesterone treated females (Klingerman et al., 2010). The effects are not attributable to changes in circulating levels of ovarian steroids, but to the response to those steroids (Klingerman et al., 2010). In other words, ovarian steroids had obvious measurable effects on appetitive behaviors in females with limited food availability, but these were attenuated in females fed *ad libitum*. Furthermore, at these short durations of mild food restriction, steroid-energy availability interaction was apparent only in appetitive, not consummatory behaviors, and they occurred in response to exogenous steroid treatment.

*Together, these experiments are consistent with the idea that an important role of estradiol is to orchestrate the choice between ingestive and sex behavior under conditions where energy availability fluctuates and that this decision occurs at the level of behavioral motivation rather than performance.* Furthermore these experiments show that the effects are not limited to food deprivation, but they extend to any situation in which overall availability of energy

is limited, for example, when the need for energy expenditure is elevated relative to energy intake.

These results lead to additional testable hypothesis about the mechanisms by which estradiol orchestrates behavioral choice. For example, we hypothesize that during the early follicular phase of the estrous cycle when circulating estradiol levels are low, sexual motivation is tonically inhibited by one or a number of putative "orexigenic" peptides, such as ghrelin, GnIH, NPY, agouti-related protein (AgRP), endocannabinoids, or some combination of these. Furthermore, we hypothesize that periovulatory levels of estradiol disinhibit the effects of ghrelin, and/or other neuropeptides in order to couple reproductive motivation with fertility and inhibit food hoarding. These hypotheses lead to testable predictions about the effects of estradiol on neural activation in identified neurons, e.g., those that contain ghrelin or endocannabinoid receptors and secrete GnIH, NPY, or AgRP. Consistent with this idea, changes in neural activation in GnIH cells are more closely associated with appetitive than consummatory sex and ingestive behaviors (Klingerman et al., 2011b).

These studies illustrate that, when studying the effects of energy availability, it is imperative to differentiate appetitive from consummatory behaviors. Appetitive behaviors are more sensitive, and occur prior to effects on gonadal steroids. By exclusive focus on food intake estrous cyclicity and pulsatile gonadotropin secretion, the effects of energy availability will be missed. Furthermore, it is well known that appetitive aspects of behavior often involve different brain areas and different hormonal stimulation (Ball and Balthazart, 2008), and these are often more relevant to human behavior. Most relevant to metabolic control of reproduction and ingestive behavior, cellular activation that corresponds with NPY/AgRP effects on food hoarding occurs in the subzona incerta and central nucleus of the amygdala, whereas cellular activation that corresponds to effects on food intake involve the typical activation of paraventricular nucleus of the hypothalamus (PVH), and other areas, but not the subzona incerta or central nucleus of the amygdala (Teubner et al., 2011). Food hoarding species, such as Siberian and Syrian hamsters should receive a great deal more attention in the future, now that it has been documented that our own species is more like hamsters than rats in that they do not show postfast hyperphagia to the same degree as laboratory rats and mice (Hetherington et al., 2000; Al-Hourani and Atoum, 2007; Levitsky and DeRosimo, 2010), but instead show significant changes in grocery shopping, i.e., carrying food from a source outside the home and storing it in their home prior to consumption (Dodd et al., 1977; Tom, 1983; Beneke and Davis, 1985; Beneke et al., 1988; Mela et al., 1996). These ideas are extensively covered in a recent, lucid review of the appetitive behavior, food hoarding, by Bartness et al. (2011).

## SENSE AND NONSENSE, THE LIPOSTAT HYPOTHESIS

The above-mentioned results suggest that not all hormones and neuropeptides function to keep body weight within limits that we imagine to be healthy and fashionable. Rather, they function to respond to changes in environmental energy availability by modulation of reproductive and ingestive behaviors. In nature, energetic demands differ in males and females, within groups of males and females, over seasons, and over the entire lifespan.

This is important because translational research programs are often built upon the idea that "normal" individuals have a healthy "set point" for body weight and adiposity, whereas overweight and obese individuals do not. The "lipostat" hypothesis purports that factors secreted from adipose tissue dictate the level of food intake in service of maintaining this set point in adiposity. A modern version of the lipostat hypothesis purports that factors such as leptin are secreted from fat cells in proportion to overall body adiposity, induce satiety, and therefore decrease meal size. When extended to reproduction, the lipostat hypothesis is called the "critical body fat" hypothesis, which suggests that puberty is delayed or adult reproduction is inhibited when body fat content and levels of the lipostatic hormone falls below a particular threshold.

The lipostat, set point, and the critical body fat concepts are intuitively satisfying descriptions that are seldom tested directly but are often reinforced with circular reasoning. In contrast, science progresses by testing hypotheses that can conceivably be ruled out by a realistic experimental outcome (Popper, 1963). The set point model is refuted whenever an experimental group fails to defend a set point for body weight (e.g., due to a change in diet, reproductive cycle, photoperiod, or ambient temperature). Instead, any significant increase in body weight is interpreted as evidence for a "resetting" or "sliding" set point. Like the existence of God, the sliding set point hypothesis cannot be refuted. It is difficult to imagine how natural selection (based on individual survival and superior reproductive success) would favor defense of a set point in environments where food availability fluctuates or is unpredictable. When food is scarce and energy demands are high, the maintenance of a particular set point for body weight or adiposity should receive low priority compared to the break down of lipids in adipose tissue to usable metabolic fuels necessary for survival. Conversely, if females encounter a windfall of energy rich food, why abstain from overeating in order to preserve a putative set point for body weight when pregnancy and lactation are so energetically demanding and the food supply so unreliable? Why not eat heartily and store the excess energy as lipids in adipose tissue depots especially designed to provide fuels for milk production that will feed your genetic contribution to the next generation? Females might develop obesity but would be more likely to get their genes into the next generation, particularly if the unhealthy consequences of obesity accrue after the reproductive years.

The set point is a seductively satisfying label or analogy that has been criticized because it terminates, rather than stimulates further investigation. To name a phenomenon is not, in and of itself, progress in understanding the phenomenon. The set point is used as an analogy, but that does not translate into a testable hypothesis. The lipostat and the set point idea are inspired by the engineers' design of the mechanical thermostat. A homeowner's thermostat contains a physical object, a thermometer, that can measure temperature and can be calibrated and set by the homeowner. The room temperature varies above or below the set point, and when it deviates too far from set point, the heating or air conditioning corrects the error. In animals, there is no physical object that corresponds to the thermostat. We have not identified the location and biochemical nature of the detectors for metabolic fuel availability, and we have no idea how such a set point for

fuel availability or for body fat content might be set or calibrated. The set point theory has been repeatedly refuted on both empirical and theoretical grounds in numerous excellent reviews that are highly recommended (Wirtshafter and Davis, 1977; Van Itallie and Kissileff, 1990; Bronson and Manning, 1991; Bronson, 1998). Due to its intuitive and seductive nature, however, it is bound to come up whenever a new investigator enters the field of metabolic control of reproduction.

In contrast to the lipostat idea, the hypothesis that putative orexigenic and anorectic hormones function to optimize reproductive success in environments where energy availability fluctuates leads to testable hypotheses. It is in line with data showing that putative satiety peptides ensure overeating when those peptides are low, but are often ineffective in curbing appetite when they are high (Ahima et al., 1996; Flier, 1998). The hypotheses are reminiscent of Optimal Foraging Theory, which has its origins in ecology and is based on the ubiquitous observation among wild animals that food intake decreases with increasing cost in terms of time and energy expenditure. The corollary is that energy intake increases when food is cheap, plentiful, and requires little energy to obtain and digest (Emlen, 1966; MacArthur, 1966). When animals encounter diets of different caloric density that can be consumed at a particular energetic cost, intake increases as cost decreases and as net calories increase, and this choice ultimately results in accumulation of body fat (Collier et al., 1972; Houston and McNamara, 1989). This effect is linear, and thus, any lipostatic explanation would have to include a new set point reached for every excess calorie consumed. Instead of stability, body weight displays remarkable plasticity that allows anticipation of future metabolic challenges and permits the learning and memory formation that occurs when postingestional cues reinforce sensory cues from food. It is important to embrace this fact of life, and study the mechanisms whereby cheap and calorically dense food elicits changes in metabolism that allow excess storage, rather than search for an elusive and possibly non-existent mechanism that is suppose to maintain one set point for body weight throughout adult lifespan.

Like body fat content, food intake is not held at a set point. For example, when laboratory chow is diluted with non-nutritive bulk, laboratory rodents do not maintain their food intake at a set point. Quite the contrary, they show a controlled increase in food intake in proportion to the caloric dilution, and reproductive function is related to a threshold of usable fuels, not a particular level of bulk intake (Adolph, 1947; Peterson and Baumgardt, 1971; Nance and Gorski, 1977; Louis-Sylvestre, 1987; Szymanski et al., 2009). In contrast, changing the vitamin, mineral or essential fatty acid content of food while keeping calories constant elicits little or no change in food intake (Adolph, 1947). Furthermore, when animals must exercise or expend energy to obtain food, or when animals must expend more energy to keep warm at cold ambient temperatures, animals increase their food intake to compensate for the extra energy expended (Kraly and Blass, 1976; Browne and Borer, 1978; Tsai et al., 1982; Bart ness et al., 1984; Rowland, 1984; Louis-Sylvestre, 1987; Manning and Bronson, 1990; Schneider and Wade, 1990b). Females of many species will more than double their food intake during lactation to meet the energetic demands of milk production (Cripps and Williams, 1975; Fleming, 1976a,b, 1978; McLaughlin et al., 1983; Louis-Sylvestre, 1987; Woodside et al., 2000). Lactating female mice, *Peromyscus leucopus*, increase their food intake 230% when housed in cold ambient temperature compared to their prepregnant food intake at laboratory temperatures (Perrigo, 1987).

A particularly convincing argument for metabolic, rather than lipostatic control of food hoarding, comes from studies of Siberian hamsters fed a diet diluted with non-nutritive cellulose. Hamsters increase their food hoarding (but not their food intake) in proportion to the dilution and decrease their food hoarding in proportion to increases in caloric density. The change in food hoarding is immediate, and thus cannot be mediated by changes in body fat content, but is more likely controlled by postingestive cues that occur when the hamsters taste the diet just prior to hoarding (Wood and Bartness, 1996). Like internally stored energy, externally stored energy is flexible and varies with the energetic demands of the individual, its life history stage, and the environment. Rather than maintain a set point, food intake, food hoarding, body weight, and adiposity change dramatically in response to environmental cues (energy, ambient temperature, and photoperiod) in order to maintain the availability of oxidizable metabolic fuels for survival and reproduction (Friedman, 2008).

Contrary to commonly held dogma, stability in body weight is not a consistent feature in *ad libitum*-fed mammals, especially female mammals. The myth of stability in body weight is based on longitudinal studies, but is not supported by studies in which the same individuals are studied over many weeks. Far from the stability of body weight predicted by the lipostat hypothesis, body weight in animals with unlimited access to food increases steadily over time (Ahren et al., 1997). When genetically heterogeneous (outbred) strains of rats are singly housed with unlimited access to standard, laboratory chow, and monitored for more than 100 weeks, they fail to maintain "body weight homeostasis." Instead they not only gain significantly more body weight and adiposity than food-limited rats, but they increase their body fat content from 6–7% at 6–7 weeks of age, to 25% at 14 weeks, to 36–42% at 106 weeks of age. They also develop significant hypertriglyceridemia and hypercholesterolemia relative to food-limited rats (Keenan et al., 2005). Male rats show the largest increase in body weight during the first year; females show the largest body weight gain in the second year after cessation of their estrous cycles. The study by Keenan et al. (2005) illustrates two points. First, rats in isolation and in a confined space do not self-regulate their intake, and do not avoid the negative consequences of obesity. Second, reproductive factors create differences in the propensity to store body fat. In females, the body weight gain was exaggerated after the postmenopausal decrease in ovarian steroid secretion, whereas in males, body weight gain occurred early in the life history cycle (Keenan et al., 2005). Thus, in addition to mechanisms that maintain fuel homeostasis for individual survival, there lies another layer of control that promotes internal fuel storage as body fat to anticipate the need to forego ingestive behavior in favor of reproductive behavior necessary for Darwinian fitness.

This brings us to the lipostatic hypothesis of reproduction popularized by Rose Frisch (Frisch and McArthur, 1974; Frisch, 1990), which purports that there is a critical level of body fat necessary for the initiation of puberty and menarche, the maintenance

of adult menstrual cycles and fertility and for the restoration of reproductive function in animals that have become anestrous or hypogonadotropic after food restriction or deprivation. Little evidence actually supports this hypothesis beyond the obvious correlation between body fat content and reproductive function; but correlation is not causation. Both body fat content and reproductive competence depend upon a third factor, the availability of oxidizable metabolic fuels, thus, body fat content and reproduction are correlated. When strong inference hypothesis testing is used as a direct test of the "critical body fat" hypothesis, the critical body fat hypothesis is refuted. For example, when females are rendered hypogonadotropic by food deprivation and are then re-fed, the restoration of estrous cycles and pulsatile LH secretion occurs more rapidly than the recovery of body fat levels, and without significant increases in plasma leptin concentrations in sheep and hamsters (Schneider et al., 2000a; Jones and Lubbers, 2001; Szymanski et al., 2007). Finally, most animals respond rapidly to changes in fuel availability, too rapidly for the changes in reproduction to be due to the slow process of lipid accumulation (Bronson, 1986, 1998, 2000; Armstrong and Britt, 1987). Thus, contrary to the critical body fat hypothesis, LH pulses can be restored whenever overall metabolic fuel availability increases, even when body fat levels and levels of plasma leptin concentrations lag behind and remain at the same level as hypogonadal food-restricted females.

## SENSE AND NONSENSE, THE METABOLIC HYPOTHESIS

> "The simplest way in which this lipostasis could be achieved is by sensitivity to the concentration of circulating metabolites."
>
> (Kennedy, 1953)

Gerald Kennedy is often credited with coining the word *lipostat*, which, over time, became associated with the set point hypothesis. It is important to note, however, that Kennedy's idea of a fat-derived signal came not from circulating hormones or neural signals from adipose tissue, but rather from circulating metabolites associated with either lipolysis or lipogenesis (Kennedy, 1953). Furthermore, he proposed that the ability to sense metabolic fuel availability was critical for the control of food intake and reproductive development. Kennedy's lipostat was more in line with the large body of data showing that the productive system is responsive to the availability of oxidizable metabolic fuels (Schneider and Wade, 1989; Wade and Schneider, 1992; Foster et al., 1995; Nagatani et al., 1995; Murahashi et al., 1996). Gerald Kennedy was perhaps the first to speculate that if we understood the mechanisms that switched animals from lipolysis and free fatty acid oxidation to lipogenesis and fat storage we would understand the onset of puberty and the control of food intake (Kennedy, 1953; Kennedy and Mitra, 1963).

Metabolic control of reproduction has a long history spearheaded by ingestive behavior researchers and reproductive endocrinologists working together. Inspired by the original papers by Kennedy, M. I. Friedman, S. Ritter, and others (Ritter, 1986; Friedman, 1989), Schneider and Wade (1989) used pharmacological inhibitors of glucose or free fatty acid oxidation to differentiate between the effects of fuels vs. the effects of body fat content on estrous cyclicity. They studied Syrian hamsters because a mere 48 h period of food deprivation induces anestrous in this species (Morin, 1986). Schneider and Wade (1990a) compared the effects of 48 h deprivation in fat vs. lean females and demonstrated that body fat content could buffer against food deprivation-induced anestrous. However, as Kennedy predicted, fat hamsters were protected not by their body fat content *per se*, but by the metabolic fuels hydrolyzed and mobilized from lipids in adipocytes, i.e., free fatty acids. This was demonstrated when 48 h of food deprivation-induced anestrous in lean, but not fat female hamsters, and the protective effects of fat were blocked by treating fat hamsters with the inhibitor of fatty acid oxidation, methyl palmoxirate (MP; Schneider and Wade, 1989). Food-deprived hamsters treated with MP became anestrus even though they did not differ in body fat content from the estrous-cycling, food-deprived hamsters treated with vehicle. Thus, the HPG system is not controlled directly by body fat content, but rather, by the peripheral free fatty acids released from adipocytes, although the effect of those peripheral fatty acids might be to spare glucose for the brain. Since the publication of Schneider and Wade (1989), control of reproductive processes by the availability of oxidizable metabolic fuels has been documented in other model systems (Dickerman et al., 1990; Berriman et al., 1992; Bucholtz et al., 1996; Murahashi et al., 1996; Nagatani et al., 1996; Medina et al., 1998; Temple et al., 2002; I'Anson et al., 2003a; Moriyama et al., 2003; Shahab et al., 2006).

The above-mentioned research was focused on changes in the periphery. More recent research is focused on CNS fatty acid oxidation and synthesis, and the bulk of this work is concerned with ingestive behavior. Prior to diving into this field of research, it is important to examine the role of glucose sensing, as well as the idea that food intake and reproduction are controlled by the availability of ATP, or the ratio of ATP:AMP (Friedman, 2008). Glucose and fatty acid oxidation do not occur independently, but rather they are interrelated. The availability of glucose and the ratio of ATP to AMP and ADP, for example, determine the extent to which cells engage in fatty acid oxidation. This is important because translational research aimed at one metabolic pathway will have to account for compensatory coordinated changes in the other, and detection of these different fuels may take place in different parts of the brain.

Cellular detectors of glucose availability important for control of estrous cycles have been localized to the brainstem, just as those for food intake, adrenal glucocorticoid secretion, and counterregulatory responses to glucoprivation are localized in the brainstem (Ritter et al., 2011). There are "glucose-sensitive" cells in many brain areas involved in other diverse functions (including neuroprotection, circadian rhythms, and reward, to name a few; Levin et al., 2004), but those outside the hindbrain require more than 10-fold higher concentrations and 100-fold higher volume of inhibitors of glucose oxidation for significant effects on ingestive behavior (Ritter et al., 2011). Ingestive behavior is stimulated (Smith and Epstein, 1969; Ritter, 1986), and reproductive processes are inhibited by treatments that block glucose oxidation given systemically (Schneider and Wade, 1989) or into the third or fourth ventricle (Ritter et al., 1981; Murahashi et al., 1996), and the effects of glucoprivation are prevented by lesions of the AP in the caudal most part of the hindbrain (Ritter and Taylor, 1990; Schneider and Zhu, 1994; Panicker et al., 1998). For both ingestive behavior and reproduction, catecholaminergic projections from

hindbrain to the PVH are necessary for the effects of glucoprivation (Ritter and Calingasan, 1994; I'Anson et al., 2003b; Bugarith et al., 2005).

For the HPG system and sex behavior, we are likely to find that brainstem structures are far more sensitive to changes in glucose availability than are forebrain structures. This is foreshadowed by work on ingestive behavior. Microinjections of an inhibitor of glucose utilization into hundreds of brainstem areas in the NTS increased food intake, whereas only a few did so when administered to hypothalamic areas (reviewed by Ritter et al., 2011). Small (200 nl volumes of 12–24 g per animal) of glucoprivic agents microinjected unilaterally into discrete hindbrain regions increase food intake and initiated counterregulatory responses. The same doses and even higher doses are not effective when injected into hypothalamic sites or even into the fourth ventricle (Ritter et al., 2000). Furthermore, small microinjections of glucoprivic agents that increase food intake also increase mRNA for the orexigenic peptide NPY. The hyperphagia and increase in NPY mRNA are significantly decreased by immunotoxic destruction of the catecholaminergic/NPYergic cells that originate in the hindbrain. This is not true in the hypothalamus. In contrast to brainstem lesions, NPY-saporin-induced lesions of Arc NPY neurons do not impair glucoprivic feeding or hyperglycemic responses (Ritter et al., 2006). Careful mapping of metabolic stimuli that affect food intake strongly suggests that the important signals are detected in the caudal brainstem, and this type of mapping should be done for metabolic control of reproduction. These brainstem structures and projections to the PVH are not required for the effects of food deprivation on food intake and reproduction, only for changes elicited by glucoprivation. Thus, glucoprivic control cannot explain all effects of natural energetic challenges on food intake and reproduction. However, the parallels between glucoprivic control of food intake and glucoprivic control of estrous cycles are striking and worth remembering when trying to unravel the effects of intracellular fuel metabolism on reproduction.

Another important line of research showed that food intake is not controlled by either glucose or free fatty acid availability, *per se*, but by the general availability of oxidizable fuels or a metabolic event in the final common pathway to ATP production, perhaps ATP content itself. This idea is supported by the results of experiments in which pharmacological agents that reduce hepatic (liver) ATP increase food intake, and treatments that prevent depletion of hepatic ATP content also preclude increases in food intake (Rawson and Friedman, 1994; Rawson et al., 1994; Ji and Friedman, 1999; Ji et al., 2000). There is evidence that detectors of fatty acid oxidation and hepatic ATP content are in the periphery, most likely in the liver, because effects of fatty acids and of hepatic ATP content on food intake are blocked by treatments that disconnect the communication between the liver and brain via the vagus nerve (Ritter and Taylor, 1990; Tordoff et al., 1991). Metabolic inhibitors that decrease hepatic ATP status increase intracellular sodium and calcium concentrations in hepatocytes *in vitro* (Friedman et al., 2003; Rawson et al., 2003). Future research on metabolic control of food intake and reproduction will have to contend with the possibility that changes in the brain have effects on these peripheral ATP detectors.

The metabolic hypothesis was diluted in the literature after the discovery that leptin, the protein product of the *ob* gene, decreased food intake and restored reproductive capabilities in obese, hyperphagic, infertile *ob/ob* mice (mice homozygous for a mutation in the *ob* gene; Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Most of the literature in the decade from 1995 to 2005 portrays leptin action as nothing more than endocrine signaling, with less attention to leptin's effects on intracellular fuel oxidation or to the metabolic events that control leptin synthesis and secretion. However, some investigators gathered evidence from diverse sources, which together suggested that peripheral hormones like insulin and leptin act via intracellular fuel oxidation. M. I. Friedman pointed out that effects of insulin and leptin on ingestive behavior. . .

> ". . . is an indirect response to a shift in fuels from storage to oxidation, not a direct response to a satiety signal associated with the level of adiposity. Many experimental treatments that affect eating behavior, whether restricted to the central nervous system or not, alter peripheral metabolism. Because changes in fuel partitioning can affect food intake, knowing the metabolic consequences of such experimental manipulations may be necessary to understand their effect on eating behavior." (Friedman, 1998)

Inspired by conversations with Friedman, in the year 2000, J. E. Schneider et al. pointed out that. . .

> "There are at least two possible ways that leptin might interact with the metabolic sensory system that controls reproduction. First, leptin synthesis and secretion in various tissues might be sensitive to metabolic fuel availability. . . as a *mediator* of the metabolic signal. Second, leptin might affect reproduction indirectly by way of *modulating* the metabolic signal that is known to influence reproduction. Leptin's effects on reproduction coupled with its unique effects of fuel metabolism, i.e., it's ability to promote *in situ* fuel oxidation without increasing the general availability of metabolic fuels in circulation, might provide new clues to the nature of the metabolic stimulus that controls reproduction." (Schneider et al., 2000b)

Leptin decreases food intake and stimulates reproductive process in a wide variety of species (reviewed by Schneider, 2000), but contrary to the lipostatic hypothesis, leptin acts on estrous cycles by modulating the intracellular availability of oxidizable fuels. The first evidence for the notion that leptin *modulates* intracellular fuel availability was demonstrated *in vitro* by the laboratories of Ungar and Rossetti (Rossetti et al., 1997; Shimabukuro et al., 1997; Wang et al., 1998). Does leptin modulate ingestive behavior and/or reproduction by modulating the intracellular availability of glucose or free fatty acids?

Treatment with leptin prevents food deprivation-induced anestrus in Syrian hamsters, and, thus, follow-up experiments were designed to ask whether follow-up experiments show that leptin's ability to prevent food deprivation-induced anestrous is dependent upon the ability to increase glucose and/or fatty acid oxidation (Schneider et al., 1998). Syrian hamsters were either food deprived or fed *ad libitum* and treated with doses of MP, the

inhibitor of free fatty acid oxidation, or 2-deoxy-D-glucose (2DG), an inhibitor of glucose utilization. Leptin was given either systemically or intracerebroventricularly. Doses of MP and 2DG were used that do not inhibit estrous cycles in *ad libitum*-fed females. The stimulatory effects of systemic and intracerebroventricular leptin on estrous cycles are blocked by treatments that blocked intracellular glucose or fatty acid oxidation (**Figure 4**; Schneider et al., 1998; Schneider and Zhou, 1999). This, to the best of our knowledge, was the first experiment to demonstrate the interaction between leptin and intracellular fuel oxidation on reproduction.

Whereas the above-mentioned experiments examined the effects of fatty acid *oxidation*, later work examined the effects of fatty acid *synthesis*. Generally, fatty acid synthesis is stimulated by excess fuel availability, i.e., when there is more than ample substrate availability for fuel oxidation and the formation of new ATP. Fatty acid oxidation occurs is predominant during fasting when the primary fuel available in the periphery is in the form of free fatty acids released from triacylglycerides in adipose tissue. If inhibition of fatty acid oxidation increases food intake and inhibits reproduction, does inhibition of fatty acid synthesis have the opposite effects? Inhibition of fatty acid synthase (FAS) in the brain and periphery decreases food intake (Loftus et al., 2000). FAS is the multienzyme protein that catalyzes the synthesis of fatty acids from the substrate malonyl-CoA under conditions of excess fuel availability. The discovery that food intake is inhibited by agents that inhibit the activity of FAS created a renewed interest in metabolic control of ingestive behavior focused on the effects of metabolic challenges (such as starvation and diabetes) and peripheral hormones such as leptin and ghrelin on enzymes and substrates involved in fatty acid synthesis in the brain.

The inhibitory effects of centrally administered FAS inhibitors on food intake was a surprise to many neuroscientists, given that glucose is the primary substrate oxidized in the CNS. It turns out that free fatty acids, and some particular amino acids also reach the brain. The same metabolic pathway for synthesis of fatty acids that functions in peripheral cells also exists in the CNS.

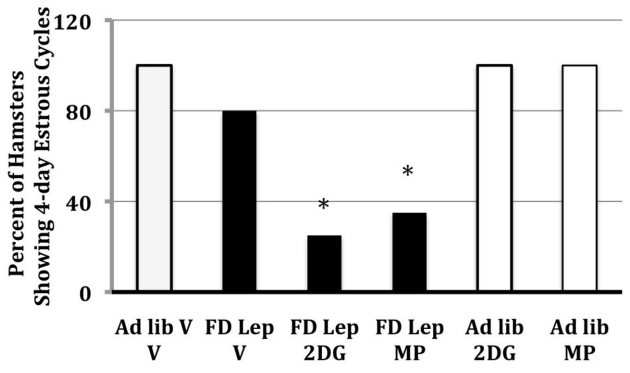


**FIGURE 4 | The frequency of hamsters that showed regular, 4-day estrous cycles after 48 h of food deprivation on days 1 and 2 of the estrous cycle and treatment with either leptin (intraperitoneal injection) or vehicle. Half of each group was treated with 2-deoxy-d-glucose (2DG), methyl palmoxirate (MP), or the vehicles of these inhibitors of glucose or fatty acid oxidation respectively.** * = significantly different from *ad libitum* vehicle at $P < 0.05$. After Schneider et al. (1998).

To review some of these basic pathways, fatty acids (i.e., LCFacyl-CoA, used in formation of triacylglycerides) are synthesized from malonyl-CoA (the reaction catalyzed by FAS). Malonyl-CoA is synthesized from acetyl-CoA [catalyzed by acetyl-CoA-carboxylase (ACC)]. ACC is inhibited by 5′-adenosine monophosphate-activated protein kinase (AMPK) as well as palmitate. AMPK is sensitive to energy availability, specifically the ratio of ATP to AMP. The formation of fatty acids (LCFacyl-CoA) for storage as triacylglycerides is an ATP-using process. Thus, stimulation of this pathway is appropriate under conditions of high energy availability.

There is a very large and confusing body of literature on the effects of fatty acid synthesis intermediates on control of food intake. Understanding these data requires that we step back and remember that these cerebroventricularly applied metabolic inhibitors might not mimic the endogenous events that control normal ingestion. Furthermore, these artificial CNS manipulations are likely to have effects on peripheral fuel metabolism that could, in turn, affect the behaviors in questions (Cha et al., 2005; Lam et al., 2005; Bartness et al., 2010; Bachman et al., 2002). Keeping this in mind, the general outcome of these studies is that most factors that decrease food intake tend to decrease the CNS activity of AMPK, the nutrient-sensitive kinase that inhibits ACC. For example, ICV leptin, insulin, and GLP-I decrease the CNS activity of CNS AMPK, which would be expected to increase the synthesis of malonyl-CoA and cause the accumulation of newly synthesized fatty acids. In line with this idea, factors that decrease food intake also increase the activity of CNS ACC, the key enzyme that catalyzes the rate-limiting step in malonyl-CoA synthesis. Factors that decrease food intake inhibit a brain-specific CPT-I, the enzyme that transports free fatty acids into mitochondria for oxidation in the periphery but is hypothesized to have a nutrient sensing function in brain. Factors that decrease food intake also increase mammalian target of rapamycin (mTOR), another nutrient-sensitive kinase involved in regulation of protein synthesis and energy balance. Conversely, factors that *increase* food intake tend have the opposite effects in hypothalamic fatty acid synthesis and oxidation, i.e., activation of AMPK and CPT-I, and inhibition of ACC, malonyl-CoA, and mTOR (Minokoshi et al., 2002, 2008; Cota et al., 2006, 2008; Pocai et al., 2006; Proulx et al., 2008; Wolfgang et al., 2008).

The relative importance of each of these intermediates, the mechanisms involved, and their importance for ingestive behavior in brain vs. periphery, hypothalamus vs. brainstem is still in question. For example, agents that inhibit (e.g., leptin, Compound C) and stimulate (ghrelin, AICAR) the activity of AMPK in hypothalamus decrease and increase food intake respectively. However, these exogenously applied agents are likely to have myriad side effects. For example, transgenic knockout of AMPKα2 exclusively in NPY neurons results in a lean phenotype, whereas knockout of AMPKα2 in POMC neurons results in an obese phenotype, and both types of neurons with these knockouts show normal electrophysiological responses to leptin but are insensitive to glucose (Claret et al., 2007). The effects of energetic challenges and ghrelin on AMPK in liver and adipocytes are the opposite of that in

brain, and thus, hypotheses about the role of AMPK and other intermediates in control of ingestive behavior must consider the whole organism. AMPK is an ancient and ubiquitous enzyme. Some specificity might be added by consideration of other proteins involved in fatty acid oxidation and mitochondrial respiration, such as uncoupling protein-2 (UCP-2). For example, studies that compare knockouts for UCP-2 with wild type mice show that effects of leptin and ghrelin depend upon a functional gene for UCP-2, whereas substrate-mediated effects of AMPK occur with or without a functional gene for UCP-2 (Andrews, 2011; Diano and Horvath, 2012).

The importance of taking a broad perspective that includes the whole organism (brain and periphery) is exemplified by the exaggerated diet-induced obesity that occurs in knockout mice that lack the functional gene for CPT-Ic (Wolfgang et al., 2008). This is not predicted by the theory that inhibition of CPT-Ic in brain decreases appetite, but is instead consistent with the idea that decreased fatty acid oxidation produces a deficit in fuels for oxidation that leads to peripheral mechanisms that conserve energy by inhibition of energy expenditure and promote fuel storage.

Given that food restriction tends to increase food intake and inhibit the HPG system and sex behavior, it might be expected that central inhibition of fatty acid oxidation might inhibit the reproductive system. Investigators have begun to explore the potential role of AMPK, mTOR, and glucokinase (GK) in metabolic control of reproduction. Central treatment with inhibitors of free fatty acid oxidation inhibit the HPG system (Sajapitak et al., 2008). Pulsatile LH secretion is suppressed in a dose-dependent manner by fourth ventricular treatment with an inhibitor of fatty acid oxidation in ovariectomized female rats that were either treated with estradiol or vehicle. These results suggest that central inhibition of free fatty acid oxidation inhibits the HPG system. In other studies, activation of hypothalamic mTOR reverses food restriction-induced inhibition of LH secretion (i.e., stimulated LH secretion). Furthermore, blockade of mTOR delayed reproductive maturation, prevented the restorative effects of leptin on puberty, and suppressed KiSS1 mRNA levels in the Arc (Roa et al., 2009). Still other lines of research have focused on intermediate metabolism in GnRH neurons (in slice preparation). GnRH neurons are differentially responsive to glucose according to the steroid milieu, and these effects are mediated by AMPK (Roland and Moenter, 2011a,b,c). These direct effects of glucose availability on GnRH neurons are particularly interesting in light of evidence that GnRH neurons are privy to circulating metabolites from the periphery (Herde et al., 2011). These results must be reconciled with the fact that glucoprivic control of reproduction requires and intact AP. Why, if GnRH secretion is influenced directly by glucose availability, are the effects of systemic inhibition of glucose oxidation on estrous cycles and LH secretion prevented by lesions of the AP/NTS or by selective immunotoxic destruction of NE/NPY neurons from the brainstem to the PVH? In other words, if there are multiple sites of glucose detection in brain and periphery: which are the most relevant when metabolic challenges orchestrate ingestive and reproductive choices?

If these mechanisms function to maintain fuel homeostasis, how can animals overeat, store fat, and survive winter, migration, famine, and lactation? Enzymes such as AMPK, mTOR, and GK are linked to ATP formation, making them critical for maintaining intracellular fuel homeostasis, but what about the need to anticipate future increases in energy expenditure for reproduction? When ATP content is sufficient for energetic needs, the biochemical pathways leading to formation of ATP are halted, thus, it might be predicted that this would blind AMPK to the availability of oxidizable fuels. Thus, another type of energy sensor that is uncoupled from ATP formation might be required to enable animals to anticipate future food shortages and increase energy demands of reproduction by storing energy as adipose tissue. These sensors might also play a role in moment-to-moment decisions about the choice between conflicting behaviors (whether to forage for food or for mates, or whether to risk predation in order to consume or hoard food). The sodium–glucose cotransporter (SGLT) might fill this role because it appears to function independently of energy metabolism. SGLTs are transmembrane proteins that transport glucose and other sugars by coupling the uptake of a sugar molecule to the influx of one or two $Na^+$ions (Wright, 2001). Because glucose is electroneutral, SGLT activity generates a net inward current that causes direct membrane depolarization and increased electrical activity without the need for glucose metabolism (Gribble et al., 2003). It has been known for more than 30 years that phloridzin, a SGLT antagonist, increases food intake in rats when given intracerebroventricularly (Glick and Mayer, 1968). Furthermore, SGLT is expressed in peripheral glucose-sensitive cholinergic neurons (Diez-Sampedro et al., 2003). Most glucose-sensitive neurons that are depolarized in response to non-metabolizable glucose analogs (such as α-MDG) are SGLTs (O'Malley et al., 2006), and furthermore, the effects of both glucose and α-MDG are abolished by phloridzin or by the removal of extracellular $Na^+$. Together these results are consistent with the possibility that generation of ATP is not an essential prerequisite for sugar sensing in glucose-sensitive neurons. Thus, it is plausible that the SGLT and other similar metabolic sensors might allow orchestration of the energy economy under conditions of excess fuel availability. SGLT has not been explored with regard to reproduction, to the best of our knowledge.

Despite the excellent work in this field, the role of these intermediates in control of ingestive behavior is not understood. With regard to reproduction, the initial probes have been launched only recently. Virtually nothing is known about the role of these intermediates and metabolic substrates in the control of appetitive behavior and underlying motivation. These metabolic events might be important pivot points for decisions about whether to eat food or engage in reproductive activities.

## BEYOND THE HYPOTHALAMUS

A final warning to new investigators would be to double check all assumptions about the importance of a neuropeptide, hormone, or brain area based on the number of published articles concerned with that topic. The vast majority of research on metabolic control of reproduction examines projections to and from the hypothalamus, and of those, most concern Arc NPY/AgRP, POMC, and more recently the Kisspeptin/GnIH system that includes the Arc, PVH, preoptic area, dorsomedial hypothalamus (DMH), and AVPV (Estrada et al., 2006; Franceschini et al., 2006; Kriegsfeld et al., 2006; Roa et al., 2006; Smith et al., 2008; Yeo and Herbison, 2011). In fact, a large body of elegant work in metabolic

control of reproduction and ingestive behavior supports a widely distributed neural network that includes the caudal brain stem, midbrain, and forebrain (Schneider et al., 1993, 1995; Horn et al., 1999, 2001; Ritter et al., 2001; I'Anson et al., 2003b; la Fleur et al., 2003; Hudson and Ritter, 2004; Bugarith et al., 2005; Hayes et al., 2009; Skibicka and Grill, 2009). This work has been the subject of thoughtful and scholarly reviews (Grill and Kaplan, 1990, 2002; Grill, 2006; Friedman, 2008; Grill and Hayes, 2009). In the 1980s and 1990s, neuroanatomical characterization of POMC NPY cells located these molecules and their receptors in the brain stem as well as in other areas, and the notion of distributed neuroanatomical control of energy balance was well accepted (Sawchenko et al., 1985; Bronstein et al., 1992).

Agents that increase or decrease voluntary food intake do so when microinjected into the hindbrain, and, in many cases, these agents can have the same effects on passive intake in decerebrate animals (reviewed by Grill and Hayes, 2009). This applies to leptin (Grill et al., 2002; Harris et al., 2007). Furthermore, these hindbrain effects on food intake involve the activity of AMPK, and fail to occur when leptin-induced inhibition of AMPK is prevented (Hayes et al., 2009). Furthermore, the stimulatory effects of 2DG on food intake activate AMPK, and 2DG-induced hyperphagia does not occur without AMPK activation (Li et al., 2011). The main point for reproductive endocrinologists is that it would be a mistake to imagine that metabolic control of ingestive and reproductive behavior is restricted to the Arc or even the entire hypothalamus.

Both food intake and energy expenditure, and presumably energy expenditure for reproduction, are under the influence of the peptide systems in these widely distributed interconnected nuclei. For example, Skibicka and Grill (2009) measured food intake, core body temperature, heart rate, and spontaneous activity in rats that received two different picomolar doses of an MC4R agonist (MTII) into six different brain subnuclei distributed along the neuraxis. The MTII doses were titrated to be well below the threshold dose known to be effective when infused intracerebroventricularly. The results were unequivocal. In each case when the MTII infusion was demonstrated histologically to hit its intended target, five of the six brain areas, including the NTS and midbrain, showed increases in body temperature and heart rate as well as decreased food intake. The data confirm a growing body of data demonstrating that melanocortinergic effects on food intake and energy expenditure occur in the caudal brain stem (Grill et al., 1998; Williams et al., 2000, 2003; Grill and Kaplan, 2001, 2002).

The important implication for researchers interested in metabolic control of reproduction is that the brain is organized into a network of areas that display a great deal of redundancy of function, most of which impact aspects of energy expenditure as well as food intake. At this point, it should be obvious that a significant portion of total energy expenditure is allocated for reproduction. Investigators new to this field of research will no doubt come across published articles that employ "expression of a particular neuropeptide in the Arc" as evidence for the function of that peptide in control of ingestion. After reading this review, it should be obvious that an equally probably function is control of reproduction, as well as general activity, thermogenesis, body temperature, heart rate, and oxygen consumption.

## SUMMARY

This review is intended to provide a foundation for future research at the interface of ingestive behavior and reproduction. At the interface of ingestive behavior and reproduction lies a metabolic sensory system that detects changes in fuel availability and initiates changes in motivation, appetitive sex, and ingestive behaviors, in addition to changes in the HPG system. The observed changes in behavior, hormones, and metabolic fuel partitioning are best understood within the metabolic hypothesis:

> A sensory system monitors the availability of oxidizable metabolic fuels and orchestrates behavioral motivation to optimize reproductive success in environments where energy availability fluctuates or is unpredictable.

This hypothesis leads to testable predictions. For example, it predicts that orexigenic and anorectic hormones and neuropeptides will have different effects on ingestive and sex behavior depending on the energetic challenges faced by the animals. This prediction was realized with regard to the effects of estradiol on food hoarding and courtship in Syrian hamsters (**Figures 1** and **2**). It also predicts that animals housed alone, with unlimited food, with limited behavioral options are not the control group, but the experimental group. It predicts that behavioral motivation will be more sensitive than food intake or copulation (**Figures 1–3**), and that mechanisms that function to in short term choices between food and sex might not be capable of maintenance of long term stability in body fat content in environments where energy availability and energetic demands fluctuate.

There is now recognition that so-called lipostatic hormones, once thought to maintain a set point in body fat content, are actually modulators of metabolic fuel availability, more specifically, fuel oxidation, and synthesis. The challenge is to incorporate what is known about these mechanisms in fuel homeostasis to the demonstrated ability to engage in opportunistic overeating in anticipation of the high energetic demands of reproduction (as well as seasonal and unpredictable changes in energy availability).

Finally, all of the above concepts must be studied in the context of a distributed neural network with multiple, redundant function. More specifically, metabolic control of reproduction must include the hindbrain (AP/NTS), midbrain (e.g., the lateral parabrachial nucleus and VTA), and forebrain (e.g., hypothalamus and striatum), as well as areas such as the ventral premammillary nucleus (Donato et al., 2009) in sharp contrast to the more common exclusive focus on Arc. In fact, the PMV and medial amygdala are good candidates for mediation of metabolic control of motivation for ingestive and sex behavior (Donato et al., 2010).

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# A potential mechanism for the sexual dimorphism in the onset of puberty and incidence of idiopathic central precocious puberty in children: sex-specific kisspeptin as an integrator of puberty signals

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The major determinants of the variability in pubertal maturation are reported to be genetic and inherited. Nonetheless, nutritional status contributes significantly to this variability. Malnutrition delays puberty whereas obesity has been associated to a rise in Idiopathic Central Precocious Puberty (ICPP) in girls. However, epidemiology data indicate that contribution of obesity to early puberty varies significantly among ethnic groups, and that obesity-independent inheritable genetic factors are the strongest predictors of early puberty in any ethnic group. In fact, two human mutations with confirmed association to ICPP have been identified in children with no history of obesity. These mutations are in kisspeptin and kisspeptin receptor, a ligand/receptor pair with a major role on the onset of puberty and female cyclicity after puberty. Progressive increases in kisspeptin expression in hypothalamic nuclei known to regulate reproductive function has been associated to the onset of puberty, and hypothalamic expression of kisspeptin is reported to be sexually dimorphic in many species, which include humans. The hypothalamus of females is programmed to express significantly higher levels of kisspeptin than their male counterparts. Interestingly, incidence of ICPP and delayed puberty in children is markedly sexually dimorphic, such that ICPP is at least 10-fold more frequent in females, whereas prevalence of delayed puberty is about 5-fold higher in males. These observations are consistent with a possible involvement of sexually dimorphic kisspeptin signaling in the sexual dimorphism of normal puberty and of pubertal disorders in children of all ethnicities. This review discusses the likelihood of such associations, as well as a potential role of kisspeptin as the converging target of environmental, metabolic, and hormonal signals, which would be integrated in order to optimize reproductive function.

**Keywords: central precocious puberty, kisspeptin receptor signaling, reproduction, LH surge, sexual differentiation of the brain**

## INTRODUCTION

A mystery that still puzzles scientists is what initiates puberty. The age at onset and duration of pubertal development is primarily driven by genetics (Palmert and Boepple, 2001; Palmert and Hirschhorn, 2003; Parent et al., 2003). However, timing of sexual maturation is influenced by other factors such as nutrition, environment, and sex (Parent et al., 2003). This review focuses on the role of the G protein-coupled kisspeptin receptor—KISS1R/Kiss1r—and its endogenous ligand, kisspeptin, on the onset of puberty and etiology of pubertal disorders. Potential roles of nutrition and sex on kisspeptin signaling are also discussed.

The onset of puberty is first detected as an increase in pulses and frequency of gonadotropin-releasing hormone (GnRH), which leads to mirroring increases in the secretion of gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gland. Failure to increase GnRH or gonadotropin secretion at puberty is the underlying cause of idiopathic hypogonadotropic hypogonadism (IHH), which is characterized by impaired sexual maturation and infertility (Seminara et al., 1998). Conversely, premature activation of GnRH secretion leads to idiopathic gonadotropin-dependent (or central) precocious puberty (ICPP).

Most of the advances into the mechanisms of re-activation of the hypothalamic-pituitary-gonadal (HPG) or reproductive axis at puberty have been provided by the characterization of genetic mutations associated with reproductive disorders in humans. The majority of mutations studied to date were identified in patients with IHH, a disorder far less frequent than ICPP. Prevalence of premature puberty has been predicted to be 0.2% in one population (Teilmann et al., 2005), whereas incidence of IHH is estimated to be 1–10 cases per 100,000 births (or 0.001–0.01%) (Seminara et al., 1998). Despite of the higher prevalence and of an estimated 30% familial cases (de Vries et al., 2004), the first

genetic mutation associated to ICPP was published just four years ago, in 2008 (Teles et al., 2008).

Two striking distinctions between ICPP and IHH are the inheritance mode and the marked sexually dimorphic distribution of these disorders. The pedigree of families with history of IHH (with a normal sense of smell) show or suggest an autosomal *recessive* mode of inheritance (Bianco and Kaiser, 2009). This was confirmed in reported cases, which show that only individuals carrying the associated mutation in the *homozygous* (or compound heterozygous) state exhibit the IHH phenotype, whereas *heterozygous* parents and siblings have no obvious reproductive abnormalities (de Roux et al., 2003; Seminara et al., 2003; Bhagavath et al., 2006; Topaloglu et al., 2006; Bedecarrats and Kaiser, 2007; Nimri et al., 2011). On the other hand, the pedigree of families with a history of ICPP indicate an autosomal *dominant* mode of transmission (de Vries et al., 2004) that is supported by two reports of human mutations with confirmed association with ICPP, which were identified in the *heterozygous* state in affected children (Teles et al., 2008; Silveira et al., 2010). Interestingly, females are at least ten times more likely to develop premature puberty than males (de Vries et al., 2004). This likelihood increases if only idiopathic cases of early puberty are considered (Teilmann et al., 2005; Teles et al., 2011). Conversely, incidence of hypogonadism is predicted to be about 5-fold elevated in males (Sykiotis et al., 2010). Severity of the hypogonadism is also influenced by sex as males quite often exhibit more severe symptoms than females carrying the same mutation.

## SEXUAL MATURATION OF THE BRAIN

Sexual maturation of the brain is driven by exposure to sex steroids at specific windows of sensitivity during development. The stage of brain development at the time of exposure appears to be a critical determinant of brain *masculinization* or *feminization*. However, there is species specificity in the timing of brain sensitivity to these effects. In rodents, brain masculinization occurs just before and after birth and is heavily dependent on local conversion of androgens to estradiol by brain aromatases (McCarthy, 2008; McCarthy et al., 2009). In contrast, sexual maturation of the brain in humans and other primates occurs earlier in development, around mid- to late-gestation. Androgens (rather than estrogens) are the determinants of brain masculinization in primates, although brain aromatization also plays a role (Michael et al., 1987; Wallen, 2005). Manipulation of brain exposure to androgens during certain critical developmental windows may produce subjects with the genitals of one sex but with behaviors that are typical of the opposite sex (Wallen, 2005). Accordingly, abnormal prenatal exposure of female embryos to androgens has been described to result in male-typical behavior and decreased female-typical behavior in species such as Rhesus monkeys (Wallen, 2005), guinea pig (Phoenix et al., 1959) and rats (Simerly, 2002). Interestingly, abnormal exposure of mouse embryos to sex steroids has been associated to corresponding abnormalities in the sexual dimorphism of kisspeptin expression in the hypothalamus (Kauffman et al., 2007a,b; Gonzalez-Martinez et al., 2008).

Consequences of abnormal exposure of the developing human brain to sex steroids are evidenced by anomalous puberty, fertility, sexual behavior and even sexual identity in patients carrying certain genetic mutations (Deladoey et al., 1999; Cohen-Bendahan et al., 2005; Lin et al., 2007; Zirilli et al., 2008). A classical example is the brain masculinization that is consistently reported for girls born with congenital adrenal hyperplasia. Hyperplastic adrenal glands in affected girls produce excessive amounts of androgens, which flood the developing brains of affected females (Cohen-Bendahan et al., 2005). Additionally, affected girls can develop precocious puberty, an effect also observed in female rodents exposed to androgen (Witham et al., 2012). Precocious Puberty may sound inconsistent with masculinization or incomplete feminization of the female brain, which would rather be expected to cause delayed puberty. Nonetheless, premature puberty is a common symptom in girls with a family history of another disorder strongly associated to female androgenization: polycystic ovary syndrome (PCOS) (Franceschi et al., 2010). Elevated androgens in these cases could be contributing to premature puberty by abnormally stimulating the hypothalamic-pituitary-gonadal (HPG) or reproductive axis. Incidentally, serum kisspeptin is reported to be elevated in adolescents with PCOS. Also, serum kisspeptin positively correlated to serum LH and testosterone in affected adolescents, suggesting an involvement of kisspeptin in the etiology of PCOS in these girls (Chen et al., 2010b).

Genetic mutations leading to aromatase deficiency are also reported to cause abnormal sexual maturation of the brain in humans and in mice (Lin et al., 2007; Bakker et al., 2010). This is due to insufficient aromatization of androgens to estrogens resulting in elevated serum androgens. Among the cases of congenital aromatase deficiency in humans is the 46XX (female) who exhibited boy-typical behavior and male gender identity from early age, despite estrogen supplementation started at age 3 to correct for low bone density and delayed bone age (Lin et al., 2007). Similar masculinization of the brain is described for female mice with congenital aromatase insufficiency (Bakker et al., 2010). Interestingly, deficiencies of sexual differentiation of the brain in these mice were associated to an absence of sexual dimorphism in the hypothalamic expression of kisspeptin (Bakker et al., 2010).

## SEXUAL DIMORPHISM OF PUBERTY AND OF PUBERTAL DISORDERS

During pubertal transition to sexual maturity, the reproductive axis is activated in humans (Delemarre-van de Waal, 2002) and other non-human primates (Plant et al., 1989). The pubertal rise in gonadal sex steroids is coordinated with the appearance of secondary sexual features, which culminates with the attainment of reproductive competence. The onset of puberty in girls occurs 1–2 years earlier than in boys; menarche happens even earlier than sexual maturity in boys (Iuliano-Burns et al., 2009). A similar phenomenum is observed in Rhesus monkeys: menarche occurs around age 2 years in females, whereas males only reach sexual maturity during the breeding season of their fourth year of age (Wilen and Naftolin, 1976; Resko et al., 1982; Mann et al., 1998). These observations are consistent with sexual dimorphism of the major drivers of puberty.

Prevalence of pubertal disorders in humans is also sexually dimorphic: incidence of precocious puberty is disproportionally

higher in girls when compared to boys (de Vries et al., 2004). The ratio for idiopathic CPP is estimated to be 15–20 females for each male with the disorder (Teles et al., 2011). On the other hand, incidence of IHH is 5-fold elevated in males when compared to females (Seminara et al., 1998; Sykiotis et al., 2010).

The basis for the sexually dimorphic presentation of puberty and pubertal disorders in humans is not known, but the underlying mechanisms could involve sexually dimorphic signaling pathways with a role on GnRH secretion, rather than sexual dimporhism of GnRH neurons. This would be compatible with the involvement of KISS1R signaling, which has been shown to be sexually dimorphic in the hypothalamus of mice (Wray and Gainer, 1987; Kauffman et al., 2007b), rats (Kauffman et al., 2007a), and sheep (Schwanzel-Fukuda et al., 1981). On the other hand, GnRH neurons were not found to be sexually dimorphic in experimental animals such as rats or guinea pig (Clarkson and Herbison, 2006; Cheng et al., 2010). While there are no reports of sexually dimorphic pubertal disorders on experimental animals, investigation of animal models may help to elucidate the underlying causes of the dimorphism in humans, provided that appropriate consideration is given to species diversity.

Of note, requirement of GnRH neurons for fertility in mice has been reported to be sexually dimorphic (Herbison et al., 2008). The majority of GnRH neurons were not essential for puberty or fertility in male or female mice under ideal conditions (Herbison et al., 2008). Male mice expressing 12% of total GnRH neurons had normal reproductive function and fertility. Females with 12% of total GnRH neurons had normal puberty, but subsequently develop infertility due to an inability to generate LH surges and ovulate (Herbison et al., 2008). This demonstrated that females require additional GnRH neurons to be fertile after puberty. Sure enough, females expressing 34% of the GnRH neurons had no fertility or cyclicity problems later on (Herbison et al., 2008). The excess of GnRH neurons not required for puberty or fertility in ideal conditions could be, instead, required to warrant or modulate GnRH pulsatility in response to environmental, nutrition, stress, lactation or other cues conveying adverse situations. If this were the case, adaptive capability of GnRH responsiveness to adversity would be incredibly high, as the majority of GnRH fibers would not be required in ideal conditions.

## IDIOPATHIC CENTRAL PRECOCIOUS PUBERTY (ICPP) FACTS

Gonadotropic-dependent or central precocious puberty (CPP) is characterized by early activation of the reproductive axis. This form of precocious puberty is further classified as *idiopathic* (ICPP) after tumors or other anatomical abnormalities are discarded (Klein, 1999). Thus, ICPP is early puberty of central origin with no obvious underlying cause. Dysfunction in these cases presumably lyes on signaling pathways regulating GnRH pulsatility, which is unmasked at puberty. Thus, ICPP cases are particularly interesting to investigate, as uncovering the underlying deffect may expose yet unkown or underappreciated brain pathways critical for puberty and/or reproductive competence.

Onset of puberty occurs earlier in girls, who are considerably more likley to develop precocious puberty, and 95% of girls reported with precocious puberty develop the idiopathic (or genetic) central form of the disorder (Klein, 1999). This sexual dimorphism could be associated to sexually dimorphic signals to puberty. The high rate of familial cases of ICPP emphasizes the genetic origin of this disorder (Palmert and Boepple, 2001; Anderson et al., 2003; Palmert and Hirschhorn, 2003; Parent et al., 2003; Prete et al., 2008; Aksglaede et al., 2009; Biro et al., 2010; Ogden et al., 2012).

Epidemiology studies suggest that age at onset of puberty in girls is decreasing over the years (Anderson and Must, 2005; Cesario and Hughes, 2007; Golub et al., 2008; Ahmed et al., 2009; Rosenfield et al., 2009; Biro et al., 2010; Burt Solorzano and McCartney, 2010); some propose that this decrease, as well as an increase in diagnosed cases of CPP in girls, would be associated to a cuncurrent rise in childhood obesity (Wattigney et al., 1999; Kaplowitz et al., 2001; Anderson et al., 2003; Lee et al., 2007; Kaplowitz, 2008). While there is positive correlation of obesity with early onset of puberty in some studies, this is not true for others. An example is a strictly controlled Copenhagen Puberty Study, in which early puberty remained significant after adjustment for body mass index (BMI) (Aksglaede et al., 2009). Another example is the study by Prete and cols, in which obesity was not a significantly contributor to premature puberty in the population studied (Prete et al., 2008).

The distinct ethnic distribution of ICPP reported in several studies emphasizes the genetic basis of this disorder. Additionally, these studies show that contribution of ethnicity to early puberty is significantly heavier than that of obesity (Anderson et al., 2003; Rosenfield et al., 2009; Biro et al., 2010; Walvoord, 2010; Ogden et al., 2012). Among girls of normal BMI, those of African American descent start puberty earlier than any other ethnicity (Biro et al., 2008; Rosenfield et al., 2009). Within the same ethnicity, a minority of overweight/obese girls develops ICPP: 72% of African American girls are overweight (BMI $>25$ and $<30$) or obese (BMI$>30$) but only 23% of these developed ICPP. Similarly, 61% of Hispanic girls are overweight or obese, but only 15% developed ICPP. Thirty-six percent white girls are overweight or obese but 10% developed ICPP (Biro et al., 2010; Ogden et al., 2012). Also, the last US National Health and Nutrition Evaluation Survey (NHANES) shows a clear contribution of obesity-independent genetic factors to early puberty (Rosenfield et al., 2009). This survey shows that obesity accelerates puberty only in early maturating girls, whereas thelarche or pubarche were not affected by obesity in late maturating girls (Rosenfield et al., 2009).

Lastly, obesity did not appear to have been a factor in the ICPP developed by the two children carrying the naturally occurring genetic mutations in KISS1R (Arg386Pro), which was identified in a girl (Teles et al., 2008) or in kisspeptin (Pro74Ser), which was identified in an unrelated toddler boy (Silveira et al., 2010).

Of note, correlation of obesity with puberty is sexually dimorphic as well. Rates of obesity are significantly higher in boys than in girls of pubertal age (age 6–11) in all ethnicities tested (White, Hispanic and African American) (Ogden et al., 2012). However,

obesity in boys is largely associated to an opposed phenotype of delayed puberty and low testosterone, whereas early sexual maturation in boys is associated to lower rates of obesity when compared to later maturing boys (Wang, 2002; Burt Solorzano and McCartney, 2010; Walvoord, 2010).

## NATURALLY OCCURRING GENETIC MUTATIONS IN HUMANS WITH ICPP

### KISSPEPTIN AND KISSPEPTIN RECEPTOR

A role for KISS1R and kisspeptin in the etiology of ICPP was revealed by the identification of two mutations with confirmed association to ICPP, one in KISS1R (Arg386Pro) and the other in kisspeptin (Pro74Ser) (Teles et al., 2008; Silveira et al., 2010). As opposed to KISS1R mutations associated to IHH; however, the two affected children carry the associated *gain-of-function* mutation in the *heterozygous* state. This is in conformity with the autosomal dominant inheritance (one mutated allele is enough for the manifestation of the phenotype) predicted for familial ICPP (de Vries et al., 2004). Nonetheless, tests performed for an additional kisspeptin mutant identified in two unrelated Brazilian girls (His90Asp) did not detect significant changes in the activity of the mutant (Silveira et al., 2010). Two additional mutations associated with ICPP in genome-wide association studies (His196Pro-KISS1R and Pro110Thr-kisspeptin) await confirmation of this association (Luan et al., 2002, 2007).

To date, mutations in KISS1R or kisspeptin appear to account for a minority of ICPP cases, as the majority of patients investigated to date have no mutations in the exons or exon-intron boundaries of *KISS1R* or *KISS1*.

### MUTATIONS IN OTHER GENES INVESTIGATED IN HUMAN ICPP

Among a plethora of proteins in which genetic mutations could potentially affect pubertal development, this section focus on genes that have been investigated in patients with ICPP, which include *TAC3*, *TACR3*, *LIN28B*, *GABRA1,* and *NPY*.

#### *TACR3 and TAC3 genes*

The *TACR3* encodes the G protein-coupled receptor neurokinin B (NKR3), and the *TAC3* gene encodes neurokinin B (NKB), the natural ligand for the NKR3. NKB and NKR3 are co-expressed with kisspeptin in a unique set of neurons named KNDy neurons, which are conserved across mammalian species and have been described for humans (Hrabovszky et al., 2010), monkeys (Ramaswamy et al., 2010), sheep (Goodman et al., 2007), goat (Wakabayashi et al., 2010), rat (Burke et al., 2006), and mice (Navarro et al., 2009). Increase in serum gonadotropins in response to stimulation of KNDy neurons by senktide (an NKB analog) has been demonstrated in monkeys (Ramaswamy et al., 2010), rats (Navarro et al., 2011), and ewes (follicular phase only) (Billings et al., 2010).

Loss-of-function mutations in NKB or in NK3R have recently implicated this ligand/receptor pair in the etiology of IHH in humans (Guran et al., 2009; Topaloglu et al., 2009; Fukami et al., 2010; Gianetti et al., 2010; Young et al., 2010; Francou et al., 2011). Also, one mutation in NK3R has been identified in a patient with ICPP (Ala63Pro-NK3R). However, association of this mutation with ICPP is yet to be confirmed, as the same mutation is present in the patient's mother, who reports normal pubertal development, and no functional assays have been performed for this mutation (Tusset, 2010; Teles et al., 2011).

#### *LIN28B*

*LIN28B* is the human homolog of a *C. elegans* gene with a role in timing larvae to adult maturation, which suggests that *LIN28B* could play a role in human sexual maturation. This is supported by genome-wide association studies indicating that polymorphisms in or near the *LIN28B* gene could be significant sources of variation in the age at menarche in girls (He et al., 2009; Ong et al., 2009; Perry et al., 2009). One mutation in *LIN28B* was indentified in the heterozygous state in a 4 year-old girl with sporadic (not inherited) ICPP. Nonetheless, functional assays performed did not detect significant changes in the activity of this mutant (Teles et al., 2011); thus, significance of *LIN28B* for human pubertal maturation remains unknown.

#### *GABRA1*

*GABRA1* encodes the gamma amino butyric acid A1 receptor α-1 subunit, which is reported to be essential for the effects of the gamma-aminobutyric acid type A ($GABA_A$) receptors on GnRH neurons (Lee et al., 2010). An interest in investigating $GABA_A$ receptors in girls with ICPP came from studies showing that a $GABA_A$ receptor antagonist (bicuculine) accelerated puberty in monkeys (Keen et al., 1999), and that this effect was mediated by kisspeptin as indicated by robust increases in kisspeptin secretion in response to bicuculine (Kurian et al., 2012). Also, the effect of bicuculine on GnRH neurons was prevented by pre-treatment with anti-kisspeptin serum (Terasawa et al., 2010; Kurian et al., 2012). However, sequencing the *GABRA1* gene in a cohort of girls with ICPP did not detect mutations (Brito et al., 2006). Additionally, selective reduction of $GABA_A$ receptors in GnRH neurons in mice did not result in visible pubertal abnormalities (Lee et al., 2010), suggesting that deficiencies in this receptor would be compensated for in rodents.

#### *NPY receptor*

The *NPYR* gene encodes the receptor for neuropeptide Y (NPY), which antagonizes GABA effects on GnRH neurons. This antagonism was shown to play a role in pubertal development in monkeys and rodents (Terasawa and Fernandez, 2001). Additionally, hypothalamic NPY-producing neurons were shown to co-express Kiss1r and respond to kisspeptin in mouse cells and sheep hypothalamic explants. These observations raised the possibility that mutations in the *NPYR* gene could play a role in the etiology of ICPP (Backholer et al., 2010; Kim et al., 2010). Nonetheless, sequencing of the NPY receptor-1 detected only a synonymous (does not result in amino acid substitution) single nucleotide polymorphism (SNP) in the heterozygous state in a girl with familial ICPP, and this polymorphism was present at a higher rate in the control population (28%). Moreover, *in vitro* assays failed to show altered activity for this mutant (Freitas et al., 2007).

## KISSPEPTIN RECEPTOR SIGNALING AND PUBERTY

The KISS1R was first linked to reproductive function in 2003, when loss-of-function mutations in this receptor were associated

to IHH in two unrelated consanguineous families (de Roux et al., 2003; Seminara et al., 2003). Affected members of both families carried the associated mutation in the homozygous state, whereas heterozygous siblings and parents had no obvious reproductive abnormalities (de Roux et al., 2003; Seminara et al., 2003). Additional loss-of-function mutations in *KISS1R* were subsequently identified in patients with IHH (de Roux et al., 2003; Lanfranco et al., 2005; Semple et al., 2005; Pallais et al., 2006; Tenenbaum-Rakover et al., 2007; Teles et al., 2010; Nimri et al., 2011). More recently, a loss-of-function mutation in the kisspeptin gene (*KISS1*) was also associated to IHH in a consanguineous family with history of IHH (Topaloglu et al., 2012). Similarly, disruption of *Kiss1r* or *Kiss1* in mice resulted in a phenotype compatible with that of IHH in humans (Funes et al., 2003; Seminara et al., 2003; Dungan et al., 2007; Kauffman et al., 2007b; Lapatto et al., 2007; Colledge, 2009). Conversely, kisspeptin treatment was shown to advance puberty in intact female mice (Navarro et al., 2004b), and two *gain-of-function* mutations (one in the *KISS1R* and the other in the *KISS1* gene) were identified in children with ICPP (Teles et al., 2008; Silveira et al., 2010). These observations validate the role of KISS1R and kisspeptin as essential regulators of GnRH secretion and onset of puberty.

### KISSPEPTIN AND GnRH SECRETION

All loss-of-function mutations in *KISS1R* or *KISS1* have been shown or are predicted to impair G protein signaling by the KISS1R, which in turn blocks stimulation of GnRH secretion by this receptor, impairing spontaneous onset of puberty (de Roux et al., 2003; Lanfranco et al., 2005; Semple et al., 2005; Pallais et al., 2006; Tenenbaum-Rakover et al., 2007; Teles et al., 2010; Nimri et al., 2011). On the other hand, kisspeptin expression was shown to be high and to increase during puberty in the infundibular nucleus of the medium basal hypothalamus (MBH) in male and female Rhesus monkeys (Hrabovszky et al., 2010). This pubertal increase in kisspeptin is accompanied by parallel changes in GnRH pulses, which suggests a connection between the increase in kisspeptin and the pubertal changes in GnRH pulses (Shahab et al., 2005). The MBH is reported to contain the majority of neuroendocrine GnRH neurons in primates and in humans (Krey et al., 1975; Plant et al., 1978; Hrabovszky et al., 2010). Additional reports show pubertal increases in hypothalamic kisspeptin expression for mice (Herbison et al., 2010), rats (Navarro et al., 2004a,b), and teleost fish cobia (Mohamed et al., 2007), which demonstrates phylogenetic conservation of the effect of kisspeptin on sexual maturation across species. Accordingly, kisspeptin has been shown to stimulate gonadotropin secretion in humans (Dhillo et al., 2005), sheep (Messager et al., 2005), pigs (Lents et al., 2008), rats (Matsui et al., 2004; Navarro et al., 2005; Pheng et al., 2009), mice (Gottsch et al., 2004), and gilts (Lents et al., 2008). Pubertal increases in *Kiss1r* expression in GnRH neurons have also been reported in mice (Herbison et al., 2010).

The role of kisspeptin signaling on GnRH pulses is emphasized by the disruptive effect of the infusion of a kisspeptin antagonist (peptide 234) on GnRH pulses in Rhesus monkeys as well as on gonadotropin secretion in the ewe (Millar et al., 2010; Guerriero et al., 2012). The detection of Kiss1r expression in GnRH neurons of cichlid fish (Parhar et al., 2004), rats (Irwig et al., 2004), and mice (Herbison et al., 2010) further supports a role for kisspeptin/Kiss1r on GnRH secretion, as well as suggest that kisspeptin would activate GnRH secretion by direct bind to receptors on GnRH neurons. Localization of kisspeptin fibers in close apposition with GnRH neurons in mice suggests that Kiss1r would be expressed at both somata and dentrites (Wray and Gainer, 1987), enabling kisspeptin to activate signaling on somata and/or dentrites of GnRH neurons in mice. In humans, kisspeptin-containing axons have been reported to be in contact with dentrites of GnRH neurons (Hrabovszky et al., 2008).

In rodents, the arcuate and the anteroventral periventricular (AVPV) nuclei of the hypothalamus are believed to regulate GnRH pulsatility. The arcuate nucleus is the target of a potent negative feedback of estrogen on gonadotropin secretion, whereas the AVPV is the target of positive feedback of estrogen on gonadotropin secretion (Mayer et al., 2010). Interestingly, virtually all kisspeptin neurons of the AVPV and of the arcuate nucleus in mice coexpress estrogen receptor-α (ER-α). Disruption of ER-α on kisspeptin neurons abolishes both negative and positive effects of estrogen on gonadotropin secretion (Smith et al., 2005a, 2006b), which suggests that kisspeptin neurons mediate estrogenic effects on gonadotropin secretion (Smith et al., 2005a, 2006b). This is supported by the absence of estradiol or androgen receptors on GnRH neurons (Herbison and Theodosis, 1992; Huang and Harlan, 1993). On the other hand, kisspeptin neurons contain ER-α (Smith et al., 2005a,b; Franceschini et al., 2006), progesterone (Smith et al., 2007) and androgen (Smith et al., 2005b) receptors, and a slow release of estrogen restraint on kisspeptin neurons of the arcuate nucleus is reported to precede the pubertal increase in kisspeptin (Takumi et al., 2011).

## SEXUAL DIMORPHISM OF KISSPEPTIN EXPRESSION AND ASSOCIATION TO REPRODUCTIVE FUNCTION

Kisspeptin neurons, kisspeptin expression and/or serum kisspeptin have been consistently shown to be sexually dimorphic in many species, including humans (Wray and Gainer, 1987; Kauffman et al., 2007a,b; Homma et al., 2009; Kauffman et al., 2009; Bakker et al., 2010; Hrabovszky et al., 2010; Jayasena et al., 2011; Pita et al., 2011a). This dimorphism has been associated to the onset of puberty and fertility in some species (Wray and Gainer, 1987; Kauffman et al., 2007a,b; Homma et al., 2009; Kauffman et al., 2009; Bakker et al., 2010; Hrabovszky et al., 2010; Jayasena et al., 2011; Pita et al., 2011a). Prenatal exposure to sex steroids may account for at least part of the sexual dimorphism in kisspeptin, and lack of kisspeptin dimorphism can lead to irreversible abnormalities of the sexual behavior in some species (Kauffman et al., 2007a,b; Gonzalez-Martinez et al., 2008). Also, circulating kisspeptin has been reported to be sexually dimorphic in humans, with women having significantly elevated kisspeptin when compared to men (Wray and Gainer, 1987; Kauffman et al., 2007a, 2009; Hrabovszky et al., 2010; Pita et al., 2011a,b). Expression of the kisspeptin receptor has also been reported to be sexually dimorphic in rats (Navarro et al., 2004a), Rhesus monkeys (Shahab et al., 2005), and teleost fish cobia (Mohamed

et al., 2007), which demonstrates phyllogenetic conservation of this effect as well.

Observations discussed below support an association between the sexual dimorphism in KISS1R/Kiss1r/kisspeptin and the sexual dimorphism of the onset of puberty and that of pubertal disorders.

## RELEVANCE OF THE SEXUAL DIMORPHISM IN KISSPEPTIN FOR THE REPRODUCTIVE FUNCTION IN RODENTS

The AVPV, which is implicated in the generation of the LH surge and ovulation in females (Smith et al., 2005b), has substantial sexual dimorphism of kisspeptin expression in rodents, with the male-typical expression pattern been established just before and after birth (Kauffman et al., 2007b). This corresponds to the timing of brain masculinization reported for mice. Kisspeptin fibers in the AVPV of sexually mature females is 12-fold elevated in rats (Kauffman et al., 2007a; Bakker et al., 2010) and ∼15-fold elevated in mice (Wray and Gainer, 1987). On the other hand, kisspeptin expression in the arcuate nucleus of mice showed steroid-dependent sexual dimorphism only before puberty (Kauffman et al., 2009; Kauffman, 2010). After puberty, hypothalamic kisspeptin expression in both sexes responded similarly to changes in serum levels of sex steroids caused by gonadectomy (Kauffman et al., 2009). This juvenile distinction could be a sign of earlier onset of puberty in female mice, which would place rodents among species with a sexual dimorphism in pubertal development, in which females maturate earlier than males.

## CONSEQUENCES OF LACK OF KISSPEPTIN SEXUAL DIMORPHISM IN RODENTS

Disruption of the *Kiss1r* gene leads to loss of the sexual dimorphism in kisspeptin expression in the hypothalamus of male and female mice (Kauffman et al., 2007b). *Kiss1r* null females fail to ovulate and are infertile (Chan et al., 2009), whereas signs of abnormal sexual maturation in null males resemble those of male mice with aromatase insufficiency (Kauffman et al., 2007b; Bakker et al., 2010). Incidentally, the lack of brain masculinization in male and female mice with aromatase insufficiency has been attributed to the absence of masculinization of kisspeptin neurons, as indicated by inverse cFos activation in kisspeptin neurons of males and females in response to urinary odors in affected mice (Bakker et al., 2010).

Postnatal administration of testosterone rescued male copulatory behavior in aromatase-insufficient as well as in *Kiss1r* null male mice; however, female preference and other kisspeptin-dependent sexually dimorphic traits could not be recovered with postnatal sex steroid replacement in either animal model (Kauffman et al., 2007b; Bakker and Baum, 2008). These observations suggest that the sexual dimorphism in kisspeptin expression is critical for typical male/female sexual responses, which in turn implicates kisspeptin in the mediation of olfactory signals to reproduction (Bakker et al., 2010).

Abnormal sexual differentiation of the brain has been atributted to the abnormal sexual dimorphism in kisspeptin expression in other animal models such as α-fetoprotein-deficient mice (Gonzalez-Martinez et al., 2008). Placental α-fetoprotein metabolizes estrogens, which protects developing females from excessive exposure to maternal estrogens (Bakker et al., 2006). Masculinization of the female brain in α-fetoprotein-deficient females has been attributed to the altered sexual dimorphism of kisspeptin expression in the AVPV of affected females, who are infertile and incapable of generating LH surges in response to steroid stimulation (Gonzalez-Martinez et al., 2008). Similar masculinization of the female brain is described for neonatal female mice abnormally exposed to sex steroids, who exhibit "male-typical" low kisspeptin expression in the AVPV and an inability to generate LH surges (Kauffman et al., 2007a). Conversely, castration of neonatal male mice right after birth prevented brain masculinization. This resulted in males with female-typical kisspeptin expression in the AVPV, who exhibited an unusual ability of mounting LH surges in response to steroid stimulation (Homma et al., 2009).

## SERUM KISSPEPTIN AND SEXUAL DIMORPHISM IN HUMANS

Recent studies have investigated expression and/or secretion of kisspeptin in adults as well as in pubertal children. While not definitive, the results of these studies consistently show sexually dimorphic differences in serum levels as well as in the expression of kisspeptin in humans. In one study, sexually dimorphic differences were identified in the distribution and number of immunolabeled kisspeptin in hypothalamic areas relevant for reproductive function in humans, with females exhibiting heavily labeled kisspeptin in the infundibulus, whereas very few, if any, were present in males (Hrabovszky et al., 2010). Brain samples analyzed in this study were obtained from healthy human subjects who died of sudden death (Hrabovszky et al., 2010). Results were confirmed with an additional antibody from a distinct source, the analysis was blinded, and the age of research subjects did not influence the results (Hrabovszky et al., 2010). The homogeneity of the data in the female group was reassuring against the potential variability of (unknown) sex steroid levels at the time of death among research subjects. Nonetheless, findings should be confirmed in brains from subjects with similar levels of sex steroids at the time of death.

Although the source of circulating kisspeptins has not been established, experimental studies show that intravenously injected kisspeptins can effectively stimulate GnRH/gonadotropin/steroid secretion in humans (Dhillo et al., 2005; George et al., 2011) and animal models such as Rhesus monkeys (Ramaswamy et al., 2007), rats (Matsui et al., 2004; Pheng et al., 2009), and mice (Mikkelsen et al., 2009). Systemic injections of physiologically relevant concentrations of kisspeptin synchronizes LH surge in cycling ewes and induces ovulation in non-cycling ewes on the anestrous season (Jayasena et al., 2010). Similarly, kisspeptin injected peripherally to women is capable of inducing desensitization of the LH response (Jayasena et al., 2009) as well as of bypassing the suppression of LH in patients affected with hypothalamic amenorrhea (Caraty et al., 2007). These observations demonstrate that circulating kisspeptins are physiologically relevant and likely to play a role in the regulation of the HPG axis in many species, including humans.

Serum kisspeptin in adult, sexually mature women was significantly elevated when compared to adult men of similar age in two

studies of different populations from distinct ethnic backgrounds (Pita et al., 2011a,b). This endorses the sexually dimorphic character of kisspeptin differences, as opposed to other ethnic-specific genetic factors.

In healthy children, serum kisspeptin is reported to positively correlate with rises in LH and testosterone during all stages of puberty in boys (Bano et al., 2009). Likewise, serum kisspeptin in pubertal girls is reported to positively correlate to bone age, peak/basal LH, and LH/FSH ratios (Rhie et al., 2011). Additionally, healthy pubertal girls from an unrelated population were reported to have significantly elevated serum kisspeptin when compared to tanner grade-matched healthy boys, which are, in average, one year older (Pita et al., 2011a). These observations endorse serum kisspeptin as faithful indicator of onset and progression of puberty in healthy children as well as provide support for the involvement of kisspeptin in the mediation of the onset and progression of puberty in children.

## KISSPEPTIN AS AN INTEGRATOR OF NUTRITIONAL, HORMONAL, AND OTHER SIGNALS TO PUBERTY—A HYPOTHESIS FOR THE ETIOLOGY OF ICPP

### LEPTIN AS THE MAIN MEDIATOR OF NUTRITIONAL SIGNALS TO PUBERTY

Fat-produced leptin is regarded as the main mediator of nutritional signals to reproduction. A role for leptin on the onset of puberty is evident in leptin-deficient *ob/ob* mice, which have arrested puberty and infertility (Swerdloff et al., 1976; Batt et al., 1982). Similar phenotype is observed in humans with congenital leptin deficiency, who present with hypogonadotropic hypogonadism and other symptoms of leptin deficiency such as hyperphagia and early onset obesity. All abnormalities are at least partially rescued with leptin supplementation in humans (Montague et al., 1977; Clement et al., 1998; Kiess et al., 1998; Strobel et al., 1998; Farooqi et al., 1999, 2002; Ozata et al., 1999; Gibson et al., 2004; Licinio et al., 2004) and in *ob/ob* mice (Halaas et al., 1995; Barash et al., 1996; Chehab et al., 1996; Mounzih et al., 1997; Kiess et al., 1998).

Co-regulation of reproductive function by nutrition is thought to improve species survival by suppressing reproduction during adversities such as negative energy balance. The absence of leptin in *ob/ob* mice leads to a genetically induced state of negative energy balance. A similar state of negative energy balance is associated with the suppression of serum leptin in intact humans and experimental animals subjected to fasting (Nagatani et al., 1998; Licinio et al., 2004; Welt et al., 2004). Negative energy balance with suppression of serum leptin is also associated with loss of body fat due to extreme exercise routines or eating disorders in humans (Licinio et al., 2004; Welt et al., 2004). The negative energy balance in these cases is also reversed with leptin supplementation (Licinio et al., 2004; Welt et al., 2004).

While there is no doubt that leptin plays a role in the onset of puberty, current evidence is not enough to define the precise nature of this effect. Some argue that the initiation of puberty in humans (Frisch and Revelle, 1970) and rodents (Kennedy and Mitra, 1963) would require a critical fat mass, and that the resulting increase in fat-produced leptin would be the signal to initiate puberty once this critical fat mass is achieved (Barash et al., 1996; Chehab et al., 1996). The fact that injection of leptin accelerates puberty in normal female mice would support the requirement of a critical fat mass for the onset of puberty (Ahima et al., 1997; Chehab et al., 1997). However, Cheung and cols (Cheung et al., 2001) found unchanged pre-pubertal and pubertal serum leptin in male and female rats, and serum leptin only correlated with body weight *after* puberty in all tested animals (Cheung et al., 2001).

A similar pattern is observed in children. The three largest epidemiology studies in children show that leptin levels are similar in pre-pubertal boys and girls. Sexually dimorphic differences in leptin only appear at later stages of pubertal development, when boys and girls are at tanner grades 2–5. At the late stages, girls exhibit elevated serum leptin when compared to boys. This increase in serum leptin in females could be attributed to the pubertal rise in circulating estradiol, as clinical data shows that estrogen increases leptin in women independently of body fat content (Lavoie et al., 1999). After correction for fat mass, women have higher serum leptin than men; premenopausal women have higher leptin than postmenopausal women, and short-term estrogen replacement increases serum leptin in postmenopausal women independently of changes in fat mass. This effect is estrogen-specific, as progesterone replacement did not affect serum leptin (Lavoie et al., 1999). These observations suggest that the pubertal increase in leptin could be a consequence (rather than the trigger) of puberty in healthy children (Blum et al., 1997; Clayton et al., 1997; Ahmed et al., 1999).

Additional clinical evidence demonstrates that, despite undetectable *serum* leptin, patients from a family with history of lipoatropic diabetes who have virtually no subcutaneous or visceral fat have normal sexual maturation and menarche (Andreelli et al., 2000). Despite severe leptin deficiency, serum gonadotropins, gonadal steroids, subsequent menstrual cycles, and fertility were not affected in these patients (Andreelli et al., 2000). These phenotypic characteristics are in contradiction to those of patients homozygous for mutations that inactivate leptin or the leptin receptor, which develop early onset obesity and IHH. This inconsistency exposes a gap in our knowledge of how leptin regulates energy metabolism and reproductive function. Nonetheless, the explanation for the inconsistencies may lie in one fundamental difference: despite suppressed, any endogenous leptin produced in patients with lipoatropic diabetes is biologically active, whereas patients with homozygous loss-of-function mutations in leptin or the leptin receptor are simply incapable of activating leptin receptor signaling.

### KISSPEPTIN AS THE MAIN TARGET OF LEPTIN SIGNALS TO REPRODUCTIVE FUNCTION

Kisspeptin is a recognized target of leptin (Smith et al., 2006a; Backholer et al., 2010). In fact, kisspeptin is believed to be the main mediator of pubertal effects of leptin. Kisspeptin expression is highly sensitive to variations in serum leptin or in the nutritional state (Castellano et al., 2005; Smith et al., 2006a; Luque et al., 2007; Kalamatianos et al., 2008; Roa et al., 2008; Quennell et al., 2011), which is compatible with the involvement of kisspeptin in the transmission of nutritional signals

to reproduction. Accordingly, expression of kisspeptin is suppressed in animal models of congenital leptin deficiency or negative energy balance (Castellano et al., 2005; Luque et al., 2007; Kalamatianos et al., 2008), and kisspeptin administration increases gonadotropin secretion in leptin-deficient or fasted rodents (Navarro et al., 2004b; Castellano et al., 2005; Roa et al., 2008). In humans, kisspeptin administration has been shown to counteract the suppression of serum gonadotropins associated with hypothalamic amenorrhea, a human model of negative energy balance (Caraty et al., 2007).

Interestingly, the above effects of kisspeptin occur in spite of the unaltered state of negative energy balance and suppression of serum leptin, as well as the absence of changes in body weight or food intake in affected women or experimental animals. This indicates that kisspeptin is able to bypass the negative state of energy balance to activate the HPG axis, suggesting that stimulation of the reproductive axis by kisspeptin is downstream of metabolic signals such as leptin. This is further supported by the rescue of vaginal opening, serum gonadotropins, and estradiol in fasted female mice treated with kisspeptin (Castellano et al., 2005). Moreover, administration of kisspeptin accelerates puberty in pre-pubertal female rodents despite prior treatment with anti-leptin antibody or negative state of energy balance due to fasting or leptin-resistance (Navarro et al., 2004b, 2005; Castellano et al., 2005).

In rodents, leptin effects on puberty have been presumed to result from binding of leptin to receptors located in kisspeptin neurons of the arcuate nucleus. However, a study using mice with selective ablation of leptin receptors in kisspeptin neurons is challenging this presumption. Ablated mice had normal pubertal development and were fertile (Donato et al., 2011). Additionally, lesions targeting neurons within the hypothalamic ventral premammillary nucleus (PMV) blocked the progression of puberty in *ob/ob* mice by exogenous leptin. These observations are against the requirement of a direct effect of leptin on kisspeptin neurons. They also suggest that the main target of leptin would not be kisspeptin neurons of the arcuate nucleus. Instead, reproductive effects of leptin would be mediated through the PMV (Donato et al., 2011). These observations challenge our perception of how leptin stimulates puberty, and suggest an indirect effect of leptin on kisspeptin, which would be triggered by binding of leptin to receptors within the PMV (rather than in the arcuate) nucleus. This is supported by the absence of leptin receptors in the majority of kisspeptin neurons in the arcuate nucleus (Herbison et al., 2010) as well as by observations that fasting suppresses kisspeptin expression in the AVPV (which do not express leptin receptors) but not in the arcuate nucleus in rats (Kalamatianos et al., 2008). Nonetheless, current experimental and clinical data consistently point to kisspeptin as (indirect?) mediator of leptin signals to reproduction.

## PROPOSED HYPOTHESIS

The sexual dimorphism in the distribution of kisspeptin neurons, in kisspeptin expression, and/or serum kisspeptin is proposed to contribute to the sexual dimorphism in the onset of puberty reported for humans and experimental animals. Additionally, the effect of kisspeptin on the onset and progression of puberty is proposed to depend on the contribution of puberty signals from nutritional, hormonal, environmental, and other sources, which would be integrated at the kisspeptin level in order to customize/optimize reproductive function. **Figure 1** shows a schematic representation of this hypothesis, in which pubertal stimuli would converge into kisspeptin neurons to fine-tune the effect of these on GnRH stimulation by kisspeptin. **Figure 1** also shows the proposed effects on girls and boys of progressive increases in kisspeptin secretion along puberty. A hypothetically elevated number of kisspeptin fibers is proposed to contribute to the earlier onset of puberty in girls when compared to age-matched boys. This is also proposed to contribute to the significantly elevated incidence of CPP in girls.

The above hypothesis is supported by the sexual dimorphism in serum kisspeptin reported for pre-pubertal girls (tanner grade 1), who exhibit elevated serum kisspeptin when compared to age- or tanner grade-matched boys (Pita et al., 2011a). This sexual dimorphism persists in adulthood and is corroborated by elevated expression of kisspeptin in the hypothalamus, which is reported for females of many species, including humans (Wray and Gainer, 1987; Cheng et al., 2010; Pita et al., 2011a,b). The sexual dimorphism in kisspeptin could be associated to the reported sexual dimorphism in gonadotropin secretion in humans. Serum gonadotropins are elevated in healthy, pre-pubertal girls (tanner grade 1) when compared to age- or tanner grade-matched boys (Nottelmann et al., 1987). Notably, boys in tanner grade 1 are in average one year older than tanner grade-matched girls.

A role for the sexual dimorphism in kisspeptin in the etiology of ICPP is supported by the elevated serum kisspeptin reported for girls with ICPP when compared to age-matched healthy girls[1] (de Vries et al., 2009; Chen et al., 2010a; Rhie et al., 2011). These studies selected precocious girls from distinct ethnic backgrounds presenting with classic idiopathic CPP (advanced bone age and elevated LH and FSH peak responses) after other known causes of the disorder were discarded. Serum kisspeptin in precocious girls positively correlated with peak LH and LH/FSH ratio but not with BMI (de Vries et al., 2009; Rhie et al., 2011). In one study, serum kisspeptin in girls with ICPP correlated with urinary levels of monobutyl phthalate, suggesting that acceleration of puberty induced by this endocrine disruptor could be mediated by kisspeptin (Chen et al., 2010a).

The involvement of kisspeptin in the etiology of ICPP is also supported by two mutations with confirmed association to the disorder, which were identified in kisspeptin (Pro74Ser) or the KISS1R (Arg386Pro). Of note, both mutations lead to surprisingly modest increases in signaling, and require KISS1R activation by kisspeptin, which demonstrates that these mutants lack constitutive activation. The small magnitude of the effects of these mutants is presumed to be the basis for the *gain-of-function*, which for both mutants is due to a prolongation of the *endogenous* stimulation of KISS1R by kisspeptin (Teles et al., 2008; Silveira et al., 2010).

[1]Relevance of serum kisspeptin to reproductive function is discussed in the second paragraph of section "Consequences of Lack of Kisspeptin Sexual Dimorphism in Rodents."

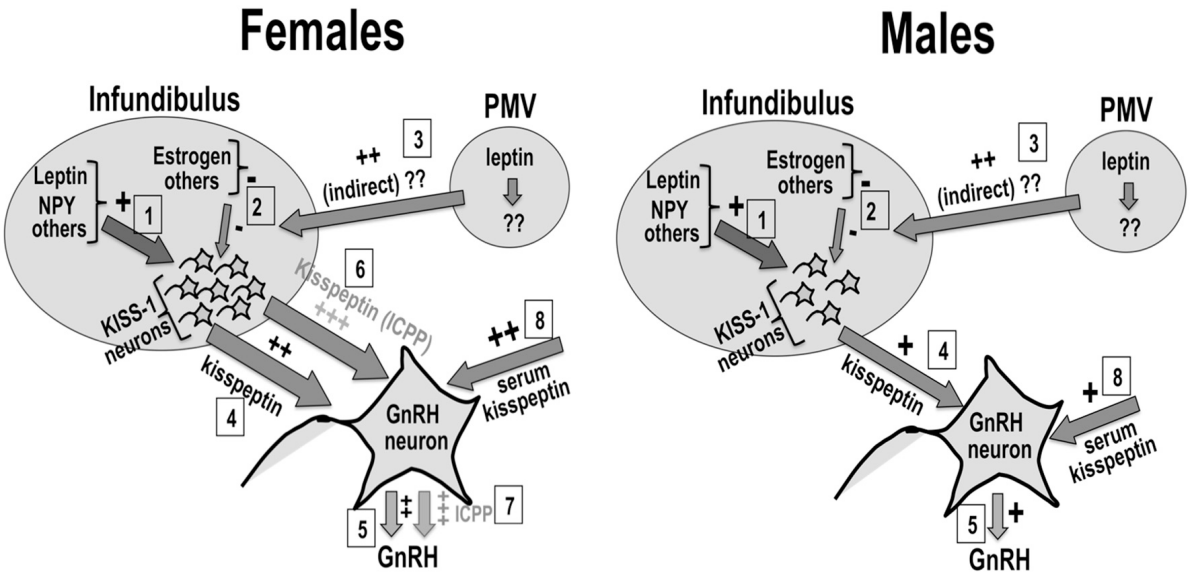


**FIGURE 1 | Kisspeptin, sexual dimorphism, and puberty: Kisspeptin neurons (KISS-1) in the infundibular nucleus would be regulated by positive (1) and negative (2) inputs from nutritional, hormonal and environmental sources.** Possible leptin input on kisspeptin neurons is shown in (1) and (3). Kisspeptin from KISS-1 neurons (4) or the circulation (8) stimulate GnRH neurons to secrete GnRH (5). The number of KISS-1 neurons in the infundibulus is reported to be elevated in women (left panel) when compared to men (right panel). This sexual difference in kisspeptin is proposed to contribute to earlier onset of puberty in healthy girls when compared to boys (6), as well as to the higher incidence of idiopathic CPP in girls (7).

## FINAL CONSIDERATIONS

Precocious or late puberty have implications for affected children, often requiring long-term counseling. Additionally, both disorders are associated with increased risk of other diseases. Early puberty is associated to obesity, polycystic ovaries, metabolic syndrome (for girls), and a variety of cancers (Deardorff et al., 2005; Franks, 2008; Golub et al., 2008; Burt Solorzano and McCartney, 2010; Franceschi et al., 2010), whereas late puberty is associated with metabolic syndrome (for boys), osteoporosis and osteoporotic fractures later in life (Finkelstein et al., 1992; Francis, 1999; Golub et al., 2008). New efficient strategies for early detection and prevention of pubertal disorders and their associated health risks require the understanding of the pathophysiological mechanisms underlying normal and abnormal puberty. For that, the identification and characterization of upstream regulators of GnRH pulsatility is of upmost importance.

One additional study comparing serum kisspeptin in pre-pubertal obese, pre-pubertal age-matched (normal), and ICPP girls found increased serum kisspeptin in the obese but not in the ICPP group (Pita et al., 2011a). A likely source of discrepancy with results of studies previously discussed here is the high cut-off peak LH adopted for the selection of the ICPP group: premature girls with peak LH <7.0 IU/L were excluded, whereas above studies excluded girls with peak LH <5.0U/L (de Vries et al., 2009) or did not adopt a LH cut-off value to exclude potential subjects (Rhie et al., 2011). Incidentally, the girl carrying the first genetic mutation with confirmed association to ICPP (Arg386Pro-KISS1R) would have been eliminated from this but not the other studies, as her peak LH was 6.4 IU/L (Teles et al., 2008). Additional support against the exclusion of girls with LH <7.0 IU/L is provided by the six-month follow-up of girls in the previous studies, which found no differences in kisspeptin or any other clinical, laboratorial or tanner stage parameters between ICPP girls with a peak LH >5.0 IU/l and those with a peak LH <5.0 IU/l (de Vries et al., 2009). Thus, the high LH cut-off value likely eliminated girls with true ICPP from the study by Pita and cols.

Further characterization of the complex relationship of reproductive function with energy metabolism may uncover the basis for inconsistencies such as the opposite effect of obesity on pubertal maturation in boys and girls (Wang, 2002; Burt Solorzano and McCartney, 2010; Walvoord, 2010), and the intriguing correlation of serum leptin with nocturnal but not with diurnal gonadotropin secretion in pubertal girls and adult women (Matkovic et al., 1997; Licinio et al., 1998). Equally intriguing is the inverse correlation of nocturnal serum leptin with weight gain in pubertal girls (Matkovic et al., 1997), and the puzzling phenotype of patients with lipoatropic diabetes, who have normal puberty despite the undetectable serum leptin. Investigation of serum kisspeptin during puberty and adulthood in these patients may provide mechanistic insights into phenotypic inconsistencies between these patients and those

carrying homozygous loss-of-function mutations in leptin or leptin receptor.

Finally, despite the failure to identify genetic mutations in receptors for neurotransmitter such as NPY and GABA in ICPP girls, the possibility of the involvement of these or other receptors and signaling pathways in the etiology of ICPP cannot be discarded.

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# Cross-talk between metabolism and reproduction: the role of POMC and SF1 neurons

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Energy homeostasis and reproduction require tight coordination, but the mechanisms underlying their interaction are not fully understood. Two sets of hypothalamic neurons, namely pro-opiomelanocortin (POMC) neurons in the arcuate nucleus and steroidogenic factor-1 (SF1) neurons in the ventromedial hypothalamic nucleus, are emerging as critical nodes where metabolic and reproductive signals communicate. This view is supported by recent genetic studies showing that disruption of metabolic signals (e.g., leptin and insulin) or reproductive signals (e.g., estradiol) in these neurons leads to impaired regulation of both energy homeostasis and fertility. In this review, we will examine the potential mechanisms of neuronal communication between POMC, SF1, and gonadotropin-releasing hormone neurons in the regulation of metabolism and reproduction.

**Keywords: hypothalamus, energy homeostasis, reproduction**

## INTRODUCTION

Since animals under metabolic stress must invest their energy in survival rather than reproduction, the reproductive axis has the capacity to respond to changes in caloric status. The hypothalamic signals driving the reproductive axis are suppressed when a mammal is in negative energy balance, whether that state is caused by inadequate food intake, excessive locomotor activity, or heavy thermoregulatory costs. Likewise, the energy demands of maintaining fertility and successful reproduction require increased food consumption and appropriate regulation of energy expenditure. Therefore, hypothalamic control of metabolism must be responsive to the reproductive state of the animal. However, despite the passage of 40 years since the discovery of gonadotropin-releasing hormone (GnRH; Schally et al., 1971), the afferent neuronal groups and pathways through which gonadal steroids and nutrient signals regulate GnRH release remain unresolved.

Much attention has focused on the role of hypothalamic neurons expressing kisspeptin in coordinating GnRH neuronal function and the physiological state of the animal. However, while the central role of kisspeptin in steroid feedback to the hypothalamus is clear, evidence of it conveying metabolic signals to the reproductive axis is equivocal. In addition, data suggest that other hypothalamic neurons also convey gonadal steroid input to GnRH circuitry, either directly or via the kisspeptin network.

Here we will discuss new findings resulting from genetically modifying pro-opiomelanocortin (POMC) and steroidogenic factor-1 (SF1) neurons of the hypothalamus. While primarily understood to function as metabolic regulators, these neurons are emerging as critical nodes of communication that respond to both metabolic and reproductive cues and directly interact with reproductive circuitry. In particular, we will focus on their ability to transmit information gleaned from circulating factors, specifically leptin, insulin, and estradiol ($E_2$).

## CANONICAL REPRODUCTIVE CIRCUITS

Hypothalamic GnRH neurons produce the final output of a complex neuronal system regulating fertility. Adult mammals possess a loose field of GnRH neurons stretching from the olfactory bulbs to the medial basal hypothalamus with a highly dense population of GnRH neurons within the preoptic area (POA) and adjacent to the organum vasculosum of the lamina terminalis (OVLT). The axons of GnRH neurons project to the median eminence (ME) where GnRH is secreted in pulses into the pituitary portal bloodstream. GnRH neurons can possess dendrites extending millimeters away from their cell bodies (Campbell et al., 2005; Cottrell et al., 2006), with the average extending over 550 μm (Roberts et al., 2006). Interestingly, many GnRH dendrites follow routes similar to those of their axons toward the ME. Dendrites of GnRH neurons frequently initiate action potentials due to their expression of voltage-gated sodium channels (Rhodes and Llinas, 2005). As a result of the morphology of these dendrites, highly proficient action potential initiation in the distal dendrites is possible even when synaptic potentials are quite small (Witkin and Silverman, 1985; Witkin et al., 1995). Distal portions of the GnRH dendrite, for instance segments located in the arcuate nucleus of the hypothalamus (ARC), thus provide synaptic input and can potently

affect GnRH hormone secretion (Campbell and Suter, 2010; Zoli et al., 1998; Herbison, 2006).

A great deal of the recent focus on afferent control of GnRH secretion has been on kisspeptin neurons in the hypothalamus, based on the ability of mutations of the kisspeptin receptor to cause hypogonadotropic hypogonadism in humans and animal models (de Roux et al., 2003; Seminara et al., 2003). Kisspeptin exerts an extremely powerful and long-lasting depolarizing stimulus upon GnRH neurons (Gottsch et al., 2004; Han et al., 2005; Messager et al., 2005; Pielecka-Fortuna et al., 2008) and plays a central role in the physiological regulation of GnRH release (Oakley et al., 2009; Ohkura et al., 2009). Confocal images have shown kisspeptin terminals in direct apposition to GnRH cell bodies (Clarkson and Herbison, 2006; Ramaswamy et al., 2008). In addition, in the rat and monkey, kisspeptin terminals form close contacts with GnRH terminals in the ME (Krajewski et al., 2005, 2010; Ciofi et al., 2006; Decourt et al., 2008; Lehman et al., 2010). Recent studies on the stimulatory effects of kisspeptin are consistent with actions at both the ME (d'Anglemont de Tassigny et al., 2008) and GnRH cell bodies in the POA (Pielecka-Fortuna et al., 2008; Moenter and Pielecka-Fortuna, 2010). Two populations of kisspeptin neurons exist in the hypothalamus; one is located in the anteroventral periventricular nucleus (AVPV; Smith et al., 2005), and the other is located in the ARC. Most ARC kisspeptin neurons co-express neurokinin B (NKB) and dynorphin (Dyn) in rat (Burke et al., 2006), mouse (Navarro et al., 2009), sheep (Goodman et al., 2007), goat (Wakabayashi et al., 2010), and possibly human (Rance, 2009) leading to these neurons acquiring the moniker of KNDy neurons (Cheng et al., 2010). Each of these three neuropeptides has been strongly implicated in the feedback regulation of GnRH neurons (Rance and Young, 1991; Sahu and Kalra, 1992; Rance and Bruce, 1994; Goodman et al., 2004; Foradori et al., 2005). Since the kisspeptin network is not the focus of this review, readers interested in this topic are referred to some excellent recent publications (Oakley et al., 2009; Navarro and Tena-Sempere, 2011).

## POMC NEURONS LINK METABOLIC AND REPRODUCTIVE CIRCUITS

### POMC NEURONS INNERVATE REPRODUCTIVE CIRCUITS

Besides kisspeptin neurons, other hypothalamic populations may also provide afferent signals on the hypothalamo-pituitary gonadal (HPG) axis to regulate reproduction. Particularly, recent evidence suggests that hypothalamic sites that regulate energy balance have important inputs to GnRH neurons. One example is POMC neurons in the ARC. POMC neurons have long been believed to be a primary central regulator of energy homeostasis (Huszar et al., 1997; Cone, 1999). The anorexigenic property of POMC neurons has been well established, as the deletion of POMC gene causes hyperphagia and obesity (Yaswen et al., 1999).

Evidence also suggests that POMC neurons innervate the reproductive circuits in the central nervous system (CNS). For example, POMC neurons make direct synaptic contact with GnRH neurons (Leranth et al., 1988; Thind and Goldsmith, 1988; Chen et al., 1989a). The major neurotransmitters released from POMC neurons include two POMC gene products: the anorectic peptide α-melanocyte-stimulating hormone (α-MSH) and the endogenous opioid β-endorphin (Cheung et al., 1997; Broberger et al., 1998; Hahn et al., 1998; Vrang et al., 1999). Fibers specifically immunoreactive for β-endorphin have been identified within the immediate vicinity of GnRH neurons, and based upon electron microscopic evidence, the β-endorphin-immunoreactive terminals synapse on the GnRH neuron soma in the rat (Leranth et al., 1988; Chen et al., 1989b), sheep (Goodman et al., 2004), and monkey (Thind and Goldsmith, 1988). About 20–30% of POMC neurons in the ARC co-express estrogen receptor-α (ERα; Lehman et al., 1993; de Souza et al., 2011; Xu et al., 2011), and an ERα-positive subpopulation of ARC POMC neurons has been shown to project to the POA, where GnRH neurons are concentrated (Simonian et al., 1999). Furthermore, GnRH neurons express receptors for β-endorphin. For example, μ-opioid receptors, have been indentified in GnRH neurons of the guinea pig (Lagrange et al., 1995). In addition, δ-opioid receptors have been identified in mouse GnRH neuron-derived GT-1 cells and in a fraction of rat GnRH nerve terminals, including in the ME (Pimpinelli et al., 2006). Collectively, these findings indicate that POMC neurons are well positioned to provide synaptic inputs to GnRH neurons.

At the functional level, neurotransmitters released from POMC neurons have been shown to regulate the HPG axis. In both monkeys and rats, β-endorphin and other opioids exert inhibitory effects on GnRH and LH secretion (Bruni et al., 1977; Kinoshita et al., 1982; Gilbeau et al., 1985; Leadem and Kalra, 1985b,a; Wiesner et al., 1985; Leadem and Yagenova, 1987; Wardlaw and Ferin, 1990; Kalra and Kalra, 1996). This inhibitory effects appear to be tonic, as administration of naloxone, an opiate receptor antagonist, to rats increases LH production (Babu et al., 1987). Reducing the opioid inhibition also facilitates the production of the GnRH surge on proestrus (Lustig et al., 1988; Masotto et al., 1990; Hashimoto and Kimura, 1991; Zhang and Gallo, 2002). This inhibition by opioids can be interpreted as increased overall suppression of GnRH and LH release or as augmentation of steroid negative feedback. Indeed, opioid antagonism can also stimulate GnRH release independent of $E_2$ levels (Babu et al., 1987; Karahalios and Levine, 1988; Goodman et al., 1995). However, during the rat estrous cycle, β-endorphin levels fluctuate in the ARC, POA, and ME. The maximum levels are seen during diestrus while the lowest occurs on proestrus leading up to the LH surge (Gallo and Drouva, 1979), suggesting a role in negative feedback. While these studies strongly support an inhibitory effect of opioids on GnRH release, genetic evidence is less convincing. Mutant mice lacking the classical endogenous opioids (dynorphin, enkephalin, and endorphin; Konig et al., 1996; Rubinstein et al., 1996; Sharifi et al., 2001) as well as mutants of the three opioid receptors (Matthes et al., 1996; Sora et al., 1997; Roy et al., 1998; Simonin et al., 1998; Schuller et al., 1999; Zhu et al., 1999; Filliol et al., 2000) are fertile, although μ-opioid receptor-deficient mice display reduced spermatogenesis and impaired sexual function (Tian et al., 1997). Thus, at least when absent during the development of hypothalamic circuitry, opioid inhibition of GnRH release is not required for fertility.

In contrast, it has been demonstrated that α-MSH exerts an excitatory effect on the GnRH system, likely by acting on central melanocortin receptors (MCs; Celis, 1985; Backholer et al., 2009). A robust connection has recently been demonstrated between MCH neurons, which express MC4 receptors and receive direct input from POMC neurons, and GnRH neurons (Wu et al., 2009). Agouti-related peptide (AgRP), an endogenous receptor antagonist of MCs, decreases the magnitude of the LH surges in normally

fed rats (Watanobe et al., 1999; Schioth et al., 2001; Schioth and Watanobe, 2002), while anti-AgRP serum partially but significantly enlarges the LH surge (Schioth et al., 2001). Indeed, melanocortin 4 receptor (MC4R) deficient mice exhibit erectile dysfunction and changed sexual behavior in males secondary to obesity (Van der Ploeg et al., 2002) and reduced ovulation rates and fertility accompanied by increased follicular atresia by 6 months of age in females (Sandrock et al., 2009). Furthermore, *agouti* mice, which congenitally overproduce AgRP, have adult-onset infertility (Granholm et al., 1986). Collectively, these findings point to α-MSH as a potential afferent signal to the HPG axis.

In addition to neuropeptides, POMC neurons release classical neurotransmitters GABA and glutamate (Hentges et al., 2004, 2009), both of which have been shown to regulate GnRH neurons (Kusano et al., 1995; Shepherd, 1996; Spergel et al., 1999; Sim et al., 2000; Sorra and Harris, 2000; Simonian and Herbison, 2001; DeFazio et al., 2002; Fiala et al., 2002; Han et al., 2002; Kuehl-Kovarik et al., 2002; Ottem et al., 2002; Moenter and DeFazio, 2005). In addition to direct synaptic inputs, POMC neurons may also influence GnRH neurons via kisspeptin intermediary neurons. Supporting this possibility, kisspeptin fibers have been shown in close apposition with ARC POMC neurons in ewes (Backholer et al., 2009, 2010), and double-label fluorescent immunohistochemistry showed that reciprocal connections exist between kisspeptin neurons and POMC neurons (Backholer et al., 2010).

The studies reviewed above emphasize the critical "choice" of POMC neurons to express β-endorphin or α-MSH. The former, possibly acting in concert with other opioids such as dynorphin (via its own kappa-opioid receptor; Navarro et al., 2009), is intimately involved in the negative feedback regulation of GnRH release. The latter, through activation of second-order metabolic circuitry, is involved in the gating of fertility during times of energy deprivation. The control of β-endorphin vs. α-MSH production by POMC neurons is an area of ongoing study (Wardlaw, 2011).

## LEPTIN AND INSULIN ACT ON POMC NEURONS TO REGULATE BOTH REPRODUCTION AND ENERGY HOMEOSTASIS

Emerging evidence indicates that POMC neurons respond to metabolic cues to provide coordinated control of metabolism and reproduction. One example of these metabolic cues is leptin. Leptin is a circulating adiposity-related factor that informs the CNS regarding energy stores. Released by adipocytes when stored fat is plentiful, leptin acts in the hypothalamus to suppress body weight gain and to improve insulin sensitivity (Morton et al., 2003, 2005; Balthasar et al., 2004; Coppari et al., 2005; Dhillon et al., 2006; van de Wall et al., 2008). Mice lacking leptin or leptin receptors (LepRs) develop hyperphagic morbid obesity, insulin resistant diabetes, and hypothermia (Coleman, 1978). Leptin reduces food intake and body weight when administered to leptin-null mice (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995), and brain-specific deletion of LepRs leads to obesity (Cohen et al., 2001).

Leptin is also a prerequisite for pubertal development and successful reproduction. Humans and mice carrying leptin gene mutations fail to go through puberty, have low LH levels, and are infertile (Montague et al., 1997), and leptin administration, but not weight loss alone, allows pubertal progression and restores their fertility (Barash et al., 1996; Chehab et al., 1996; Mounzih et al., 1997; Ziotopoulou et al., 2000). Leptin also overrides the fasting-induced suppression of LH secretion and fertility (Nagatani et al., 1998, 2000; Gonzalez et al., 1999; Kohsaka et al., 1999). In anorectic females and in athletes with extreme decreases in body adiposity, leptin can increase levels of luteinizing hormone (Licinio et al., 1998) and restore the menstrual cycle (Welt et al., 2004). Re-expression of LepRs in the brain of LepR-null mice restores fertility completely in males and partially in females (Kowalski et al., 2001; de Luca et al., 2005). In addition, AAV-induced expression of the LepR gene in the POA or ARC of LepR-null rats normalizes their estrous cycle length and increases GnRH concentrations in the hypothalamus (Keen-Rhinehart et al., 2005). Collectively, this evidence indicates that leptin, while primarily acting as a metabolic signal to maintain normal energy and glucose homeostasis, also plays essential roles in reproduction.

Insulin, another circulating factor related to adiposity, is also implicated in the coordinated control of metabolism and reproduction. Insulin levels in the circulation are proportional to adipose tissue in most mammals (Woods et al., 1979). Intracerebroventricular (icv) insulin administration results in a dose-dependent reduction in food intake and body weight (Woods et al., 1979), and neuron-specific deletion of insulin receptors (IRs) leads to increased body fat deposition (Bruning et al., 2000).

A variety of mouse models have demonstrated insulin's essential role in the central control of reproduction. For example, increased circulating levels of insulin during a hyperinsulinemic clamp stimulate LH secretion (Burcelin et al., 2003a). In addition, mice lacking IRs in the brain exhibit decreased spermatogenesis and follicular maturation, resulting in only 42–46% of matings successfully producing offspring (Bruning et al., 2000). The primary deficit in these mice was found to be a reduction in GnRH release and consequent reductions in pituitary gonadotropin secretion and gonadal function. In another study, expression of IRs in liver and pancreas alone was sufficient to maintain fertility in males, but females also required IR expression in the brain (Okamoto et al., 2004). Finally, a study of IR substrate 2 (IRS-2) knockout mice found that only 9% of IRS-$2^{-/-}$ females and 89% of IRS-$2^{-/-}$ males were fertile. IRS-$2^{-/-}$ females showed a supranormal response to GnRH, consistent with hypothalamic hypogonadism (Burks et al., 2000). These studies show that, particularly in the female, IRs in the brain are required for fertility.

While it is clear that both leptin and insulin could signal the brain to coordinate energy status and reproductive demands, the exact brain sites where these signals are integrated remained unclear. GnRH neurons do not appear to be the direct target of these hormones. For example, double-labeling experiments using *in situ* hybridization and immunohistochemistry have shown few GnRH neurons, if any, to express LepRs in rats (Burcelin et al., 2003a) and monkeys (Finn et al., 1998). Insulin has a direct stimulatory effect on the output of GnRH in hypothalamic cells *in vitro* and *in vivo* (Kovacs et al., 2002; Burcelin et al., 2003b). However, mice lacking IRs specifically in GnRH neurons displayed normal pubertal timing and fertility (Divall et al., 2010), suggesting that the insulin responsiveness of GnRH neurons is low. While early reports suggested that 40% of kisspeptin mRNA-expressing cells in the ARC of mice express LepRs (Smith et al., 2006), other

laboratories have found fewer than 5% of kisspeptin neurons exhibit LepRs (Donato et al., 2011; Louis et al., 2011). The latter results appear to be borne out by the lack of a reproductive phenotype in mice with a targeted deletion of LepRs from kisspeptin neurons (Donato et al., 2011). Similarly, our preliminary data suggest that insulin sensing directly by kisspeptin neurons plays a minor role in mouse fertility (Qiu et al., 2011). Thus, leptin/insulin sensing outside of the dedicated reproductive circuitry is likely to play a role in their effects on reproduction.

POMC neurons are well positioned to be a direct target of leptin and insulin signals. POMC neurons express LepRs (Cheung et al., 1997; Elmquist et al., 1998; Baskin et al., 1999) and IRs (Benoit et al., 2002). LepRs in POMC neurons mediate a portion of leptin actions on energy homeostasis, as mice lacking LepRs specifically in POMC neurons are mildly obese and hyperleptinemic (Balthasar et al., 2004). Although deletion of IRs from POMC neurons does not affect body weight (Konner et al., 2007), simultaneous deletion of IRs and LepRs from POMC neurons produces more severe insulin resistance and diabetes than deletion of each individual receptor alone (Hill et al., 2010). Therefore, POMC neurons appear to be one important site where insulin and leptin signals interact to regulate energy and glucose homeostasis.

Our recent studies also pinpointed POMC neurons as a target of leptin/insulin actions important for fertility. We have reported that female mice lacking both leptin and IRs in POMC neurons (IR/LepR$^{POMC}$) exhibit lengthened reproductive cycles, follicular arrest, hyperandrogenemia, and infertility. These mice lack IRs and LepRs in POMC-expressing cells in the hypothalamus and pituitary corticotrophs and melanotrophs, but retain them in other cell types and tissues, such as liver and ovary (Hill et al., 2010). These results were confirmed by an absence of altered IR and LepR expression in these tissues using qPCR. Despite the expression of POMC in corticotrophs, we found no alteration in corticosterone release. Pup numbers born to experimental females older than 4 months were significantly reduced (Hill et al., 2010). These females also showed a lengthened estrous cycle. In addition, the percentage of matings not producing a litter was higher for experimental females across all maternal ages. These reproductive deficits were not caused by abnormal prolactin or $E_2$ levels, and no pups born to IR/LepR$^{POMC}$ dams died after birth. While hypothalamic GnRH gene expression was comparable among the groups, LH levels were significantly increased in IR/LepR$^{POMC}$ females. Histological examination of their ovaries showed that double knockout females exhibited more degenerating follicles. Serum testosterone levels were significantly elevated in experimental females, accompanied by a significant elevation in the expression of ovarian 3β-HSD I gene, which produces androstenedione. CYP17 gene expression was also slightly increased ($p = 0.0530$; Hill et al., 2010). Interestingly, males also exhibit reduced numbers of successful pairings with wild-type females despite an enthusiastic mounting response and increased testes weights (**Figures 1A–C**). In addition, a subset of males exhibited dramatically increased LH levels (**Figure 1D**) with normal FSH concentrations (data not shown). The heterogeneity in these mice may be due to their mixed strain background. Collectively, these results suggest that the absence of leptin and insulin signaling in POMC neurons may reduce the inhibitory opioid tone on GnRH neurons and cause basal LH levels to increase, disrupting reproductive function. Indeed, the absence of leptin and insulin signaling would both be expected to reduce β-endorphin production from its POMC precursor (Wardlaw, 2011).

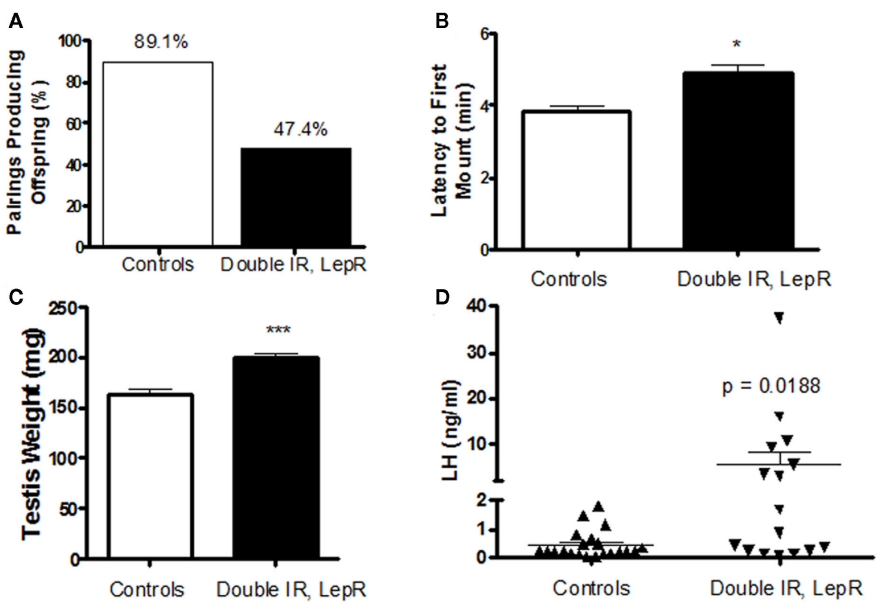


**FIGURE 1 | Reproductive phenotype of 4-month-old IR, LepR$^{POMC}$ mice.** **(A)** Male mice lacking insulin and leptin receptors in POMC neurons were paired with genetically normal control females and the number of pairings producing pups were quantified. **(B)** The amount of time before experimental and control males attempted to mount a novel control female was quantified. Latency was scored during a 10-min period after male was introduced to the cage of a singly housed female in Proestrus under red lighting during the first half hour after lights out (1800 h). **(C)**. Testes weights were measured at time of sacrifice. **(D)**. Serum levels of luteinizing hormone were measured by RIA by the University of Virginia Ligand Assay and Analysis Core.

Further studies are required to determine whether β-endorphin production in these mice is reduced, and the signaling mechanisms involved.

### ESTROGENS ACT ON POMC NEURONS TO REGULATE BOTH REPRODUCTION AND ENERGY HOMEOSTASIS

Steroid hormones such as $E_2$ exert potent feedback at both the neural and pituitary levels to regulate the HPG axis. During much of the female reproductive cycle, $E_2$ reduces the GnRH pulse amplitude (Sarkar and Fink, 1980; Caraty et al., 1989; Chongthammakun and Terasawa, 1993; Evans et al., 1994) and inhibits LH release via negative feedback actions on the hypothalamus as well as the pituitary gonadotropes (Shupnik et al., 1988; Shupnik, 1996). A similar mechanism appears to be at work in males via aromatization of testosterone to $E_2$ (Veldhuis and Dufau, 1987; Finkelstein et al., 1991a,b; Bagatell et al., 1994; Hayes et al., 2000; Schnorr et al., 2001). In the course of the later stage of the follicular phase, circulating $E_2$ levels rise resulting in a biphasic effect on GnRH secretion, causing a noticeable suppression of pulsatile GnRH and LH secretion, followed by an induction of a high amplitude GnRH surge supplementary to the LH surge, a process termed positive feedback (Crowder and Nett, 1984; Moenter et al., 1990). Lesion studies performed in the rat and hamster (Shander and Barraclough, 1980; Wiegand et al., 1980) have implicated anatomically distinct brain regions for the conveyance of the positive and negative feedback. These studies localized negative feedback to the ARC and ME and positive feedback to the POA and the suprachiasmatic nucleus.

Both positive and negative feedback regulation are primarily mediated by ERα, but not by estrogen receptor-β (ERβ). This is demonstrated by observations that global deletion of ERα in mice abolishes the positive and negative feedback responses, while mice with global ERβ deficiency appear to retain normal feedback responses (Couse et al., 1995, 2003; Wintermantel et al., 2006). However, the sites where estrogens act to regulate the HPG axis have not been fully revealed. Wintermantel et al. (2006) demonstrated that female mice lacking ERα only in the brain show blunted positive feedback and impaired fertility. While these findings identified brain ERα as the key mediator for the positive feedback, the exact ERα populations in the brain responsible for this regulation remain unknown. Singh et al. (2009) showed that deletion of ERα in the pituitary LH cells blunts $E_2$-induced inhibition on LH secretion and results in infertility in female mice. Therefore, pituitary ERα is at least partly responsible for the negative feedback, but redundant ERα populations likely exist in the brain. Given that GnRH neurons do not appear to express ERα (Shivers et al., 1983), interneurons must exist to relay $E_2$/ERα signals on GnRH neurons.

Recent evidence suggests that POMC neurons may serve as interneurons mediating $E_2$ actions. First, POMC neurons co-express ERα (Miller et al., 1995; de Souza et al., 2011), suggesting that POMC neurons could be the direct target of estrogenic actions. $E_2$ treatment increases POMC expression (Hammer et al., 1994; Cheung and Hammer, 1997). β-endorphin positive fibers and opioid receptors in the POA also increase upon $E_2$ treatment (Hammer et al., 1994). In addition, $E_2$ regulates the excitability of POMC neurons. Using electron microscopy, Gao et al. (2007) reported that the number of excitatory synaptic inputs to ARC POMC neurons rises as mice enter proestrus when $E_2$ levels are high. Further, central $E_2$ administration rapidly increases the excitatory synapses on POMC neurons, an effect that is also reflected by increased miniature excitatory postsynaptic current recorded from POMC neurons (Gao et al., 2007). Similarly, Malyala et al. (2008) reported that $E_2$ administration in hypothalamic slices increases excitability of POMC neurons by rapidly uncoupling $GABA_B$ receptors from their G-protein-gated inwardly rectifying $K^+$ channels. These studies demonstrated that $E_2$ directly acts on POMC neurons and regulates their cellular activity.

The physiological relevance of $E_2$ sensing by POMC neurons was further established by our recent findings in mice lacking ERα specifically in POMC neurons. First, we found that mutant females have a modest increase in plasma $E_2$, raising the possibility of impaired negative feedback on the HGP axis (Xu et al., 2011). Thus, while $E_2$ replacement suppresses FSH and LH expression in the pituitary of ovariectomized (OVX) wild-type mice, this $E_2$-induced suppression is blunted in OVX mutant mice (Xu et al., 2011). Interestingly, only 30% of female mice lacking ERα in POMC neurons successfully delivered pups (Xu et al., 2011). The averaged size of litters from these 30% mutant dams was significantly reduced compared to those from control dams (Xu et al., 2011). In addition, the mating time required for these mutant females to conceive was significantly increased (Xu et al., 2011). These findings indicate that $E_2$/ERα signals in POMC neurons are at least partially required to mediate the negative feedback regulation of the HPG axis and to maintain normal fertility.

Besides being a reproductive cue, estrogens also exert important anti-obesity effects in women (Flegal et al., 2002; Freedman et al., 2002; Carr, 2003) and female mammals (Drewett, 1973; Blaustein and Wade, 1976; Wallen et al., 2001; Rogers et al., 2009). $E_2$ reduces food intake and body adiposity and increase energy expenditure in animals and humans of both sexes through a hypothalamic mechanism (Dubuc, 1985; Wade et al., 1985; Hess et al., 1997; Heine et al., 2000). Effects of $E_2$ on energy balance are primarily mediated by ERα, as women or female mice with mutations in the ERα gene display hyperadiposity (Heine et al., 2000; Okura et al., 2003), whereas ERβ-null mice have no body weight phenotype (Ohlsson et al., 2000). Interestingly, we recently found that a portion of anti-obesity effects of estrogens are mediated by ERα expressed by POMC neurons. For example, we found that female mice lacking ERα only in POMC neurons develop chronic hyperphagia and increased body weight (Xu et al., 2011). In addition, the leptin-induced suppression in food intake was blunted in female mice lacking ERα in POMC neurons (Xu et al., 2011). Collectively, these findings indicate that $E_2$/ERα signals within POMC neurons are not only important to mediate the negative feedback and maintain normal reproduction, but also are physiologically relevant in the regulation of feeding behavior. Therefore, $E_2$ signals within POMC neurons may coordinate the regulation of energy balance and reproductive demands.

## SF1 NEURONS LINK METABOLIC AND REPRODUCTIVE CIRCUITS

### SF1 NEURONS INNERVATE REPRODUCTIVE CIRCUITS

In addition to POMC neurons in the ARC, steroidogenic factor-1 (SF1) neurons, located in the ventromedial hypothalamic nucleus

(VMH) may serve as an important connection point relaying metabolic and reproductive cues.

The physiological relevance of VMH neurons to the regulation of body weight homeostasis is well recognized. Ikeda et al. (1995) discovered that a transcription factor, SF1, is expressed exclusively in the VMH neurons within the brain. SF1 neurons constitute the majority of VMH neurons (Stallings et al., 2002). During early development, SF1 is essential for the formation of the VMH architecture, as mice with embryonic deletion of SF1 gene do not form a VMH (Dellovade et al., 2000). These SF1 knockout mice develop massive obesity (Majdic et al., 2002). It is important to note that SF1 is also abundant in a number of endocrine organs, such as the pituitary gland, the adrenal gland and gonads (Zhao et al., 2001). Therefore, the obesity phenotype in global SF1 knockout mice may have been confounded by the dysfunctions of these tissues (Majdic et al., 2002). To circumvent this issue, a brain-specific SF1 knockout mouse line was generated to determine the role of VMH SF1 neurons in the context of body weight control (Kim et al., 2011). This brain-specific SF1 deletion also leads to disruption of the VMH structure and obesity in mice (Kim et al., 2011). Thus, these findings demonstrate that VMH neurons are physiologically important for the regulation of energy homeostasis.

VMH neurons may also regulate the central reproductive circuits. For example, the VMH has well defined projections to the medial central gray and periaqueductal gray regions that have been implicated in lordosis (Canteras et al., 1994). In addition, VMH neurons are also found to project to GnRH neurons (Boehm et al., 2005; Yoon et al., 2005). Further, numerous studies have suggested that VMH neurons regulate sexual behaviors (Blaustein and Feder, 1980; Rubin and Barfield, 1983a,b; Canteras et al., 1994; Mani et al., 1997; Sinchak et al., 2007). Mice with genetic ablation of VMH SF1 neurons show a significantly blunted lordosis quotient and receptivity (Kim et al., 2010). The role of the VMH in reproduction may be more extensive than just behavior. For example, the mutant female mice with genetic ablation of VMH SF1 neurons show severely irregular estrus cycles, and are infertile or subfertile (Kim et al., 2010). The impaired female fertility is likely due to defective ovulation, as demonstrated by decreased or absent corpora lutea in the ovary (Kim et al., 2010). Interestingly, exogenous administration of gonadotropins induced normal ovulation in these mice, demonstrating that the ovaries are functionally intact (Kim et al., 2010). Further, when the mutant females were stimulated with a synthetic GnRH agonist after priming, they exhibited markedly reduced LH secretion compared with wild-type littermates, arguing that disorganization in and around the VMH caused by SF1 ablation interferes with the GnRH priming process or gonadotrope LH capacity (Kim et al., 2010).

Collectively, these findings indicate that functional VMH SF1 neurons are required to maintain not only normal energy balance but also reproduction. Therefore, these SF1 neurons could serve as an import point of intersection for these two systems.

## ESTROGENS ACT ON SF1 NEURONS TO REGULATE BOTH REPRODUCTION AND ENERGY HOMEOSTASIS

$E_2$ acts in the VMH to regulate energy balance. Abundant ERα is found in the ventrolateral subdivision of the VMH (Osterlund et al., 1998; Merchenthaler et al., 2004; Schlenker and Hansen, 2006). To determine whether ERα in the VMH mediate the anti-obesity effects of estrogens, Musatov and colleagues used shRNA-mediated gene silencing approach to knock-down ERα in the VMH in female rodents. They found that VMH-specific ERα knock-down leads to obesity and metabolic syndrome primarily due to decreased energy expenditure (Musatov et al., 2007). We recently crossed ERα floxed mice (Feng et al., 2007) to SF1-Cre transgenic mice (Dhillon et al., 2006), which resulted in mice lacking ERα specifically in VMH SF1 neurons (Xu et al., 2011). This SF1-specific deletion of ERα results in modest body weight gain and hyperadiposity solely due to decreased energy expenditure in female mice (Xu et al., 2011). We further demonstrate that both basal metabolic rate and diet-induced thermogenesis in these mice are reduced, while the energy expenditure associated with physical activity is not altered (Xu et al., 2011). Interestingly, female mice lacking ERα in SF1 neurons also show increased visceral fat distribution, while the subcutaneous fat distribution is reduced (Xu et al., 2011). Consistent with this abdominal obesity, these mice predictably develop glucose intolerance (Xu et al., 2011). Collectively, our findings support a model in which $E_2$ acts on ERα in VMH SF1 neurons to stimulate energy expenditure and inhibit visceral fat expansion. As we also found that norepinephrine is decreased in mice lacking ERα in SF1 neurons (Xu et al., 2011), the effects of $E_2$/ERα in SF1 neurons on energy expenditure and fat distribution are presumably mediated by elevated sympathetic outflow.

The actions of $E_2$ in VMH neurons are also important for reproduction. For example, administration of $E_2$ in the VMH has been shown to modulate female sexual behaviors (Rubin and Barfield, 1983a). In addition, $E_2$ actions in the VMH may mediate induction of progesterone receptors in the VMH (McGinnis et al., 1981; Olster and Blaustein, 1989; Kalra, 1993; Moffatt et al., 1998), thereby permitting progesterone to reduce basal GnRH and LH release (Chappell et al., 1997). The role of VMH $E_2$ sensing in reproduction was further supported by our findings from mice lacking ERα only in SF1 neurons. These mutant female mice have irregular estrus cycles (**Figure 2A**). Further, most of mutant females (9 out of 10 mice tested) are infertile (**Figure 2B**). The only mutant dam that successfully conceived and delivered had smaller litter size than controls (**Figure 2C**). The impaired fertility in these females is likely due to anovulation, demonstrated by the lack of corpora lutea in the ovaries (**Figure 2D**). One caveat of this model is that ERα may also be deleted from SF1 cells in the pituitary, adrenal gland, and gonads, in addition to SF1 neurons in the VMH, which makes it difficult to attribute the fertility and ovarian phenotypes solely to the loss of ERα in SF1 neurons in the VMH. However, we did not found any significant reduction of ERα mRNA in the pituitary, adrenal gland, and ovaries from the mutant mice (Xu et al., 2011), which argues that the infertility/subfertility and anovulation phenotypes are likely due to the loss of ERα in VMH neurons. Interestingly, the brain-specific ERα knockout model shows the same fertility and ovarian phenotypes as in our mice lacking ERα in SF1 cells (Wintermantel et al., 2006), suggesting that ERα in the brain, such as in VMH SF1 neurons, is required to trigger ovulation in the ovaries. Collectively, these findings support a model in which adequate $E_2$ signaling in VMH SF1 neurons is required both for fertility and normal energy balance.

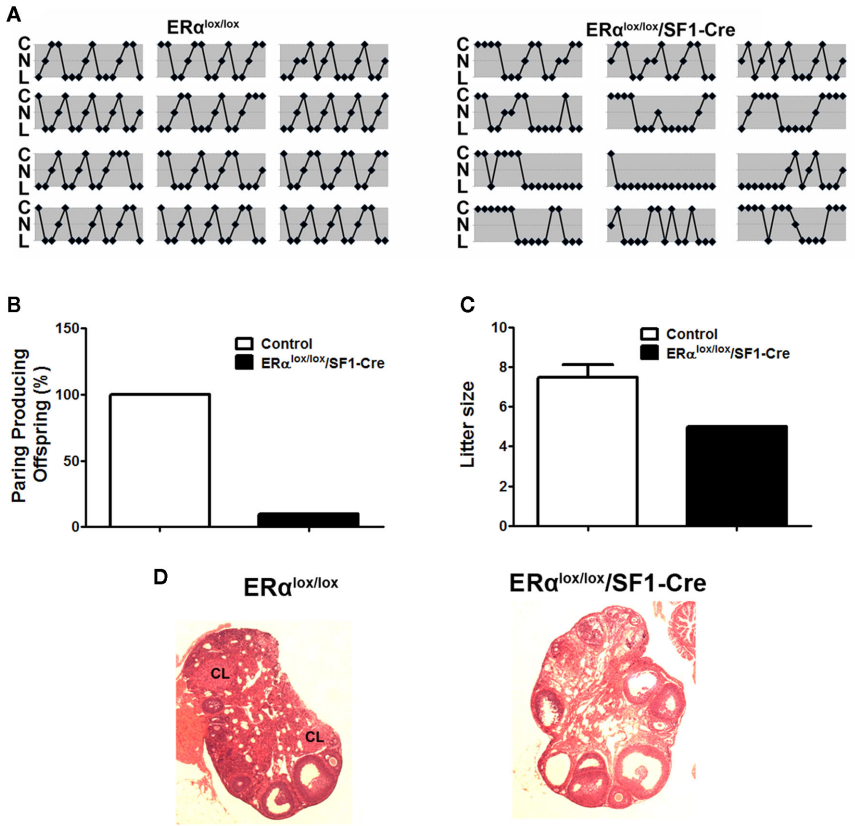


**FIGURE 2 | Reproductive phenotype of female mice lacking ERα in SF1 neurons (ERα$^{lox/lox}$/SF1–Cre). (A)**. Estrus cycles from 12-week-old female mice lacking ERα in SF1 neurons and their wild-type controls were evaluated by vaginal cytology. Note: C, cornified cells (estrus); N, nucleated epithelial cells (proestrus); L, leukocytes (diestrus). **(B)**. Female mice lacking ERα in SF1 neurons and their wild-type controls were paired with genetically normal control males and the numbers of pairings producing pups were quantified. **(C)**. Litter size from dams that successfully delivered pups in **(B)**. **(D)** H&E staining of ovaries from adult female mice. There was no corpus luteum in the ovary from mice lacking ERα in SF1 neurons.

## CONCLUSION

While POMC and SF1 neurons have previously been thought to serve primarily in the control of food intake and energy balance, new genetic rodent models have highlighted their crucial roles in the maintenance of fertility. While additional work is needed to clarify the mechanisms by which these neuronal circuits modulate the GnRH system, these studies highlight the profound integration of reproductive and metabolic control. In particular, POMC and SF1 neurons are emerging as critical sites that both respond to circulating factors and directly interact with reproductive circuitry. Future studies of POMC and SF1 neurons may shed light on the nature of positive and negative steroid feedback as well as integration of signals of adiposity in the control of the reproductive axis.

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# The ventral premammillary nucleus links metabolic cues and reproduction

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The amount of body fat and the energy balance are important factors that influence the timing of puberty and the normal reproductive function. Leptin is a key hormone that conveys to the central nervous system information about the individual energy reserve and modulates the hypothalamus–pituitary–gonad (HPG) axis. Recent findings suggest that the ventral premammillary nucleus (PMV) mediates the effects of leptin as a permissive factor for the onset of puberty and the coordinated secretion of luteinizing hormone during conditions of negative energy balance. In this review, we will summarize the existing literature about the potential role played by PMV neurons in the regulation of the HPG axis.

**Keywords: leptin, puberty, GnRH, luteinizing hormone, hypothalamus, adiposity**

## INTRODUCTION

It has long been known that nutritional status is a critical factor in determining the timing of the onset of puberty (Kennedy, 1969; Frisch and McArthur, 1974). Classical studies suggested that a minimum amount of body fat is required to attain sexual maturation (Kennedy, 1969; Frisch and McArthur, 1974; Frisch, 1985). When prepubertal animals, including primates, are exposed to energy deprivation the onset of puberty is delayed or even blocked, until a favorable energy balance is achieved (Kennedy and Mitra, 1963; Kennedy, 1969; Foster and Olster, 1985). In adults, severe energy deficits are a frequent cause of hypothalamic amenorrhea (Warren et al., 1999; Welt et al., 2004; Ribeiro et al., 2007). Recent studies not only confirm the importance of the adiposity in influencing the onset of puberty, but also suggest that excess of body fat in children cause early onset of puberty (Biro et al., 2010). Epidemiological data have indicated a high prevalence of obesity among US children and adolescents (Flegal et al., 2010). Concomitantly, a higher proportion of girls have shown signs of pubertal development at earlier ages (Biro et al., 2010).

An important challenge is to understand how metabolic cues control the reproductive system. A breakthrough in the field occurred after the discovery of the gene that encodes the adipocyte-derived hormone leptin (Zhang et al., 1994). Soon after that, leptin became the missing piece that would complete the lipostatic theory, proposed several decades earlier (Kennedy, 1953). The lipostatic theory suggests that changes in fat deposition trigger a feedback system aiming to restore the balance between food intake and energy expenditure. Leptin levels reflect the body fat content in rodents and humans (Frederich et al., 1995a; Maffei et al., 1995; Considine et al., 1996). Overfeeding increases leptin levels, whereas food deprivation causes a strong decrease in the circulating concentration of leptin (Frederich et al., 1995b; Ahima et al., 1996; Considine et al., 1996). It has been proposed that the body interprets a high concentration of leptin as a signal of energy abundance, whereas falling levels of leptin signals starvation. Therefore, low leptin levels increase hunger and decrease energy expenditure, partly because energy-demanding physiological functions are suppressed in leptin-deficient states, presumably as a way to save energy and prolong survival (Ahima et al., 1996; Friedman and Halaas, 1998; Rosenbaum and Leibel, 1998). Reproduction is one of the physiological functions strongly affected by leptin (Barash et al., 1996; Ahima et al., 2000). Because lack of leptin is interpreted as a signal of starvation, leptin-deficient individuals become hyperphagic, massively obese and infertile, and exhibit a series of metabolic dysfunctions (Zhang et al., 1994; Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Montague et al., 1997; Strobel et al., 1998). Leptin treatment rescues the alterations in body weight, metabolism, and the reproductive system (Ahima et al., 1996; Barash et al., 1996; Chehab et al., 1996; Mounzih et al., 1997; Farooqi et al., 1999). In addition, exogenous leptin administration causes early onset of puberty in mice (Ahima et al., 1997; Chehab et al., 1997), and a similar mechanism could account for the trend observed in overweight children (Biro et al., 2010).

After the discovery of leptin, many studies focused on deciphering the mechanism by which leptin regulates the reproductive system. Although leptin receptors (LepR) are expressed in many organs, including pituitary gland and gonads (Zamorano et al., 1997), it is now clear that the main target of leptin is the brain (Cohen et al., 2001; de Luca et al., 2005). The expression of LepR is found in innumerous brain nuclei, particularly in specific populations of hypothalamic neurons (Schwartz et al., 1996; Elmquist et al., 1998; Scott et al., 2009). However, defining the key neuronal population that mediates the effects of leptin on reproduction has proven to be a challenging task (Hill et al., 2008; Castellano et al., 2010; Donato et al., 2011a; Louis et al., 2011). Recent findings suggest that the ventral premammillary nucleus (PMV)

is the long sought area in which leptin modulates the activity of the reproductive system (Donato et al., 2009, 2011b; Leshan et al., 2009; Louis et al., 2011). In this review, we will summarize the existing literature about the potential role played by PMV neurons in the regulation of the hypothalamus–pituitary–gonad (HPG) axis.

## THE NEUROCHEMICAL PROFILE OF PMV NEURONS HIGHLIGHTS A KEY INTEGRATIVE FUNCTION

Ventral premammillary nucleus neurons exhibit a broad expression of receptors for hormones related to the regulation of the energy balance (**Table 1**). PMV neurons express LepR (Elmquist et al., 1998; Scott et al., 2009), the ghrelin receptor, in mice but not in rats (Zigman et al., 2006) and the insulin receptor (**Figure 1A**). Innumerous receptors for neurotransmitters involved with the regulation of energy balance are also found in the PMV, including the cannabinoid receptor 1 (**Figure 1B**), the melanocortin-4 receptor in mice (Liu et al., 2003) but not in rats (Kishi et al., 2003), the orexin receptor 1 and 2 (Marcus et al., 2001), the neuropeptide Y Y1 receptor (higher expression in mice than rats; Kishi et al., 2005), and the vasopressin receptor in hamsters (sexually dimorphic and dependent upon photoperiod length; Dubois-Dauphin et al., 1991).

Ventral premammillary nucleus neurons are potential targets of sex hormones (**Table 1**), as they express a dense amount of androgen receptor (AR) and a moderate to low amount of estrogen receptor α and β (ERα and ERβ) and progesterone receptor (Simerly et al., 1990; Yokosuka and Hayashi, 1996; Merchenthaler et al., 2004; Intlekofer and Petersen, 2011). The relatively high ratio of AR to ER and the lack of definitive data demonstrating the expression of aromatase in the PMV indicate a potential role for androgens in PMV neuronal biology (Yokosuka and Hayashi, 1996; Wu et al., 2009). Despite the strong presence of sexual steroid receptors in the PMV, it has not been determined whether changing levels of sexual hormones affect the neuronal activity or gene expression in this area.

In addition to sex steroid receptors, several neurotransmitters are expressed by PMV neurons (**Table 1**), including glutamate (Ziegler et al., 2002). The expression of glutamatergic markers, such as vesicular glutamate transporter 2 (vGluT2), can be found in the entire extension of the PMV and show a 94% colocalization with neurons that express LepR (Donato et al., 2011b). On the other hand, there is virtually no expression of GABAergic markers in the PMV, such as glutamic acid decarboxylase 67 (GAD67) or vesicular GABA transporter (vGaT; Donato et al., 2010a; Vong et al., 2011). Thus, the projections originated from PMV neurons are thought to be excitatory due to their glutamatergic component.

Peptidergic neurotransmitters are also expressed by PMV neurons (**Table 1**), including cocaine and amphetamine regulated transcript (CART), substance P (a tachykinin family member) and

**Table 1 | Neurochemical characteristics of the ventral premammillary nucleus (PMV).**

| | Expression level | Studied species | Sexual dimorphism or specie-related differences in the expression level |
|---|---|---|---|
| **NEUROTRANSMITTERS/HORMONES** | | | |
| Glutamate | +++ | M, R | |
| Nitric oxide | +++ | M, R, S | |
| CART | ++ | M, R, S | Higher expression in rats and in sheep, compared to mice |
| Substance P | ++ | R | Sexually dimorphic (higher in male) |
| Enkephalin | ++ | R, Hu | |
| Dopamine | – | M, R, B | Described only in birds. Absent in rodents |
| Melatonin | – | B | Described only in birds |
| **RECEPTORS** | | | |
| Leptin receptor | +++ | M, R | |
| Insulin receptor | ++ | M | |
| Ghrelin receptor | ++ | M, R | In mice but not in rats |
| Androgen receptor | +++ | M, R | |
| Estrogen receptor α | ++ | M, R | |
| Estrogen receptor β | + | M | |
| Progesterone receptor | + | R | |
| Cannabinoid receptor 1 | +++ | M | |
| Melanocortin receptor 4 | ++ | M, R | In mice but not in rats |
| Neuropeptide Y Y1 receptor | +++ | M, R | Higher expression in mice than rats |
| Orexin receptor 1 | ++ | R | |
| Orexin receptor 2 | +++ | R | |
| Vasopressin receptor (binding) | +++ | Ha | Sexually dimorphic and dependent upon photoperiod length |
| Melatonin receptor (binding) | +++ | S | Described only in sheep |
| Melanopsin (photopigment) | – | B | Described only in birds |

*Expression level: +++, high; ++, moderate; +, low; –, not described.*
*Species: B, birds; Ha, hamsters, Hu, humans; M, mouse; R, rat; S, sheep.*

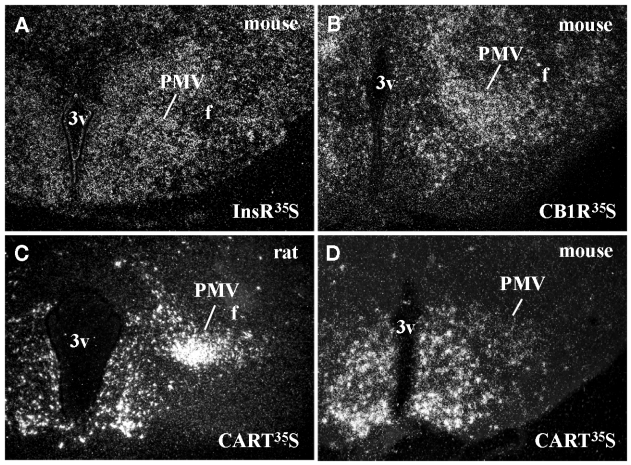


**FIGURE 1 | Neurochemical characteristics of the ventral premammillary nucleus (PMV). (A,B)** Darkfield photomicrographs demonstrating the distribution of insulin receptor (InsR) mRNA **(A)** and cannabinoid receptor type 1 (CB1R) mRNA **(B)** in the mouse PMV. **(C,D)** Darkfield photomicrographs demonstrating the distribution of cocaine and amphetamine regulated transcript (CART) mRNA in the rat **(C)** and in the mouse **(D)** PMV. Note the abundance of CART mRNA in the PMV of rats compared to mice.

enkephalin (Wamsley et al., 1980; Shimada et al., 1987; Larsen, 1992; Douglass et al., 1995; Sukhov et al., 1995). However, sexually dimorphic or species-related differences exist. While the PMV of rats densely expresses CART mRNA and peptide (Douglass et al., 1995; Koylu et al., 1997), the PMV of mice exhibits low CART expression (**Figures 1C–D**). In addition, the number of neurons that express tachykinin peptides in the PMV is higher in male than in female rats (Akesson, 1993). PMV neurons also densely express neuronal nitric oxide synthase (nNOS), which catalyzes the synthesis of nitric oxide (NO; Vincent and Kimura, 1992).

## PMV NEURONS ARE NEUROCHEMICALLY AND NEUROANATOMICALLY WELL POSITIONED TO REGULATE REPRODUCTION

Many of the neurotransmitters expressed in the PMV are involved in the neuroendocrine regulation of reproduction. For example, glutamatergic inputs were shown to induce gonadotropin-releasing hormone (GnRH) release and subsequent activation of HPG axis (Brann and Mahesh, 1994; Gargiulo and Donoso, 1995; Dhandapani and Brann, 2000; Mahesh and Brann, 2005). Moreover, glutamate facilitates the expression of sexual behaviors (Gargiulo and Donoso, 1995) and has regulatory effects on the timing of puberty (Zamorano et al., 1998; Terasawa and Fernandez, 2001; Ojeda et al., 2006). Of note, intracerebroventricular administration of glutamate receptor agonists, such as NMDA, elicits secretion of GnRH and luteinizing hormone (LH), even in *Kiss1* and *Kiss1r* knockout mice, suggesting a role for glutamatergic neurotransmission outside the Kiss1 neuronal system (d'Anglemont de Tassigny et al., 2010). CART peptide was shown to mediate the stimulatory effects of leptin on GnRH secretion *in vitro* and *in vivo* (Lebrethon et al., 2000, 2007; Parent et al., 2000). In addition, NO has been implicated in the regulation of sexual behaviors and HPG axis (Moretto et al., 1993; Rettori et al., 1993; Mani et al., 1994; Benelli et al., 1995; Nelson et al., 1995). A complete disruption of *Nos1* gene results in hypogonadism and infertility (Gyurko et al., 2002). Furthermore, several studies found that NO is a key neurotransmitter that mediates leptin-induced GnRH/LH secretion (Yu et al., 1997; McCann et al., 1999; Watanobe and Schioth, 2001; Reynoso et al., 2007). Recently, we reported that 73% of leptin responsive cells in the PMV express NO-synthesizing enzymes (Donato et al., 2010b). Leptin does not affect the expression of *Nos1* mRNA in the PMV, but low leptin levels, as in fasting or in *ob/ob* mice, cause a reduction in the number of PMV neurons expressing the phosphorylated form of $nNOS^{S1412}$ (pnNOS). The phosphorylation of nNOS at Ser1412 increases nNOS enzymatic activity (Parkash et al., 2010) and acute injection of leptin restores the number of pnNOS neurons in the PMV of fasted mice (Donato et al., 2010b).

The projections of PMV neurons were first described in rats using the neurotracer *Phaseolus vulgaris* leucoagglutinin (Canteras et al., 1992b). It was demonstrated that PMV neurons project mainly to the periventricular zone of the hypothalamus, which is composed of nuclei involved in the regulation of anterior pituitary function. PMV neurons also project to major nuclei of the sexually dimorphic circuitry, including the ventrolateral part of the ventromedial nucleus of hypothalamus (VMH), medial preoptic nucleus, bed nuclei of the stria terminalis (BST), ventral lateral septal nucleus, posterodorsal part of the medial nucleus of the amygdala (MeA), and posterior nucleus of the amygdala (Canteras et al., 1992b). It is interesting that the major neuronal inputs to the PMV originate from neurons located in the sexually dimorphic circuitry, highlighting the intense intercommunication between this circuitry and the PMV (Simerly and Swanson, 1988; Canteras et al., 1992a,b, 1994, 1995; Coolen and Wood, 1998). For example, PMV is densely innervated by neurons located in the MeA, including cells that express urocortin 3 (Canteras et al., 1995; Coolen and Wood, 1998; Cavalcante et al., 2006b).

More recent studies in mice and in rats using genetic tools in combination with tracing techniques highlighted a putative role of the PMV in the regulation of the HPG axis. It was shown that PMV neurons project directly to GnRH perikarya in the medial preoptic area (MPA; Rondini et al., 2004; Boehm et al., 2005; Leshan et al., 2009) and to GnRH fibers in the median eminence (Donato et al., 2011b). Interestingly, among all neurons that express LepR, only those in the PMV and a subpopulation of neurons in the MPA seem to project directly to GnRH neurons (Louis et al., 2011). In addition, PMV neurons project to the anteroventral periventricular nucleus (AVPV; Canteras et al., 1992b; Rondini et al., 2004; Hahn and Coen, 2006), a key site for female reproductive function (Wiegand and Terasawa, 1982; Gottsch et al., 2004; Herbison, 2008). The AVPV contains a subpopulation of kisspeptin neurons, which is critical for the preovulatory LH surge (Smith et al., 2006; Herbison, 2008; Cravo et al., 2011). We have recently found that fibers from PMV neurons make apparent synaptic contact with kisspeptin neurons in the AVPV (Donato et al., 2011b). The arcuate nucleus (ARH) also receives a dense projection from PMV neurons (Canteras et al., 1992b), but whether kisspeptin neurons in the ARH or a specific population of ARH neurons is selectively targeted

by PMV inputs is still unknown. Overall, PMV neurons potentially regulate the reproductive system directly through inputs to GnRH neurons and also to upstream neuronal populations, such as kisspeptin cells.

## PMV NEURONS ARE RESPONSIVE TO CONSPECIFIC BEHAVIORS AND SOCIALLY RELEVANT CUES

Previous studies using electrolytic lesions described a potential role for PMV neurons in odor-induced LH secretion in rats (Beltramino and Taleisnik, 1985). Olfaction is a critical sense used by rodents to discriminate socially relevant cues and to trigger social behaviors, including sexual behaviors (Romero et al., 1990; Halpern and Martinez-Marcos, 2003; Yoon et al., 2005; Brennan and Zufall, 2006). In response to conspecific odors, males and females of different species exhibit increased circulating levels of gonadotropins and sex steroids (Maruniak and Bronson, 1976; Kamel et al., 1977; Beltramino and Taleisnik, 1983; Coquelin et al., 1984). Rats and mice exposed to conspecific odors show a large number of neurons expressing Fos immunoreactivity (Fos-ir) in the PMV, which suggests that the PMV is involved in the neuronal circuitry that conveys olfactory information (Yokosuka et al., 1999; Cavalcante et al., 2006a; Leshan et al., 2009; Donato et al., 2010a). Moreover, roughly 50% of PMV neurons activated by opposite-sex odor express CART and, in male rats, CART mRNA increases after exposure to female odors (Cavalcante et al., 2006a). Most of the CART neurons in the PMV express the enzymes that synthesize NO. Besides, a parcel of nitrergic neurons is stimulated by female odors and virtually all nitrergic cells in the PMV express ARs (Yokosuka and Hayashi, 1996). Altogether, these studies indicate that PMV is apt to integrate information about circulating levels of sexual hormones and socially relevant cues (through brain areas related to pheromonal processing, such as MeA or BST) and generate appropriate neuroendocrine responses to modulate socially relevant behaviors.

The PMV may also be involved in the expression of conspecific behaviors because PMV neurons of male rats are also responsive to conspecific male odors (Donato et al., 2010a). In addition, PMV neurons express Fos-ir after mating or agonistic behavior (Kollack-Walker and Newman, 1995; Coolen et al., 1996; Pfaus and Heeb, 1997). Previous studies showed that lesions of the premammillary area increases aggression between males of the same species (Van Den Berg et al., 1983). However, these studies should be interpreted with caution due to the extension of the lesion. Restricted and/or selective lesions are required to determine the real contribution of the PMV in aggressive behaviors.

An interesting question is whether nutritional conditions may alter the responsiveness of an individual to environmental cues. PMV neurons are the target of metabolic cues and also respond to socially relevant sensory stimulation. Of note, 44% of the LepR-expressing cells in the PMV of male mice are activated by female odors, whereas in female mice, 18% of LepR cells are activated by male odors (Leshan et al., 2009). These findings suggest that food availability or energy stored affect neuronal responses to odors. However, we observed that fasting caused no changes in female odor-induced Fos-ir in the PMV and in the MeA of male rats compared to normally fed controls (Donato et al., 2010a). Although this finding may suggest a dissociation of neuronal responses to different stimuli, it is important to mention that induction of Fos protein may not be the definitive indicator of changes in neuronal activity or responsiveness. Further studies will be necessary to determine the influence of the nutritional state on the response to environmental stimulation.

## PREMAMMILLARY HYPOTHALAMIC AREA MEDIATES SEASONAL REPRODUCTION IN EWES AND BIRDS

Although most of the studies about the PMV have used rats and mice as experimental models, there are several pieces of evidence that the premammillary hypothalamic area (PMH) also plays a key role in reproductive function of seasonal breeders (i.e., sheep and birds). Seasonal reproduction is a strategy used by several species to increase survival of offspring by reproducing during a period of the year when the environment offers favorable conditions. In sheep, the major environmental cue controlling reproduction is the photoperiod or day length (Duan et al., 2007). Changes in day light exposure alter the synthesis and secretion of the pineal gland hormone melatonin, which in turn binds to hypothalamic nuclei and modulates the pulsatile secretion of GnRH (Emilsson et al., 1999; Hazlerigg and Wagner, 2006; Goodman et al., 2010). The PMH of ewes is composed of the caudal ARH, the PMV and the ventral tuberomammillary nucleus. Similarly to rats, PMV neurons in ewes express CART and nNOS (Sliwowska et al., 2004). In addition, PMH of ewes is a melatonin binding site. Bilateral microimplantation of melatonin into the PMV of ewes stimulates LH secretion (Malpaux et al., 1998), indicating that in sheep, the PMV appears to play a key role in seasonal reproduction.

The reproductive cycle of a variety of avian species is regulated by circadian mechanisms driven by intrinsic oscillators (Petersen et al., 1996; Wikelski et al., 2008; Goodman et al., 2010). These mechanisms are modulated by light-sensitive neuronal populations located in the caudal hypothalamus, in a site identified as the PMH (Kang et al., 2007). In birds, a subpopulation of PMH neurons expresses dopamine, a neurotransmitter known to affect the secretion of several reproductive hormones, including LH, FSH, and prolactin. Using a complex paradigm of light-induced GnRH neuronal activation, studies identified in turkeys a photosensitive subpopulation of dopaminergic PMH neurons likely involved in GnRH secretion (Thayananuphat et al., 2007). Dopaminergic neurons (immunoreactive to tyrosine hydroxylase) in the PMH of turkeys coexpress melatonin and its synthesizing enzymes (Kang et al., 2007). Dopamine–melatonin neurons in the PMH exhibit high activity at the photosensitive phase, which was associated with higher dopaminergic neurotransmission and GnRH activation. Additionally, these neurons express the photoreceptive molecule melanopsin, which is involved in extra-retinal photoreception in birds and non-mammalian vertebrates. In hens, the expression of melanopsin mRNA in the PMH is downregulated by light in a series of models and shows a diurnal regulation; it is high during the night and low during the day (Kang et al., 2010). The PMH of turkeys also presents a distinct circadian expression of clock genes compared to the pineal gland and the brain master clock, the suprachiasmatic nucleus. In particular, *Cry1* and *Per3* seem to mediate the photic responses associated with the control of the reproductive system (Leclerc et al., 2010).

## PMV NEURONS INTEGRATE METABOLIC CUES TO REGULATE REPRODUCTION RATHER THAN ENERGY BALANCE

The high expression of receptors of hormones related with the regulation of the energy balance might imply that the PMV is involved in the control of energy balance. However, bilateral excitotoxic lesions of the PMV did not affect body weight, mean food intake and circulating leptin levels in adult female rats (Donato et al., 2009). Nonetheless, PMV-lesioned rats exhibit an attenuated reduction in food intake between the proestrus and the estrus day (Donato et al., 2009). Female rats normally show a decreased food intake in the behavioral estrus that is linked with the high estrogen levels observed during the proestrus day (Drewett, 1973; Geary et al., 2001; Asarian and Geary, 2006). Therefore, the regulation of food intake across the estrous cycle by PMV neurons can be an indirect consequence of changes in sexual hormone levels after lesions of the PMV.

Leptin exerts a pivotal role in the long-term regulation of energy balance (Schwartz, 2006; Gautron and Elmquist, 2011). As mentioned, leptin administration to leptin-deficient mice (*ob/ob*) rescues all the metabolic and neuroendocrine deficits observed in these mice (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Chehab et al., 1996). Following the same paradigm, we generated *ob/ob* mice with bilateral lesions of the PMV (Donato et al., 2011b). Upon leptin treatment, these mice showed drastic reduction in food intake and body weight, indicating that leptin may restore the metabolic deficits of *ob/ob* female mice in the absence of PMV neurons (Donato et al., 2011b). To further investigate the role played by LepR in the PMV, we generated a LepR-null mouse model in which LepR is expressed selectively in PMV neurons. We found that endogenous expression of LepR only in the PMV did not affect food intake, body weight, and fat mass in male and female mice (Donato et al., 2011b). In agreement with this, a recent study found that after genetic ablation of LepR expression from all glutamatergic (vGluT2-positive) neurons, which includes PMV cells, only minor changes in body weight, food intake, and fat mass were observed in male and female mice (Vong et al., 2011). Overall, these results suggest that despite the presence of innumerous receptors involved with the regulation of energy balance, PMV neurons are not key players in the modulation of food intake and body weight. Rather, PMV neurons may function as a key integrative site conveying metabolic cues to the reproductive system. Accordingly, PMV lesions cause a temporary anestrus in rats (persistent leukocytes in the vaginal smears). However, after a few weeks PMV-lesioned rats recover their cyclicity, although vaginal cytology continues to exhibit an atypical mixed cell profile (Donato et al., 2009). Several weeks after lesions of the PMV, rats still show reduced concentration of LH and estradiol and decreased activation of AVPV and GnRH neurons at the time of the preovulatory LH surge but no changes in Kiss1 mRNA expression (Donato et al., 2009). Possibly secondary to decreased gonadotropin levels, the ovaries of PMV-lesioned rats display a lower number of antral follicles and a trend toward a reduction in the number of corpora lutea (Donato et al., 2009). These results indicate that the PMV is required for the normal activity of the HPG axis in female rats.

Following the same line, we hypothesized that the PMV would be apt to mediate the effects of leptin on the reproductive neuroendocrine axis. To test this model, we used a well-established paradigm in which leptin treatment can restore or increase LH levels in fasted rodents (Ahima et al., 1996; Nagatani et al., 1998; Gonzalez et al., 1999; Watanobe et al., 1999; Chan et al., 2003). Lesions of the PMV blocked the stimulatory effect of leptin on LH secretion in fasted rats (Donato et al., 2009). In order to investigate putative signaling pathways that mediate the acute effects of leptin on PMV neurons, patch-clamp recordings of hypothalamic slices were performed. Leptin caused a rapid depolarization of ∼75% of LepR-expressing neurons in the PMV through a putative TRPC channel (Leshan et al., 2009; Williams et al., 2011). The other 25% recorded LepR cells were hyperpolarized in response to leptin, and this response required the activation of a putative Katp channel. Importantly, pharmacological or genetic disruption of the phosphoinositide 3-kinase (PI3K) pathway prevented the leptin-induced changes in the activity of PMV LepR neurons (Williams et al., 2011). These results indicate that PI3K is required for the acute changes in biophysical properties of PMV neurons induced by leptin. Whether these changes in cellular activity underlie the physiological effects of leptin are under investigation.

We further assessed whether leptin signaling in PMV neurons is critical to induce the onset of puberty and restore fertility in leptin- or LepR-deficient mouse models. Lesions of the PMV in female *ob/ob* mice reduced the capacity of exogenous leptin to induce sexual maturation. Besides, acute injection of leptin did not increase LH and progesterone levels in PMV-lesioned *ob/ob* mice, as observed in PMV-intact *ob/ob* mice (Donato et al., 2011b). In addition, female LepR-null mice with endogenous re-expression of LepR in PMV neurons showed unambiguous signs of sexual maturation, such as vaginal opening, increased uterus weight and size, and ovaries with corpora lutea. After a period of 6 weeks of breeding tests, 50% of mice with selective reactivation of LepR in PMV neurons became pregnant, despite their obese and diabetic phenotype (Donato et al., 2011b). Notably, the improvement of the infertile phenotype of the LepR-null mice following PMV LepR reactivation was only observed in females, not in males. Additional studies will be necessary to tackle this sex-related difference. As previously mentioned, neurotransmitters found in the PMV, such as glutamate and NO, were shown to stimulate the release of GnRH. Moreover, earlier studies suggested that the lack of leptin signaling causes a deficient release of GnRH because *ob/ob* and *db/db* mice have high content of GnRH in the median eminence/medial basal hypothalamus (ME/MBH; Johnson and Sidman, 1979; Batt et al., 1982). Re-expression of LepR only in the PMV normalized the ME/MBH GnRH content in female LepR-null mice. Together, these findings have determined the PMV as a key site linking leptin action and the female reproductive physiology.

## CONCLUDING REMARKS

Overall, this review highlights a series of recent data demonstrating that PMV neurons are apt to mediate the effects of leptin on GnRH secretion. The stimulatory effect of PMV neurons on GnRH release is possibly mediated by the coordinated effects of

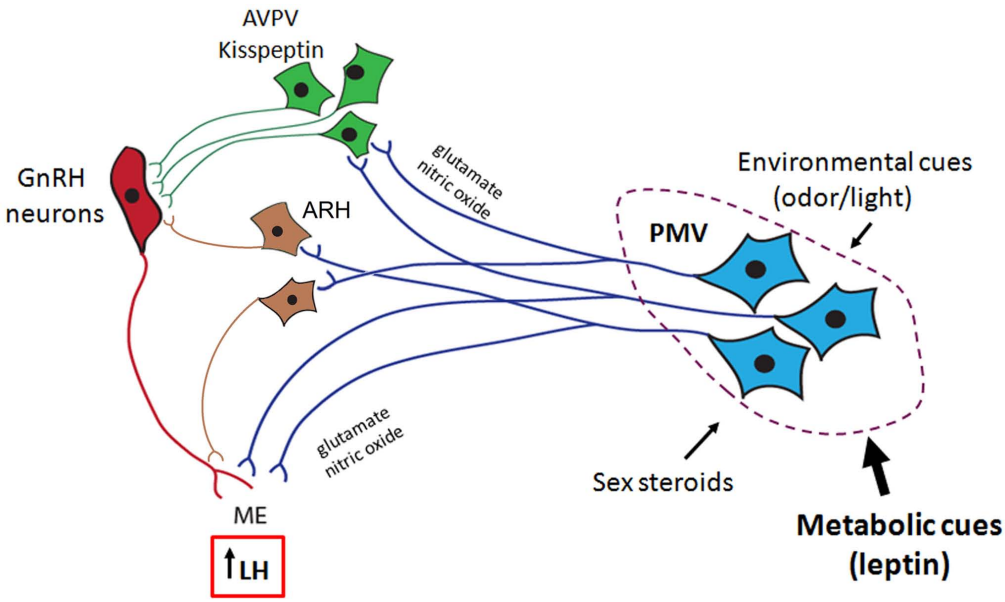


**FIGURE 2 | Proposed role for the ventral premammillary nucleus (PMV) in the female reproductive physiology.** The PMV integrates environmental cues (odors in rodents and daylight in seasonal breeders) and signals from the internal milieu related to the reproductive status (sex steroids) and energy store (leptin and insulin). PMV neurons express excitatory neurotransmitters (e.g., glutamate and nitric oxide) and directly project to the anteroventral periventricular nucleus (AVPV), to the arcuate nucleus (ARH), and to gonadotropin-releasing hormone (GnRH) neurons. Once stimulated, PMV neurons activate the target sites inducing GnRH release and LH secretion from the pituitary gland.

glutamate and NO on GnRH terminals in the median eminence (**Figure 2**). Although it is very likely that other neuronal populations also convey metabolic cues to modulate the HPG axis, the existing evidence suggests that the PMV is a key site relaying the effects of leptin on the reproductive neuroendocrine axis. We postulate that through PMV neurons, leptin modulates the influence of adiposity on the timing of puberty and the coordinated secretion of LH during conditions of negative energy balance. The data presented in this review provide the physiological and neuroanatomical basis underlying the effects of leptin on the HPG axis.

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# PI3K: an attractive candidate for the central integration of metabolism and reproduction

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In neurons, as in a variety of other cell types, the enzyme phosphatidylinositol-3-kinase (PI3K) is a key intermediate that is common to the signaling pathways of a number of peripheral metabolic cues, including insulin and leptin, which are well known to regulate both metabolic and reproductive functions. This review article will explore the possibility that PI3K is a key integrator of metabolic and neural signals regulating gonadotropin releasing hormone (GnRH)/luteinizing hormone (LH) release and explore the hypothesis that this enzyme is pivotal in many disorders where gonadotropin release is at risk. Although the mechanisms mediating the influence of metabolism and nutrition on fertility are currently unclear, the strong association between metabolic disorders and infertility is undeniable. For example, women suffering from anorectic disorders experience amenorrhea as a consequence of malnutrition-induced impairment of LH release, and at the other extreme, obesity is also commonly co-morbid with menstrual dysfunction and infertility. Impaired hypothalamic insulin and leptin receptor signaling is thought to be at the core of reproductive disorders associated with metabolic dysfunction. While low levels of leptin and insulin characterize states of negative energy balance, prolonged nutrient excess is associated with insulin and leptin resistance. Metabolic models known to alter GnRH/LH release such as diabetes, diet-induced obesity, and caloric restriction are also accompanied by impairment of PI3K signaling in insulin and leptin sensitive tissues including the hypothalamus. However, a clear link between this signaling pathway and the control of GnRH release by peripheral metabolic cues has not been established. Investigating the role of the signaling pathways shared by metabolic cues that are critical for a normal reproductive state can help identify possible targets in the treatment of metabolic and reproductive disorders such as polycystic ovarian syndrome.

**Keywords: PI3K, LH, GnRH, obesity, insulin, leptin, puberty, metabolism**

## INTRODUCTION

The achievement of reproductive competence as well as the normal sustenance of adult reproductive activity is highly affected by the availability of nutrients. This is particularly evident in female mammals, where physiological processes such as gestation, parturition, lactation, and rearing of the young are energetically demanding. Thus, the hypothalamic pituitary gonadal (HPG) axis limits fertility when nutrient availability is inadequate to support these events. Many studies suggest that negative energy balance inhibits reproduction through the alteration of gonadotropin releasing hormone (GnRH) secretion in the hypothalamus. GnRH neurons are often described as the "master regulators" of the HPG axis meaning that successful reproduction and thus the survival of the species in mammals depends on the ability of these neurons to sense changes in the internal and external environment, including changes in energy status. The intermittent discharge from these specialized neurons in the hypothalamus is in fact responsible for the pulsatile secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These in turn act on the gonads to direct gametogenesis and secretion of steroid hormones. Within the brain, the actions of gonadal steroids are then manifested as both feedback regulation of GnRH secretion and the facilitation of sexual behavior.

The mechanisms by which metabolic information is relayed to the hypothalamic GnRH network are not entirely clear. However, various peripheral metabolic signals, such as leptin and insulin, act on specific neuronal populations that in turn affect GnRH neuronal function. This is the case for multiple hypothalamic neuropeptide systems such as neuropeptide Y (NPY), products of pro-opiomelanocortin (POMC), and galanin-like peptide (GALP), among many others (Crown et al., 2007). When these neuronal populations are not able to appropriately respond to metabolic cues, for example in states of malnutrition, obesity, and diabetes, the repercussions are not limited to imbalanced metabolic homeostasis, but can include reproductive dysfunction. Therefore, metabolic hormones have a pivotal role in the central regulation of the HPG axis. Much less is known, however, about the intracellular signaling pathways that integrate the actions of metabolic signals to regulate GnRH/LH release and hence reproductive function. The variety and complexity of signaling networks that affect cell metabolism and neuronal activity make this a daunting task. However, peripheral hormones and metabolic cues such as

leptin and insulin share specific signal transduction pathways that in many cases act synergistically or in parallel to affect neuronal function. Therefore, molecules that serve as central integrators of metabolism and reproductive function might be identified by determining which signaling pathways are shared by peripheral metabolic cues known to affect both physiological functions.

The PI3K signaling cascade is well known for its role in mediating insulin and leptin effects on cell and tissue metabolism. This lipid enzyme is activated in classical metabolic tissues such as liver, pancreas, skeletal muscle, and adipose tissue, where it participates in the regulation of carbohydrate and lipid homeostasis (Foukas and Withers, 2010). In addition, central activation of PI3K plays a key role in the effect of leptin and insulin on food intake and glucose homeostasis (Morton et al., 2005; Xu et al., 2005). The critical role that PI3K plays in regulation of metabolism does not exclude the possibility that it also participates in the control of other physiological processes such as reproduction. In fact, the PI3K signaling cascade is a common pathway activated by hormones and growth factors that are also critical for the normal function of the HPG axis, including insulin and leptin. It remains to be determined whether this signaling pathway integrates the effects of these or other hormones and growth factors in the regulation of GnRH release. Furthermore, the brain sites and neuronal populations in which activation of this enzyme is necessary for the regulation of energy homeostasis might not be the same as those involved in the regulation of the HPG axis.

This article reviews the studies supporting the idea that hypothalamic control of GnRH/LH release by peripheral metabolic cues is indispensable for the normal function of the HPG axis. Then the hypothesis that PI3K is a key integrator of metabolic and neural signals regulating GnRH neurosecretion will be explored.

## DYSREGULATION OF GnRH/LH RELEASE BY METABOLIC IMBALANCE

### NEGATIVE ENERGY BALANCE

Examples of the effects that a dramatic decrease in caloric intake has on the reproductive axis can be found in patients suffering from eating disorders such as anorexia nervosa (AN) and in female athletes undergoing extreme exercise regimens (Poyastro Pinheiro et al., 2007; Attia and Roberto, 2009). In these women, hypothalamic amenorrhea and menstrual irregularities are associated with impairments in gonadotropins, specifically LH release (Attia and Roberto, 2009). These effects are likely due to decreased amplitude and/or frequency of hypothalamic pulses of GnRH.

To better understand the mechanisms by which negative energy balance disrupts reproductive function, a number of *in vivo* animal models have been developed. These include streptozotocin (STZ)-induced type 1 diabetes (Kovacs et al., 2002, 2003), extreme exercise (Manning and Bronson, 1989), chemical inhibition of metabolic pathways such as glucose oxidation (Nagatani et al., 1996), lipoprivation (Shahab et al., 2006; Sajapitak et al., 2008), as well as chronic and acute food restriction in adult (Dyer et al., 1985; Cameron and Nosbisch, 1991), and peripubertal animals (Bronson, 1986, 1988). The latter model takes advantage of the phenomenon of "catch up" growth in which stopping growth at a particular body weight halts reproductive development and subsequent reinitiation of *ad libitum* feeding results in rapid growth and reproductive development (Bronson, 1986). These negative metabolic challenges can affect the HPG axis at various levels including decreased gonadotropin secretion, slow follicular development, and inhibited synthesis of gonadal steroids (Schneider, 2004). However, it is the inhibition of the GnRH pulse generator, and hence GnRH release, that is responsible for the dysfunction of the reproductive axis. For instance, infusing GnRH in a pulsatile manner can yield close to normal onset of puberty in food-restricted female rats, even in the total absence of body growth (Bronson, 1986). Likewise, pulsatile treatment with GnRH in food-deprived or food-restricted adult mammals also reinstates HPG normal function (Rojdmark, 1987; Foster et al., 1989; Cameron and Nosbisch, 1991). These studies confirm that positive energy balance is crucial for normal reproductive function, whether the female is an adult or peripubertal, and that proper activity of the GnRH network is essential for this to occur.

Conditions of energy deficit are associated with a drastic decrease in the levels of peripherally produced metabolic cues such as insulin and the adipocyte-derived cytokine leptin. The reduction in the levels of these signals contributes to the decrease in GnRH/LH activity produced by negative energy balance. For example, central infusion of leptin or insulin restores the reproductive deficits observed using *in vivo* models in which energy balance is severely compromised such as food restriction (Nagatani et al., 1998) or type 1 diabetes (Kovacs et al., 2002), respectively. In addition, leptin treatment improves mean LH pulse frequency and ovulation when administered to AN patients (Chan and Mantzoros, 2005). These studies emphasized the importance of leptin and insulin in the metabolic control of the HPG axis. However, a decrease in the levels of other peripherally produced signals such as insulin-like growth factor-1 (IGF-1), glucose, and lipids may also contribute to the suppression of GnRH activity produced by negative energy balance.

### CHRONIC EXCESS NUTRITION: OBESITY

Fewer studies have examined the effects of a state of excess nutrition on GnRH/LH release and its impact on the rest of the HPG axis. Diet-induced obesity (DIO) in rodents has been utilized to mimic the gradual increase in body fat and body weight observed in obese humans. Similar to humans, mice with DIO display fasting hyperglycemia, hyperinsulinemia, and hyperleptinemia accompanied by insulin and/or leptin resistance (Tortoriello et al., 2004; Ghanayem et al., 2010). In female mice, DIO causes infertility, the degree of which depends on the background strain, the duration, and the percentage of fat in the diet. For example, compared to other strains such as C57BL/6, DBA/2J female mice show high susceptibility to high fat diet (HFD)-induced infertility. In these mice, a diet containing 24% fat is sufficient to significantly decrease natural pregnancy rates and follicular activity (Tortoriello et al., 2004). Their fertility defect is likely of central origin, because normal ovulatory responses and pregnancy rates are restored after exogenous gonadotropin stimulation (Tortoriello et al., 2004). Additionally, prolonged hyperleptinemia and central leptin resistance are accompanied by reduced hypothalamic GnRH gene expression (Tortoriello et al., 2004). However, whether reduction in GnRH expression caused by DIO in DBA/2J mice results in diminished hypothalamic GnRH secretion is unknown. In contrast, studies

using different strains of mice (i.e., mixed CD1/129vJ/57BL6) have found that elevated LH levels might be the primary mediator of DIO-induced infertility (Brothers et al., 2010). In these mice, 60% fat content in the diet resulted in decreased fertility, lack of regular estrous cyclicity, and reduced follicular activity (Brothers et al., 2010). The elevated LH levels observed in this model of DIO suggest that obesity affects the ability of steroid hormones to exert negative feedback on GnRH secretion. Interestingly, although this diet resulted in peripheral insulin resistance, the pituitary remained responsive to insulin and exhibited a heightened pituitary response to GnRH due to an increase in GnRH receptors (Brothers et al., 2010). In addition, DIO on this genetic background resulted in elevated androgen levels, a characteristic of polycystic ovarian syndrome (PCOS). In this context, it is valid to point out that rodent models of PCOS, such as female rats exposed to prenatal androgens, in addition to developing characteristics of the metabolic syndrome also have elevated androgen and LH levels, suggesting that an acceleration of the GnRH pulse generator mediates infertility (Demissie et al., 2008; Foecking et al., 2008). Unfortunately, studies of DIO effects on male fertility are scarce. In general, male mice are very resistant to the detrimental effects DIO on fertility, even though it also results in similar degrees of obesity, insulin resistance, and hyperleptinemia. In order to observe reduced fertility in males, both a diet with a high fat content (60%) and a prolonged exposure to it are needed (Ghanayem et al., 2010).

In women, obesity is co-morbid with menstrual dysfunction, decreased fertility, and an increased risk of miscarriages (Pasquali et al., 2007). In men, it can result in reduced testosterone (T) levels and erectile dysfunction (Lebinger, 2007). In the latter, increased estradiol-17β ($E_2$) levels from peripheral adipose tissue excess can then lead to secondary hypogonadism through negative feedback on the HPG axis (Pitteloud et al., 2005; Kasturi et al., 2008). Obesity can also exacerbate female reproductive disorders such as PCOS (Lebinger, 2007; Nestler, 2008). PCOS is the most common cause of infertility in women; it is characterized by hyperandrogenemia and elevated LH (Lebinger, 2007; Nestler, 2008). As with states of negative energy balance, alterations in the levels of peripheral metabolic cues, such as hyperinsulinemia and hyperleptinemia, characterize these metabolic disorders and are thought to play a major role in the central dysregulation of GnRH/LH pulsatility that is observed in many cases. Specifically, a decreased sensitivity of the HPG axis to insulin and leptin actions due to insulin and leptin resistance is suspected to be a major contributing factor.

### ENERGY IMBALANCE AND PUBERTAL DEVELOPMENT

The effects of eating and metabolic disorders on the reproductive axis are not limited to adults. Nutritional disturbances during early developmental stages can have adverse consequences on the reproductive axis as well. For instance, malnutrition and sickness in children can cause delayed puberty (Euling et al., 2008). On the other hand, the prevalence of obesity and other metabolic diseases in children and adolescents has increased at alarming rates in recent decades. Several studies suggest that this too represents a metabolic challenge for normal reproductive development. In girls, obesity has been associated with precocious puberty (Buyken et al., 2009), whereas the opposite has been found in boys, with obesity increasing the probability of delayed puberty (Lee et al., 2010). At the neuroendocrine level, puberty starts when GnRH neurons secrete GnRH in a pulsatile manner. Although there are no clear data linking obesity with premature activation of gonadotropin secretion in girls, an increase in adiposity, insulin, and leptin levels during childhood and adolescence can alter the secretion of other steroid hormones such as androgens from ovarian and/or adrenal origin (reviewed in Burt Solorzano and McCartney, 2010). Androgens can in turn alter the feedback mechanisms that normally restrain GnRH/LH secretion. This is one of the reasons childhood obesity has been linked to adult PCOS (Brewer et al., 2010; Burt Solorzano and McCartney, 2010).

As interest in the effects of obesity on child development has increased, so have studies investigating the effects of diet on puberty. In female rodents, states of negative energy balance delay puberty, whereas a HFD has the opposite effect, advancing pubertal onset. Female rats and mice receiving a HFD immediately after weaning showed advanced timing of vaginal opening (VO) when compared to littermates exposed to a lean diet (Boukouvalas et al., 2008; Brill and Moenter, 2009). In mice, the effect of a HFD on VO is associated with increased insulin levels (Brill and Moenter, 2009). Furthermore, treatment with the insulin sensitizer metformin blocked advancement of VO induced by HFD (Brill and Moenter, 2009). In the same study, treatment with the androgen receptor antagonist flutamide blocked pubertal acceleration while delaying VO in the lean diet fed control group, suggesting that androgens are involved in the pubertal process. It is important to point out that VO is an external marker of puberty onset that reflects exposure to pubertal sex steroid levels, but it does not necessarily imply central activation of gonadotropin secretion. Other parameters, such as day of first estrus and measurement of gonadotropin levels, are needed to further characterize these models. Nevertheless, it seems that an imbalance in insulin levels, which is known to stimulate androgen synthesis, contributes to the "precocious" pubertal onset induced by a HFD. These results also support a model where obesity alters the hypothalamic sensitivity to the restraining effects of steroid negative feedback on GnRH neuronal activity early in development.

These findings indicate that alterations in the activity of the GnRH network underlie many of the detrimental effects that metabolic imbalances have on the HPG axis. These metabolic challenges are also accompanied by profound alterations in the levels and sensitivity to peripheral hormones and metabolic signals, including IGF-1, $E_2$, leptin, and insulin. These hormones have in common the capacity to activate the PI3K pathway in various reproductive tissues including the hypothalamus.

## THE PI3K SIGNALING PATHWAY

Based on their sequence homology, similarities in function, and substrate specificity, PI3Ks are grouped into three principal categories: Classes I, II, and III (Vanhaesebroeck et al., 2005). Class I PI3Ks exist as heterodimers composed of a regulatory/adapter subunit tightly associated with a catalytic subunit. Class I is further divided into Class IA and Class IB. Three isoforms of the Class IA catalytic subunits p110 have been described, namely p110α, p110β, and p110δ, whereas three mammalian genes encode the Class IA adapter subunits p85α, p85β, and p55γ (Vanhaesebroeck et al., 2005). Class IA regulatory subunits bind to

tyrosine-phosphorylated proteins through their SH2 (Src homology 2) domains, linking them to tyrosine kinase signaling pathways. In contrast, the Class IB regulatory subunits p84 and p101 lack SH2 domains and instead couple the Class IB catalytic subunit p110γ to G protein-coupled receptors. We will limit our discussion to Class IA PI3Ks, which are the major targets of insulin and leptin actions.

PI3Ks utilize phosphatidylinositol 4,5-bisphosphate (PIP2) as the main substrate to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3), which then acts as a second messenger lipid to allosterically modify the activity and/or induce the subcellular relocalization of downstream signaling molecules by binding to their pleckstrin homology (PH) domains (Cantrell, 2001). For example, receptor-mediated phosphorylation of specific tyrosine residues in proteins such as insulin receptor substrates (IRS), produce docking sites that bind to the SH2 domains of p85, hence targeting the catalytic subunit to the membrane (Backer et al., 1992; Carpenter et al., 1993). Activated PI3K can then affect cellular functions through specific signaling pathways such as the serine/threonine protein kinase Akt. Intracellular proteins that are targets of the Akt pathway include glycogen synthase kinase-3 (GSK3β), members of the forkhead family of transcription factors, and the mammalian target of rapamycin (mTOR; Cantrell, 2001; Kozma and Thomas, 2002). These enzymes and transcription factors regulate intracellular metabolic functions such as the generation of glucose and protein synthesis (for a review see Vanhaesebroeck et al., 2005).

Non-receptor tyrosine kinases such as the cytoplasmic Janus tyrosine kinase family (JAKs) as well as other adapter proteins like GRB-2 at the plasma membrane can also initiate PI3K signaling in response to insulin, leptin, and growth factors (Cantrell, 2001). Therefore, the potential for activation by a variety of mechanisms as well as its cross-talk with other signaling pathways such as the Ras/MAPK pathway confers upon this lipid kinase the ability to be involved in a variety of biological functions including reproduction.

## PI3K SIGNALING MOLECULES AND THE CONTROL OF THE HPG AXIS

Pharmacological and genetic studies have demonstrated a role of several members of the PI3K signaling pathway in reproductive function (**Table 1**). Examples include upstream regulators of PI3K signaling such as estrogen receptor alpha (ERα), the IGF-1 receptor (IGF-1R), the insulin receptor (IR), and the leptin receptor (LepR), as well as downstream targets of the Akt branch downstream of PI3K, such as mTOR. Moreover, the steroid hormones $E_2$ and progesterone (P), which are essential for the regulation of puberty, LH release, and sexual behavior, are known to alter the expression and/or activity of members of this signaling pathway such as p85α, IRS-1, and IRS-2. Below is a summary of these findings.

### ESTROGEN RECEPTOR α

The steroid hormone $E_2$ is essential for the feedback regulation of gonadotropin release and female reproductive behavior. These $E_2$ effects are dependent on the activation of ERα in specific neuronal populations of the preoptic area–anterior hypothalamus (POA–AH) and the mediobasal hypothalamus–median eminence (MBH–ME, for reviews see (Couse and Korach, 1999; Roepke et al., 2011)). Traditionally, $E_2$ acts through its receptor to regulate the transcription of important genes for reproduction, such as the progesterone receptor. However, $E_2$ is also capable of inducing rapid non-genomic actions. This "non-classical" ERα signaling includes mechanisms in which cytoplasmic or membrane-associated receptor activation is coupled to stimulation of cytoplasmic signaling pathways, including the PI3K signaling cascade (Acosta-Martinez et al., 2009).

Cross-talk between ERα and PI3K signaling occurs at various levels. For example, *in vitro* PI3K signaling induces the phosphorylation of discrete residues of the endogenous ER, upregulating its transcriptional activity and stability (Campbell et al., 2001). Likewise, $E_2$, presumably acting on membrane ERs, induces PI3K activity, which results in activation of gene transcription in the target cell (Pasapera Limon et al., 2003). *In vivo*, $E_2$ treatment evokes rapid activation of Akt in the hypothalamus (Burt et al., 2011). ERα is required for the $E_2$ activation of hypothalamic Akt since in ERα KO mice acute $E_2$ treatment is no longer able to stimulate Akt phosphorylation (Burt et al., 2011). There is also biochemical evidence demonstrating that ERα binds to the PI3K regulatory subunit p85α in a ligand-dependent manner (Simoncini et al., 2000; Mendez et al., 2003). The interaction between ERα and p85α results in the activation of Akt and endothelial nitric oxide synthase (eNOS; Simoncini et al., 2000).

The PI3K signaling pathway participates in the $E_2$ facilitation of lordosis behavior, a reflexive posture displayed by female rodents during mating. The PI3K inhibitors wortmannin and LY294002 partially suppressed lordosis when administered during $E_2$ priming (Etgen and Acosta-Martinez, 2003). Interestingly, when both wortmannin and the MAPK inhibitor PD98059 are simultaneously infused, lordosis behavior is completely abolished (Etgen and Acosta-Martinez, 2003). Therefore, the complete inhibition of this $E_2$-dependent behavior requires concurrent suppression of both central MAPK and PI3K signaling. Future studies should examine whether central inhibition of PI3K participates in $E_2$-induced LH release.

### INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR

The actions of IGF-1 are mediated through activation of its receptor (IGF-1R), a member of the tyrosine kinase receptor family that signals through the IRS/PI3K and the MAPK signaling cascades (Nakae et al., 2001). In addition to its prominent role in the regulation of somatic growth, the IGF-1R signaling pathway participates in the regulation of GnRH release, the progression of puberty, adult reproductive function, and sexual behavior (reviewed in Daftary and Gore, 2005). Although IGF-1 can regulate the HPG axis by acting at the pituitary and gonads, it also acts centrally to stimulate GnRH release and puberty. For example, in peripubertal rats intracerebroventricular (i.c.v.) infusion of IGF-1 increases plasma LH levels, a response that is blocked by central immunoneutralization of GnRH (Hiney et al., 1996).

Recent studies suggest that the IGF-1 effect on advancing pubertal onset is mediated through direct regulation of GnRH neurons, which are known to co-express IGF-1Rs. Male and female mice with the IGF-1R specifically deleted in GnRH neurons

**Table 1 | Summary of studies on the role of upstream and downstream components of the PI3K signaling pathway in HPG axis function.**

| Molecule | Description | Experimental approach | Results | References |
|---|---|---|---|---|
| ERα | Estrogen hormone receptor | Global KO | Infertility: elevated LH and $E_2$, ovarian hemorrhage | Couse and Korach (1999), Mayer et al. (2010) |
| | Binds the PI3K regulatory subunit p85α | Kisspeptin-cell specific deletion | Advancement of puberty onset in female mice accompanied by elevated LH, abnormal ovulatory activity in the adult | |
| IGF-1R | Receptor tyrosine kinase | GnRH neuron-specific deletion | Delayed pubertal development (VO) with normal fertility | Divall et al. (2010) |
| | | i.c.v. infusion of the IGF-1-R antagonist JB-1 in $E_2$ + P-primed ovx rats | Attenuation of lordosis behavior | Quesada and Etgen (2002), Etgen and Acosta-Martinez (2003) |
| | | | Blockade of hormone-induced LH surge | |
| IR | Receptor tyrosine kinase | Neuron-specific deletion | Impaired spermatogenesis and ovarian follicle maturation, reduced LH levels | Bruning et al. (2000) |
| LepR | Class I type cytokine receptor | Global KO | Infertility: delayed/abnormal puberty, low gonadotropins | Leibel et al. (2001), Ring and Zeltser (2010) |
| | | Hypothalamic deletion | Infertility: absence of offspring | |
| IRS-2 | Adaptor protein | Global KO | Infertility: anovulatory ovaries with reduced number of follicles, reduced pituitary size, low levels of LH, prolactin, and sex steroids | Burks et al. (2000) |
| IRS-4 | Adaptor protein | Global KO | Decreased number of litters | Fantin et al. (2000) |
| mTOR | Ser/thr kinase | i.c.v. infusion of the mTOR signaling inhibitor, rapamycin in prepubertal female rats | Delayed VO, decreased LH and $E_2$ levels, ovarian and uterine atrophy | Roa et al. (2009b) |
| p85α | Class IA PI3K regulatory subunit | GnRH neuron-specific deletion | Decreased serum LH, T and sperm counts. | Acosta-Martinez et al. (2009) |

(GnRH–IGF-1R KO) experienced delayed pubertal development with normal fertility (Divall et al., 2010). Administration of IGF-1 is not able to advance puberty onset in GnRH–IGF-1R KO females, suggesting that the re-activation of the GnRH pulse generator at puberty is triggered by activation of IGF-1Rs in GnRH neurons. Compared to WT littermates, pre- and peripubertal GnRH–IGF-1R KO mice have a reduced number of GnRH neurons with complex dendritic trees and a significantly lower proportion of GnRH neurons with spines. The authors concluded that IGF-1R regulation of GnRH synaptic structure during the prepubertal period is critical for the induction of GnRH pulsatility at puberty onset (Divall et al., 2010).

Finally, central IGF-1Rs regulate adult LH release and sexual receptivity (lordosis) in female rats. Chronic i.c.v. infusion of the highly selective IGF-1R antagonist JB-1 suppresses $E_2$-induced LH release and partially inhibits lordosis behavior (Quesada and Etgen, 2002; Etgen and Acosta-Martinez, 2003). The effect of JB-1 on LH release is not observed in ovariectomized rats given no hormone replacement, suggesting that JB-1 acts primarily to suppress $E_2$-positive feedback (Quesada and Etgen, 2002). Participation of IGF-1R signaling in $E_2$-induced synaptic remodeling in the hypothalamic arcuate nucleus (ARC), which is dependent on IGF-1R activity, may underlie IGF-1 stimulatory effects on adult LH release (Fernandez-Galaz et al., 1999).

## INSULIN AND LEPTIN RECEPTORS

Transgenic mouse models and *in vivo* pharmacological studies have demonstrated that leptin and insulin receptor signaling in the brain has a major role in the regulation of the HPG axis. When male and female *db/db* mice (which have a global LepR mutation) are subjected to a neuron-specific LepR *knock in*, obesity, diabetes, and infertility are reversed (de Luca et al., 2005). The brain-specific deletion of the IR results in obesity and hypothalamic hypogonadism (Bruning et al., 2000). However, the brain region(s) and the phenotype of the neurons that are responsible for leptin and insulin actions on the reproductive axis are largely unknown. GnRH neurons are unlikely to be direct targets for leptin or insulin in rodents. Recent studies show that the GnRH neuron-specific deletion of either receptor does not affect puberty or adult HPG axis function (Quennell et al., 2009; Divall et al., 2010). Therefore, it is likely that these and other peripheral metabolic cues inform the HPG axis about energetic status via a complex network of neurons and interneurons, many of which are functionally connected to GnRH neurons. In this regard, the combined deletion of IR and LepR in POMC neurons results in high T levels, ovarian abnormalities, and reduced fertility (Hill et al., 2010). Interestingly, a reproductive phenotype is not observed in single deletions of either receptor in POMC neurons (Balthasar et al., 2004; Konner et al., 2007). In view of the fact that deletion of the LepR or the IR in all neurons results in low LH levels, it is likely that the role each receptor has in the control of the HPG axis depends on the phenotype of the neurons in which it is expressed, as well as environmental and genetic circumstances.

Insulin and leptin converge on PI3K signaling to centrally regulate feeding and glucose homeostasis (Niswender et al., 2003).

Their receptors activate PI3K through the phosphorylation of tyrosine residues on IRS proteins (Foukas and Withers, 2010). In the case of the LepR this is accomplished through the JAK/signal transducer and activator of transcription (STAT3) signaling pathway, whereas IR autophosphorylation on tyrosine residues provides docking sites for the IRS (Niswender et al., 2003; Foukas and Withers, 2010). Intracerebroventricular infusion of PI3K inhibitors blocks the ability of insulin and leptin to inhibit food intake (Niswender et al., 2003; Morrison et al., 2005). Leptin and insulin reduce appetite by decreasing the production of potent orexigenic neuropeptides such as NPY and agouti related peptide (AgRP), while increasing the expression of anorexigenic neuropeptides such as POMC (Munzberg et al., 2003; Morrison et al., 2005). Leptin regulation of NPY/AgRP transcription depends on PI3K signaling, while that of POMC requires activation of the JAK/STAT3 pathway (Munzberg et al., 2003; Morrison et al., 2005). PI3K is also involved in insulin and leptin effects on the membrane potential and firing rates of these ARC neurons (Plum et al., 2006; Hill et al., 2008; Al-Qassab et al., 2009). Since both leptin and insulin stimulate LH release, it is tempting to speculate that PI3K also serves as an integrator for the effects that these hormones have on the HPG axis.

As discussed previously, leptin and insulin resistance are thought to mediate, in part, the detrimental effects of obesity on the HPG axis. Central leptin resistance is associated with a defective PI3K pathway in the hypothalamus. For example, the ability of leptin to induce hypothalamic PI3K activity is impaired in mice fed a HFD (Metlakunta et al., 2008). These animals are hyperleptinemic, hyperinsulinemic, and have impaired glucose and insulin tolerance. Moreover, leptin fails to induce PI3K activity as early as 4 weeks after a HFD, whereas at this age, phosphorylation of STAT3 by leptin is not impaired (Metlakunta et al., 2008). A recent study using mouse lines with reduced IR expression in the brain and the ovary show that insulin resistant and hyperinsulinemic mice have impaired HPG axis activity (Nandi et al., 2010). This includes altered duration of estrous cycles, aberrant distribution, and morphology of ovarian follicles, and a decrease in pregnancy outcomes. These effects were observed in the absence of body weight gain and hyperglycemia, suggesting that abnormal insulin signaling, independent of adipose tissue mass or hyperglycemia, can affect HPG axis function. Even though these studies demonstrate the importance of IR and LepR signaling for normal HPG axis activity, it is impossible to assess whether defects in IR and LepR signaling in the brain vs. other reproductive tissues, such as the ovary and the pituitary, are responsible for the detrimental effects on the HPG axis. This remains an important question since HFD-induced obesity promotes insulin resistance in the ovary of rats and increases pituitary sensitivity to GnRH in mice (Akamine et al., 2010; Brothers et al., 2010). One way to address the question of whether impaired PI3K signaling in the brain is responsible for the effects of obesity on the HPG axis would be to investigate whether an increase in PI3K activity could rescue or lower LH levels in such models. Possible approaches include the use of brain region-specific adenoviral expression of a constitutively active mutant of PI3K to activate downstream targets such as Akt. This approach was successfully used to demonstrate that hypothalamic PI3K signaling mediates the effects of insulin treatment on glucose levels in rats with STZ-induced diabetes (Gelling et al., 2006).

## INSULIN RECEPTOR SUBSTRATES

The IRS family of proteins consists of four members named IRS-1, -2, -3, and -4 (White, 1998). Upon insulin or IGF-1 receptor activation, IRS proteins undergo rapid tyrosine phosphorylation, allowing them to recruit and activate PI3K (White, 1998, 2002). Global ablation studies have revealed a role for IRS-2 and IRS-4 in the integration of metabolism and reproduction. For example, IRS-2-null female mice are infertile, with deficits at multiple levels of the reproductive axis, including anovulatory ovaries with reduced number of follicles, reduced pituitary size, as well as very low levels of LH, prolactin, and sex steroids (Burks et al., 2000). The infertility caused by the deletion of IRS-2 is not a consequence of abnormal glucose homeostasis as these females were euglycaemic and only mildly insulin resistant. However, IRS-2-null females exhibit increased food intake, mild obesity, and have defective leptin-stimulated hypothalamic STAT3 phosphorylation, suggesting that IRS-2 may integrate the actions of both insulin and leptin on metabolism and reproduction. IRS-4-null mice also exhibit defects in reproduction, although these are not as severe as those for IRS-2. Compared to wild type breeders, the number of litters produced by IRS-4-null breeding pairs is significantly lower (Fantin et al., 2000). Because a reduced frequency of total litters was not observed upon mating the IRS-4-null males with heterozygous females, IRS-4-null females are suspected to be responsible for the decrease in fertility.

Finally, the steroid hormones, $E_2$ and P, regulate the expression and/or phosphorylation levels of specific IRS proteins. For example, *in vitro*, P induces IRS-2 gene expression in a progesterone receptor-dependent manner (Vassen et al., 1999). $E_2$, through activation of ERα, stimulates tyrosine phosphorylation and nuclear translocation of IRS-1 *in vitro* and in the uterus (Richards et al., 1996; Panno et al., 2006). The effects that growth factors such as IGF-1 have on gonadotropin release and sexual maturation are modulated and even dependent on sex steroid hormones such as $E_2$ (Quesada and Etgen, 2002; Etgen and Acosta-Martinez, 2003; Daftary and Gore, 2005). Therefore, regulation of IRS proteins may be a mechanism by which steroid hormones modulate tyrosine kinase receptor effects on the HPG axis. Future studies should examine the effect of steroid hormones on IRS proteins in the hypothalamus.

## THE MAMMALIAN TARGET OF RAPAMYCIN

Mammalian target of rapamycin is activated under nutrient-rich conditions, whereas its kinase activity is attenuated when nutrients (amino acids, glucose, and oxygen) are depleted (Howell and Manning, 2011). mTOR exists in two forms of multiprotein complexes: one that contains raptor protein (mTORC1) and another that contains the protein rictor (mTORC2; Howell and Manning, 2011). The tuberous sclerosis complex of proteins, TSC1 and TSC2, normally blocks mTORC1 activation by a variety of signals (Howell and Manning, 2011). In response to insulin, IGF-1, and other growth factors, PI3K signaling activates mTORC1, at least in part through Akt-mediated phosphorylation of TSC1 and TSC2 (Inoki

et al., 2002; Manning et al., 2002). Recent studies have linked central mTORC1 to the control of puberty onset and gonadotropin secretion. In pubertal female rats, acute activation of mTOR by L-leucine stimulates LH secretion and partially rescues the suppression of LH caused by chronic food restriction (Roa et al., 2009b). Conversely, chronic i.c.v. infusion of rapamycin, a potent mTORC1 inhibitor, delayed VO, decreased LH and $E_2$ levels, and produced ovarian and uterine atrophy (Roa et al., 2009b). Central inhibition of mTOR also abolished the positive effects of leptin on puberty onset in food-restricted animals (Roa et al., 2009b). Therefore, mTOR signaling in the brain participates in the control of puberty onset and gonadotropin secretion. Furthermore, its activation may be relevant in the suppression of GnRH/LH release and puberty caused by negative energy balance.

The suppressive effects of mTOR on puberty and gonadotropin secretion in rats were also accompanied by decreased expression of kisspeptin mRNA levels in the ARC, linking this neuropeptide system to the regulation of the HPG axis by nutrients (Roa et al., 2009b). Kisspeptin is a potent stimulator of LH secretion and is critical for the initiation and maintenance of adult reproduction (Castellano et al., 2009). Because a state of positive energy balance is needed for the normal timing and development of the reproductive axis, mTOR's role in pubertal onset and LH secretion correlates well with its known activation by positive energy status. As described earlier, IGF-1 is a potent stimulator of the PI3K/Akt pathway, and central activation of IGF-1 receptors stimulates LH release and accelerates the onset of puberty in rodents (Daftary and Gore, 2005). IGF-1 also up-regulates hypothalamic kisspeptin gene expression through the activation of Akt (Hiney et al., 2010). Therefore, it is possible that an IGF-1R/PI3K/Akt/mTOR/Kisspeptin pathway plays a role in pubertal onset and GnRH/LH secretion.

## NEUROPEPTIDE SYSTEMS THAT CONVEY ENERGY BALANCE INFORMATION TO THE GnRH NETWORK

A number of neuropeptide systems have been identified as possible mediators of metabolic signaling to the GnRH network. These include but are not limited to GALP, NPY, and products of POMC (e.g., alpha-melanocyte stimulating hormone, α-MSH) in the ARC (Crown et al., 2007), orexin neurons in the lateral hypothalamic area (LHA; Kohsaka et al., 2001; Small et al., 2003), melanin-concentrating neurons (MCH) in the LHA and zona incerta (Chiocchio et al., 2001; Williamson-Hughes et al., 2005; Murray et al., 2006), nesfatin-1 in the LHA, paraventricular (PVN), and supraoptic (SON) nuclei (Garcia-Galiano et al., 2010), and kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) and the ARC (Castellano et al., 2009). With the exception of kisspeptin, these neuropeptides serve a dual role, being important players for both the central control of feeding and energy homeostasis and of gonadotropin secretion. Importantly, many of the neurons that synthesize these neuropeptides co-express insulin and/or the leptin receptors, and their expression is regulated by metabolic hormones as well as by sex steroids. This is important because conditions of negative energy balance affect the gene and protein expression of these neuropeptides in a brain region-specific fashion. This could explain why the ability of these neuropeptides to either stimulate or suppress LH secretion in the normal female depends on the hormonal milieu (i.e., presence of estrogens), developmental stage (i.e., prepubertal vs. adult), and energetic condition (i.e., fasted vs. normally fed; **Table 2**).

Each of these neuropeptide systems represents a functional neuroanatomical pathway that can send projections to areas containing GnRH neurons, in many cases directly influencing their activity. A review of the effects that each of these neuropeptides has on the reproductive axis is beyond the scope of this article. Instead, the kisspeptin system will be used to illustrate mechanisms by which the indirect regulation of GnRH neuronal activity by metabolic signals occurs (**Figure 1**).

### KISSPEPTIN: LINKING METABOLIC SIGNALS TO THE CONTROL OF GnRH NEURONAL FUNCTION

As introduced earlier, kisspeptin and its receptor, the G protein-coupled receptor GPR-54, have a pivotal role in the regulation of the HPG axis in mammals (for review see Roa et al., 2009a; Roseweir and Millar, 2009). Kisspeptin neurons have been proposed to represent a link between systemic metabolic signals such as leptin and central maintenance of reproductive function. For example, in rodents, the detrimental effects that negative energy balance has on the maintenance or the development of the female reproductive axis is completely rescued by central kisspeptin therapy. The models studied to date include chronic subnutrition of adult rats (Clarkson et al., 2008), peripubertal female rats subjected to food restriction (Castellano et al., 2005; Clarkson et al., 2008), and STZ-induced diabetes in male and female rats (Castellano et al., 2006a, 2009). In addition to their suppressive effects on LH release and gonadal function, these states of negative energy balance are accompanied by suppression of hypothalamic kisspeptin mRNA levels (Castellano et al., 2006a, 2009; Kalamatianos et al., 2008). In some cases, i.c.v. infusion of leptin can rescue both kisspeptin expression and gonadotropin levels (Castellano et al., 2006a).

Models of obesity accompanied by leptin resistance also result in suppression of hypothalamic kisspeptin mRNA (Foukas et al., 2006; Quennell et al., 2011). Compared to WT mice, *ob/ob* mice have a significantly reduced kisspeptin mRNA level, and leptin administration increases the levels of kisspeptin mRNA in these mice (Foukas et al., 2006). Almost half of cells expressing kisspeptin mRNA in the ARC nucleus express the LepR, implying that kisspeptin neurons are direct targets of leptin (Foukas et al., 2006). However, the kisspeptin cell-specific deletion of the LepR does not affect puberty or fertility in female mice, suggesting that a leptin-kisspeptin signaling pathway is not essential for normal HPG activity (Donato et al., 2011). On the other hand, previous studies using metabolic challenges, such as undernutrition and DIO, suggest that this pathway is more relevant under such metabolic challenges.

Electrophysiological studies have recently shown that the ability of kisspeptin to stimulate GnRH neuronal activity is modulated by neuropeptide systems involved in the regulation of feeding and energy balance. This is the case for the orexigenic neuropeptide MCH. MCH fiber projections are in close apposition with a large number of GnRH cell bodies throughout the POA–AH of the rat (Williamson-Hughes et al., 2005). In addition, more than 50% of GnRH neurons co-expressed the MCH receptor, MCHR1

**Table 2 | Examples of hypothalamic neuropeptide regulation of LH release.**

| Neuro-peptide | Origin of cell bodies | Expression of receptors in GnRH neurons | Metabolic regulation of neuropeptide gene expression | Effect of neuropeptide treatment on LH release *in vivo* | Infusion site | Species and hormone treatment | References |
|---|---|---|---|---|---|---|---|
| AgRP | ARC | ? | Food restriction ↑ | Stimulatory<br>Inhibitory | 3V<br>3V | Rat: intact male<br>Monkey: OVX | Stanley et al. (1999), Vulliemoz et al. (2005) |
| NPY | ARC | NPY Y5 | Food restriction↑ | Inhibitory<br>Inhibitory | 3V<br>3V | Rat: OVX<br>Sheep: OVX | McDonald et al. (1989), Kaynard et al. (1990), Malven et al. (1992), Leupen et al. (1997) Leupen and Levine (1999), Advis et al. (2003), Tortoriello et al. (2004), Morrison et al. (2005) |
| | | NPY Y2 | HFD ↑<br>Insulin ↓<br>Leptin ↓ | Stimulatory<br>Stimulatory<br>Inhibitory | ME<br>iv.<br>3V | Sheep: intact<br>Rat: at proestrus, OVX + $E_2$ + P<br>Monkey: OVX + vehicle, OVX + $E_2$ | |
| POMC α-MSH | ARC | MC4R | Fasting ↑ | Inhibitory | ME, mPOA | Rat: OVX and ADX, $E_2$<br>Rat: OVX and ADX, $E_2$ + P | Gonzalez et al. (1997) |
| GALP | ARC | Receptor unknown | Fasting ↓<br>STZ–diabetes ↓<br>Leptin ↑<br>Insulin ↑ | Stimulatory<br>Stimulatory<br>No effect<br>Stimulatory<br>Stimulatory<br>Stimulatory | ICV<br>ICV<br>ICV<br>ICV<br>ICV<br>LV | Mice: intact male<br>Rat: adult and pubertal male<br>Rat: adult and pubertal female<br>Female mice: OVX, $E_2$ + P<br>STZ–diabetic male rat<br>Castrated male monkey | Jureus et al. (2000), Takatsu et al. (2001), Krasnow et al. (2003), Kauffman et al. (2005), Castellano et al. (2006b), Takenoya et al. (2006), Rich et al. (2007) |
| Orexin A | LHA | OR-1 | Fasting ↓<br>Leptin ↑ | Stimulatory<br>Stimulatory<br>Inhibitory<br>Inhibitory<br>Inhibitory | ICV<br>rPOA<br>ICV, 3V<br>mPOA<br>mPOA, ARC/ME | Rat: fasted, OVX, $E_2$ + P<br>Rat: OVX, E<br>Rat: OVX<br>Rat: OVX<br>Rat: OVX, $E_2$ + P | Mondal et al. (1999), Tamura et al. (1999), Lopez et al. (2000), Kohsaka et al. (2001), Bertile et al. (2003), Small et al. (2003), Gallmann et al. (2006) |
| MCH | LHA, ZI | MCH1 | Fasting ↓<br>Leptin ↓ | Inhibitory<br>Stimulatory | 3V<br>mPOA, ME | Rat: OVX + $E_2$<br>Rat: OVX + ADX + $E_2$ | Gonzalez et al. (1997), Tsukamura et al. (2000), Chiocchio et al. (2001), Williamson-Hughes et al. (2005), Murray et al. (2006) |
| Nesfatin-1 | LHA, PVN, SON | Receptor unknown | Fasting ↓ | Stimulatory<br>Stimulatory<br>No effect | ICV<br>ICV<br>ICV | Pubertal female rats<br>Fasted pubertal female rats<br>Adult female rats | Garcia-Galiano et al. (2010) |
| Kisspeptin | ARC, AVPV | GPR54 | Fasting ↓<br>HFD ↓<br>STZ–diabetes ↓<br>Leptin ↑ | Stimulatory<br>Stimulatory<br>Stimulatory<br>Stimulatory<br>Stimulatory<br>Stimulatory | LV<br>ICV<br>ICV<br>ICV<br>ICV<br>ICV | Rat: intact male<br>Rat: fasted female<br>Rat: STZ–diabetic intact male and ORX male<br>Rat: STZ–diabetic female, intact, and OVX<br>Rat: food restricted adult and pubertal female<br>Rat: adult and pubertal female | Irwig et al. (2004), Navarro et al. (2004), Castellano et al. (2005), Castellano et al. (2006a), Foukas et al. (2006), Clarkson et al. (2008), Kalamatianos et al. (2008), Castellano et al. (2009), Quennell et al. (2011) |

(Williamson-Hughes et al., 2005). Using whole-cell patch clamp recordings in brain slices from established lines of GFP mice, MCH was shown to strongly inhibit kisspeptin-activated GnRH neurons but not kisspeptin-insensitive GnRH neurons (Wu et al., 2009). These effects are mediated via a direct postsynaptic mechanism. Therefore, the MCH neuropeptide system may have a role in conveying information about energy balance to the HPG axis by modulating the activity of kisspeptin-sensitive GnRH neurons.

Kisspeptin can also interact with other appetite-regulating neuropeptide systems, such as POMC and NPY neurons in the ARC. Many kisspeptin-immunoreactive axonal boutons terminate on or near POMC neurons (Fu and van den Pol, 2010). Moreover, kisspeptin was found to potently and directly excite POMC neurons (Fu and van den Pol, 2010). In contrast, kisspeptin inhibits NPY neurons through a presynaptic mechanism based on enhancing GABA-mediated inhibitory synaptic tone (Fu and van den Pol, 2010). Since both POMC and NPY are known regulators of GnRH release, the ability of kisspeptin to regulate their neuronal function provides an additional mechanism by which metabolic information is conveyed to the HPG axis,

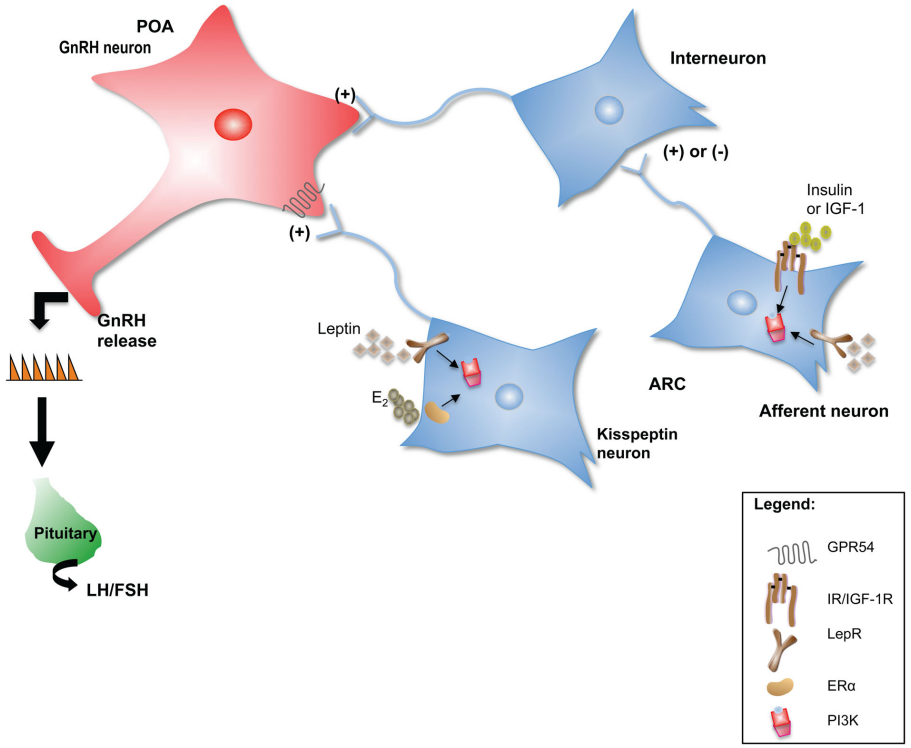


**FIGURE 1 | Neuropeptide neurons such as kisspeptin could serve as intermediaries for peripheral metabolic cues to communicate changes in energy status to the GnRH network.** In addition, metabolic signals could be "sensed" by interneurons that in turn communicate with GnRH neurons to regulate their activity. Several upstream regulators of PI3K signaling such as the LepR, IR, IGF-1R, and ERα, are critical for the normal initiation and maintenance of reproductive function in mammals. In this model, PI3K serves as an integrator of $E_2$, leptin, insulin, and IGF-1 indirect effects on GnRH neuronal function and hence on the HPG axis.

i.e., neuropeptides serving as a relay station downstream of kisspeptin.

In summary, the ability of kisspeptin to stimulate GnRH release can be directly regulated by peripheral metabolic signals such as leptin, or by other neuropeptide systems known to regulate GnRH release (**Figure 1**). Leptin and insulin directly regulate the gene expression and/or neuronal activity of many of these appetite-regulating neuropeptides, such as POMC and NPY. PI3K signaling plays a key role in the actions of both metabolic cues on these neurons. However, additional challenges include how to differentiate between PI3K metabolic and reproductive effects and how to identify in which neuronal population PI3K signaling acts to affect HPG axis activity. The *cre/loxP* system has offered a wealth of information about the phenotype of the neurons in which insulin and leptin receptors act to regulate metabolism and in some cases reproduction. In the case of PI3K, this has entailed the cell-specific ablation of its catalytic (p110) or regulatory (p85) subunits.

## TARGETING CLASS IA PI3K REGULATORY AND CATALYTIC SUBUNITS IN HYPOTHALAMIC NEURONS

The cell-specific deletion of members of the class I PI3K family has confirmed the role of this enzyme as a key integrator of the central effects of insulin and leptin on feeding and metabolic control. These studies have focused on neuronal populations in the ARC, such as POMC and AgRP neurons, well known for their involvement in the central control of feeding and body weight. The phenotype observed depends on the type of neuron being targeted (i.e., NPY vs. POMC), as well as the PI3K component (regulatory vs. catalytic subunit). For example, while mice with a POMC-specific deletion of p110α have increased body weight and adipose tissue, mice with a POMC-specific deletion of p85α showed resistance to DIO (Hill et al., 2009). These alterations in energy homeostasis are often accompanied by the prevention of insulin- and leptin-stimulated electrophysiological responses (Hill et al., 2008; Al-Qassab et al., 2009).

No reproductive phenotype has been reported in mice with POMC- or AgRP-specific deletion of class IA PI3K catalytic or regulatory subunits. The same is true in studies of mice with specific deletion of the leptin or insulin receptor in POMC- or NPY/AgRP-expressing neurons. Unfortunately, many of these studies were carried out in males, whose reproductive axis is more resistant to the effects of metabolic disturbance. Moreover, the expression of a reproductive phenotype in these models might depend on the age of the animal or might be observed only in response to a metabolic challenge. This is the case for female mice with POMC- and NPY/AgRP-specific deletion of the LepR, which show reduced fertility with age (Israel and Chua, 2010). Finally, while POMC and NPY/AgRP neurons are important for the normal regulation of the HPG axis, and alterations in their gene expression could contribute to the detrimental effects that metabolic disorders have on

fertility, gene manipulation studies in other neuronal populations that are essential for pubertal and adult GnRH/LH release are necessary in order to provide information about PI3K's role in the control of the HPG axis. Candidate neurons include GnRH and kisspeptin.

While central regulation of the HPG axis by leptin and insulin is likely due to actions on interneurons that ultimately control GnRH neurosecretion, other signals such as steroid hormones and growth factors might exert direct regulation of GnRH neurons either during development or in the adult. As indicated earlier, many of the factors that regulate GnRH release also have the capacity to activate cell signaling pathways mediated by PI3K. Examples include $E_2$ through ERα and IGF-1 through IGF-1Rs. To investigate whether PI3K signaling mediates the direct regulation of GnRH neurons by these and other factors, we used conditional gene targeting to ablate the expression of p85α in GnRH neurons.

Surprisingly, the GnRH neuron-specific deletion of p85α did not affect the female reproductive axis. Instead, we observed a male-specific phenotype consisting of decreased serum LH, T, and sperm counts (Acosta-Martinez et al., 2009). Furthermore, the same phenotype was observed when we generated the GnRH neuron-specific deletion of p85α on a global p85β KO background. Hence, p85β does not substitute for p85α activity toward PI3K function in GnRH neurons. These findings suggest that p85α in GnRH neurons participates in normal GnRH neurosecretory activity in the male mouse. It remains unknown whether p85α and PI3K activity in GnRH neurons are required in this regard during development, in adulthood, or throughout the lifespan.

Although we did not identify the cause of this male-biased effect, we speculate that PI3K signaling in GnRH neurons participates in the organizational effects of $E_2$ during development. $E_2$, which is aromatized from testicular androgens in the male, establishes sex differences in synaptic connections and neuronal morphology that are ultimately responsible for the differentiation of the male brain from the female brain in mammals (Schwarz et al., 2008). In the developing hypothalamus, activation of ERs by $E_2$ promotes dendritic spine formation via rapid, non-genomic activation of PI3K (Schwarz et al., 2008). Because fetal GnRH neurons may express ERs (Sharifi et al., 2002), it is possible that this mechanism may be activated in GnRH neurons during the prenatal/neonatal surges of T production that occur in males. The subsequent changes in synaptic connectivity might support higher levels of GnRH neurosecretory activity or increased responsiveness to synaptic inputs in adulthood. Such a mechanism might explain the observation that the GnRH pulse generator operates at a higher frequency in males than in females of many species, and that prenatal androgen exposure leads to an acceleration of GnRH pulsatility in female rodents, sheep, and monkeys (reviewed in Foecking et al., 2008).

Besides steroid hormones, PI3K signaling is activated by and regulates the function of other molecules that affect GnRH neuronal activity through direct mechanisms. This is the case of IGF-1Rs and ATP-sensitive potassium ($K_{ATP}$) channels, both of which are expressed in GnRH neurons (Zhang et al., 2007a; Brothers et al., 2010). While GnRH neuronal expression of IGF-1Rs is important for normal pubertal timing, $K_{ATP}$ channels confer responsiveness to glucose in GnRH neurons and regulate GnRH/LH release in an $E_2$-dependent manner (Zhang et al., 2007a; Huang et al., 2008). One can speculate that a reproductive phenotype in GnRH-specific p85α KO females might be observed after metabolic challenges such as fasting or DIO. On the other hand, it is unknown whether p85α's effects on male GnRH neurons are mediated through p110-independent or -dependent mechanisms. Hence, it is possible that a GnRH-specific deletion of p110 catalytic subunits might alter GnRH neuronal activity resulting in a reproductive phenotype in females.

Neuronal populations that express ERα and are important for GnRH/LH release or sexual behavior are also good candidates to investigate the role of PI3K in the control of reproduction. A good candidate for this is kisspeptin neurons. The majority of kisspeptin neurons co-express ERα, with more than 60% in the AVPV and more than 90% in the ARC (Mayer et al., 2010). In addition, the kisspeptin cell-specific deletion of ERα results in precocious puberty (advanced VO and LH hypersecretion) and the arrest of subsequent pubertal maturation (Mayer et al., 2010). Therefore, in addition to relaying metabolic information from the periphery, PI3K might participate in the direct actions of $E_2$ on kisspeptin neuronal activity and hence GnRH/LH secretion (**Figure 1**). Through the use of transgenic mouse models, experiments in our lab are underway to investigate this possibility.

## INFLAMMATION AND PI3K SIGNALING

Obesity is considered to be a chronic inflammatory state. With time, adipose tissue becomes infiltrated by macrophages, resulting in increased circulating pro-inflammatory cytokines such as transforming growth factor-alpha (TNF-α) and interleukin (IL)-6 (Thaler et al., 2010). The chronic exposure to circulating cytokines as well as to excess glucose or fatty acids can activate intracellular inflammatory pathways in other cell types and organs including the liver, muscle, endothelial cells, and neurons (Thaler and Schwartz, 2010; Thaler et al., 2010). It has been hypothesized that hypothalamic inflammation resulting from the prolonged consumption of a HFD leads to obesity through the development of central insulin and leptin resistance (Thaler and Schwartz, 2010). These are states associated with infertility in both humans and animal models of DIO. Therefore, chronic immune signals might also reduce the sensitivity of signaling pathways important for the central control of the HPG axis.

In humans, a reproductive disorder associated with a state of chronic low-grade inflammation (LGI) is PCOS (Repaci et al., 2011). Markers of LGI in PCOS patients include elevated TNF-α (Gonzalez et al., 1999), IL-18 (Escobar-Morreale et al., 2004), IL-6 (Repaci et al., 2011), and C-reactive protein (CRP; Kelly et al., 2001; Benson et al., 2008). Many of these inflammatory signals are associated with insulin resistance and high circulating insulin levels. Insulin resistance, although not part of the diagnostic criteria, is present in a large percentage of women diagnosed with PCOS, regardless of obesity (Repaci et al., 2011). It has been speculated that LGI might be the link between hyperandrogenism, insulin resistance, and the long-term consequences of PCOS. For example, TNF-α is a known inducer of insulin resistance, presumably through its ability to interfere with insulin receptor signaling (Feinstein et al., 1993; Uysal et al., 1997). While the focus of these studies has been on peripheral insulin-responsive organs such as

muscles and adipose tissue, the effects that long-term TNF-α levels might have on insulin receptor signaling in reproductive organs has not yet been investigated. This is of importance since both TNF-α and insulin are known to modulate ovarian steroid production as well as induce cell proliferation in interstitial theca cells *in vitro* (Roby and Terranova, 1990; Spaczynski et al., 1999). Interestingly, the ability of insulin to stimulate androgen synthesis by the ovary is preserved in PCOS patients (Nestler et al., 1998). In fact, the higher concentration of insulin in women with PCOS is thought to amplify the effects of LH on androgen production by theca cells. In *in vitro* studies using human ovarian theca cells, insulin augmentation of 17 α-hydroxylase activity, a key enzyme for androgen biosynthesis, is mediated by PI3K (Munir et al., 2004). Therefore, ovarian PI3K signaling could potentially contribute to the hyperandrogenemia observed in PCOS. However, whether or not inflammatory signals such as TNF-α modulate PI3K-mediated androgen production by the ovary is not known.

A role of inflammation and insulin resistance in PCOS is also supported by the marked improvement PCOS patients show after treatment with insulin sensitizers such as metformin. Long-term treatment with metformin increases ovulation, improves menstrual cyclicity, and reduces serum androgen levels (Lebinger, 2007; Nestler, 2008). Metformin treatment also significantly reduces circulating levels of CRP and white blood cells (Morin-Papunen et al., 2003; Diamanti-Kandarakis et al., 2006; Orio et al., 2007). The anti-inflammatory actions of metformin include the inhibition of IL-1β-induced release of IL-6 and IL-8 in endothelial cells, human vascular smooth cells, and macrophages (Morin-Papunen et al., 2003; Diamanti-Kandarakis et al., 2006; Orio et al., 2007).

The studies cited above focus on the role of inflammation and insulin resistance in peripheral tissues, like the ovaries and endothelial cells. However, the role that central inflammation and the resultant insulin resistance might play in the pathogenesis of PCOS, especially on GnRH/LH hypersecretion, is unknown. It is possible that central actions of metformin contribute to its beneficial effects on HPG axis function, as this drug can cross the blood–brain barrier. Here, rodent models of HFD-induced obesity and infertility could be used to determine if centrally administered metformin normalizes LH release.

The mechanisms by which dietary lipids and other nutrients become more immunogenic by overnutrition are not completely understood. It is clear, however, that many of the players involved in the immune response toward disease, by as yet unidentified mechanisms, also promote insulin and leptin resistance. In this context, it is worth mentioning that insulin has anti-inflammatory effects that are independent of its function in regulating glucose homeostasis. In *in vivo* models of endotoxemia, such as that induced by acute injection of bacterially derived lipopolysaccharide (LPS), insulin is able to reduce inflammation (decreased plasma levels of IL-6, TNF-α, monocyte chemotactic protein) and mortality (Kidd et al., 2008). In this model, pharmacological or genetic inhibition of the PI3K–Akt pathway suppresses insulin's anti-inflammatory actions (Zhang et al., 2007b; Kidd et al., 2008; Luyendyk et al., 2008). Recent studies support a role for hypothalamic inflammation and the resultant insulin and leptin resistance as triggering factors of hyperphagia and obesity (Thaler and Schwartz, 2010). Future studies should investigate if chronic levels of pro-inflammatory cytokines in the brain affect PI3K signaling in hypothalamic centers involved in the control of both reproduction and metabolism such as the ARC. This could reveal a possible mechanism by which insulin sensitizers such as metformin restore normal gonadotropin levels in PCOS patients.

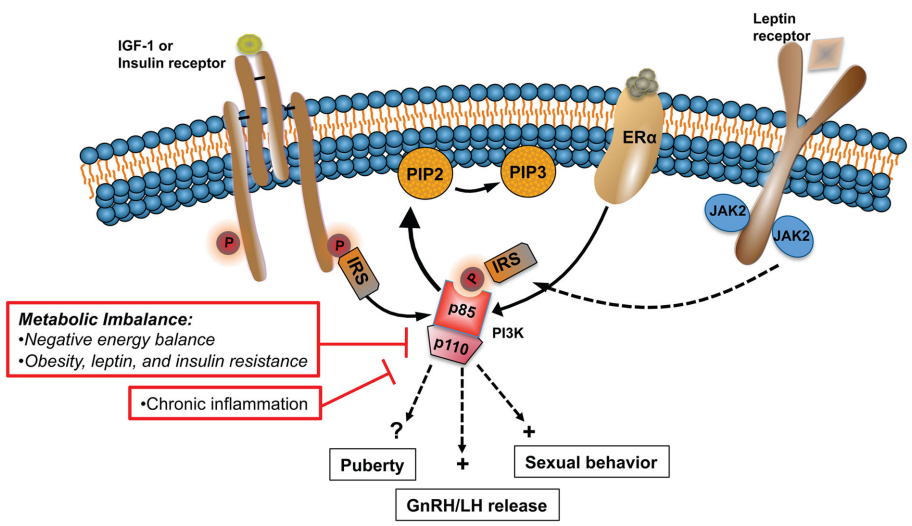


**FIGURE 2 | States of metabolic imbalance such as malnutrition and obesity have detrimental effects on the HPG axis, principally altering normal GnRH pulsatile release.** Alterations in the levels and sensitivity to peripheral hormones and metabolic signals, including IGF-1, $E_2$, leptin, and insulin, play a major role in the dysfunction of the HPG axis during chronic metabolic disturbances. PI3K is a major signaling pathway activated by the same upstream regulators that are affected during such states. In rodents, PI3K participates in the central control of sexual behavior and LH release. In this model altered PI3K signaling is linked to the negative effects that metabolic imbalance has on the HPG axis.

## SUMMARY AND CONCLUSION

Central dysregulation of GnRH neurosecretion underlies the reproductive dysfunction observed during states of metabolic imbalance, such as malnutrition and chronic nutritional excess. The inability of peripheral hormones and metabolic cues to regulate key hypothalamic centers plays a major role in the negative effects that these states have on the HPG axis. Hormones and growth factors key for the central control of GnRH release, puberty, and sexual behavior utilize the PI3K signaling pathway to regulate a variety of physiological functions including metabolism. Examples include insulin, leptin, IGF-1, and $E_2$, whose levels and capacity to act in the brain is severely compromised during conditions of disturbed energy homeostasis (**Figure 2**). In rodents, PI3K participates in the central control of female sexual behavior (Etgen and Acosta-Martinez, 2003) and gonadotropin release (Acosta-Martinez et al., 2009). A direct link between impaired PI3K signaling and the detrimental effects that a disturbed energy homeostasis has on the HPG axis has not been established. However, PI3K plays a central role mediating the effects of insulin and leptin on feeding and metabolism. Moreover, these metabolic signals also utilized PI3K to regulate the neuronal activity and expression of neuropeptide systems that are important regulators of GnRH release, such as NPY and POMC. To test the hypothesis that PI3K signaling represents a link between metabolism and reproduction, experimental approaches similar to those used to investigate its role in the central control of metabolism should be used. For example, the use of the Cre/LoxP system to alter PI3K gene expression in neuronal populations critical for the control of gonadotropin release, such as kisspeptin, will continue to shed light into the role of this enzyme in the control of reproduction. Similarly, adenoviral gene therapy could be used to investigate if the detrimental effects of negative energy balance and obesity on LH release is prevented or restored by activation of PI3K signaling in specific brain regions, like the POA and the ARC.

Finally, chronic inflammation has been identified recently as an important component of the pathophysiology of obesity and the metabolic syndrome. These and other metabolic conditions have a major impact on fertility. Therefore, it is important to identify the intracellular mechanisms that are at play during chronic inflammation within the CNS. Because a direct consequence of inflammation is central insulin and leptin resistance, signaling pathways shared by these hormones, such as PI3K, should be investigated.

The use of pharmacological agents that target different classes of PI3K is already being considered as a therapeutic strategy to treat cancer and metabolic diseases (Holmes, 2011). The insights described within this review highlight the need to also address PI3K and its role in the central control of reproduction. Disorders that span both metabolic and reproductive function, such as PCOS, may have some potential for PI3K pharmacological targeting.

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# The changes they are a-timed: metabolism, endogenous clocks, and the timing of puberty

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Childhood obesity has increased dramatically over the last several decades, particularly in industrialized countries, often accompanied by acceleration of pubertal progression and associated reproductive abnormalities (Biro et al., 2006; Rosenfield et al., 2009). The timing of pubertal initiation and progression in mammals is likely influenced by nutritional and metabolic state, leading to the hypothesis that deviations from normal metabolic rate, such as those seen in obesity, may contribute to observed alterations in the rate of pubertal progression. While several recent reviews have addressed the effects of metabolic disorders on reproductive function in general, this review will explore previous and current models of pubertal *timing*, outlining a potential role of endogenous timing mechanisms such as cellular circadian clocks in the initiation of puberty, and how these clocks might be altered by metabolic factors. Additionally, we will examine recently elucidated neuroendocrine regulators of pubertal progression such as kisspeptin, explore models detailing how the mammalian reproductive axis is silenced during the juvenile period and reactivated at appropriate developmental times, and emphasize how metabolic dysfunction such as childhood obesity may alter timing cues that advance or delay pubertal progression, resulting in diminished reproductive capacity.

**Keywords: obesity, kisspeptin, circadian, puberty, GnRH**

Normal development from childhood to adulthood involves the myriad changes accompanying puberty that most remember as awkward and unpleasant. In physiological terms, the process consists of activation of the hypothalamic–pituitary–gonadal (HPG) axis, which leads to the production of sex steroids and mature gametes as well as the ability to reproduce (Watanabe and Terasawa, 1989; Sisk et al., 2001; Harris and Levine, 2003). Normal reproductive function in mammals requires secretion of hypothalamic gonadotropin-releasing hormone (GnRH) that stimulates release of pituitary gonadotropins needed for fertility in both males and females. GnRH pulses stimulate the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), both of which act on the ovaries and testes to stimulate gonadal maturation, gametogenesis, and steroid hormone production. In adult females, preovulatory GnRH surges occur in response to increasing ovarian estrogen (E2) levels, which trigger LH surges responsible for ovulation (Levine and Ramirez, 1982; Moenter et al., 1992; Caraty et al., 1995; Herbison, 2008). The appearance of elevated LH surge secretion has been observed as a gradual process in female mammals, yet the signals responsible for this "awakening" of the reproductive axis are unclear. While the process of puberty has been well studied and work over the last century has contributed much to our understanding of associated physiological changes, molecular mechanisms have only started to be uncovered in the last decade and the trigger that initiates pubertal development is still highly debated.

## EVERYTHING IS BROKEN: ABNORMALITIES IN PUBERTAL PROGRESSION CORRELATE WITH METABOLIC DYSFUNCTION

Although, or perhaps because, the transition through puberty is central to the survival of mammalian species, it can be perturbed by a number of factors. One major factor is nutrition, a viewpoint argued by some to have been conceptually acknowledged for millennia, as evidenced by obese ancient fertility statues such as the Venus of Willendorf. This ancient intuition of the link between nutrition and reproduction has been addressed in research in multiple animal models, including humans. It appears that, in order for normal pubertal development and reproduction to occur, a minimum positive energy balance must be maintained, particularly in females (Kennedy, 1969; Frisch and McArthur, 1974; Bergendahl et al., 1991; Kile et al., 1991; Aloi et al., 1997). Eating disorders, low body weight, and even weight loss can cause ovulatory dysfunction and infertility (Frisch and McArthur, 1974; Reid and Van Vugt, 1987). It is posited that, in times of famine, the reproductive axis becomes quiescent to ensure individual survival, shunting all efforts toward maintaining critical organs over maintenance of high energy cost functions such as sexual maturation, menstruation, pregnancy, and lactation. However, it appears that an excess of energy stores can also be detrimental: obesity in women is strongly associated with infertility and irregular cycles (Rittmaster et al., 1993; Bluher and Mantzoros, 2007). Rising obesity rates both in the United States as well as countries around the world (Flegal et al., 1998; Froguel and Boutin, 2001; Ogden et al., 2006)may be

contributing to infertility rates and reproductive diseases such as polycystic ovarian syndrome (PCOS), a condition marked by cyst development in ovaries thought to be caused via overstimulation by LH (Rittmaster et al., 1993). Even more troubling, the rising rates of childhood obesity may be altering both the rate of pubertal progression as well as later reproductive function (Freedman et al., 2003; Biro et al., 2006, 2010; Ogden et al., 2006; Walvoord, 2010). The decline in reproductive health in humans, farm animals, and wildlife, as well as the rising incidence of cancers of the reproductive system, has led to an increased public desire for answers as to why these changes are occurring and how to prevent them.

The age of menarche, defined as occurrence of the first menstrual period in girls, has decreased over the last 200 years (Wyshak and Frisch, 1982), but whether or not this trend has continued or even accelerated over the last couple of decades is both debated (Walvoord, 2010) and a source of concern (Euling et al., 2008). While difficulties are encountered making direct comparisons between studies due to differences in study design and methods, it currently appears that childhood obesity is one of the main factors underlying observed increases in rates of early puberty onset and menarche (Biro et al., 2001; Kaplowitz et al., 2001; Davison et al., 2003; Freedman et al., 2003; Lee et al., 2007; Ong et al., 2009; Rosenfield et al., 2009). As the rates of childhood obesity continue to rise globally, it is possible that the cases of early puberty in girls will continue to rise as well. Some well-controlled studies from Denmark recently reported that puberty in girls today begins a full year earlier compared to girls 15 years ago. However, this change in the timing of the onset of puberty was not accompanied by changes in age at menarche (Aksglaede et al., 2009; Sorensen et al., 2010), suggesting that the multiple processes comprising puberty can be disassociated. A more thorough understanding of the developmental signals required for initiating the process of puberty is therefore crucial to determine how metabolic disorders may affect pubertal timing. This understanding starts with analyzing the observed changes in the patterns of reproductive hormonal secretion observed during the pubertal transition.

## I FEEL A CHANGE COMIN' ON: REPRODUCTIVE HORMONAL RHYTHMS IN PUBERTY

Reproductive hormone secretion in mammals over developmental time displays a unique pattern: near adult levels are in circulation at birth and in infancy, followed by dramatically decreasing concentrations for the span of several weeks, months, or years during juvenile development in a species-dependent manner, finally followed by a gradual re-awakening of the reproductive axis culminating in puberty. The origins of the intervening "juvenile pause" and what cues lead to the resumption of reproductive axis function remain as yet unclear. Two main models of pubertal progression have been proposed: one describes a "brake" mechanism that must be released for puberty to begin, while the other consists of an "accelerator" that must be fueled to start puberty (Sirinathsinghji et al., 1985; Mayer et al., 2010). The concept of a "brake" implicates elevated activity of inhibitory mechanisms underlying the juvenile pause, with a timed disinhibition responsible for initiating puberty. While some earlier studies hinted at this possibility by demonstrating an increase in hypothalamic GABAergic tone during juvenile development (Clarkson and Herbison, 2006a), and that serotonin and GABA administered centrally can be stimulatory to GnRH/LH secretion in pre-pubertal female rats, while becoming inhibitory to GnRH/LH release in adults (Mogilevsky and Wuttke, 2001), this may be difficult to reconcile with recent findings that GABA actually depolarizes a subset of GnRH neurons, thus increasing secretion (Sim et al., 2001; Moenter and DeFazio, 2005). Still other studies support the "accelerator" hypothesis, demonstrating that catecholamines and NMDA in the hypothalamus begin to rise nearing puberty, pharmacological blockade of NMDA receptor signaling can prevent E2-primed LH surges in peri-pubertal females, and central NMDA treatment can accelerate pubertal timing (Urbanski and Ojeda, 1987, 1990; Gore et al., 1996). Still other studies have suggested a role for glial factors such as prostaglandins that may prime neuroendocrine neurons to exhibit altered sensitivity to both neurotransmitters and steroid hormones at developmentally appropriate times (Ma and Ojeda, 1997; Ojeda and Ma, 1999; Ojeda et al., 2000). $PGE_2$ stimulates GnRH-activating norepinephrine (NE) release within the mediobasal hypothalamus, an effect which is potentiated by E2, suggesting that astrocytes may participate in sensitizing the neuroendocrine hypothalamus to the positive feedback effects of E2 (Ojeda et al., 1986). Hypothalamic glia may also contribute to pubertal development by production of transforming growth factor α (TGFα) and its receptors, effects which can also be modulated by E2 exposure (Rage et al., 1997; Ojeda and Ma, 1999; Prevot et al., 2005). Few of these models, however, provide insight as to what factors may signal to putative mechanisms at the appropriate developmental *times*. Since proper pubertal progression appears to require a threshold basal metabolic state, we will explore below recent literature probing how peri-pubertal metabolic cues may signal the hypothalamus to initiate a cascade of cellular events leading to puberty.

In addition to the above long-term changes in baseline peptide, glycoprotein, and steroid hormone release, a striking feature of secretion in pre-pubertal rodents (Kimura et al., 1981), primates (Plant, 1985), and humans (Wu et al., 1991) is an approximate 24-h rhythmicity of several reproductive hormones, rhythms that can begin quite early (6–8 years of age) and persist through puberty, to diminish in the adult (Oerter et al., 1990; Dunkel et al., 1992; Apter et al., 1993; Albertsson-Wikland et al., 1997; Ankarberg-Lindgren and Norjavaara, 2004). In particular, pulses of LH increase initially only at night in humans, until during later puberty when daytime LH secretion increases to match the elevated nighttime release already established (Porcu et al., 1997). Interestingly, in some post-pubertal girls with abnormally high testosterone or obesity, these rhythms appear to persist into adulthood and are often associated with decreased reproductive capacity (Porcu et al., 1997), which suggests that while these rhythms may play a role in pubertal progression, they may be unnecessary and possibly detrimental to adult reproductive patency. Other studies indicate that normal daily rhythms of LH secretion may be altered in patients with peri-pubertal obesity or PCOS, who display relative increases in daytime release in comparison to controls (McCartney, 2010). Diurnal rhythms of sex steroids, particularly E2, have also been observed during puberty in girls, but mostly these release patterns are evident only *following* (in response to) rhythmic LH secretion, and peak in the morning after the nocturnal rise in

LH (Norjavaara et al., 1996), thus suggesting that the neuroendocrine components (i.e., the hypothalamus and pituitary) of this axis are primarily responsible for initiating pubertal progression. Supporting this, studies in primates have demonstrated that pre-pubertal increases in rhythms of GnRH and LH secretion can occur even in gonadectomized (GDX) animals (Chongthammakun and Terasawa, 1993; Chongthammakun et al., 1993). Further, many other daily hormonal rhythms, including those indicative of metabolic condition, such as leptin, growth hormone (GH), and insulin-like growth factor-1 (IGF-I), occur at the same developmental time in relation to puberty whether animals have been GDX or not (Suter et al., 2000), suggesting that the presence of circulating sex steroids in primates may not be required to generate these rhythms. While some hormonal rhythms are associated with sleep–wake cycles, evidence is accumulating that most patterns may be generated by endogenous circadian clocks.

## ALL ALONG THE CLOCKTOWER: ENDOGENOUS CLOCKS WITHIN THE REPRODUCTIVE AXIS

Since this review explores potential pubertal *timing* mechanisms, it is worthwhile to consider how both central and peripheral molecular timing devices may contribute to pubertal progression, especially considering the rhythmic patterns of reproductive hormone release in early puberty. The model of molecular timing most recently elucidated is the intracellular circadian clock, consisting of transcriptional–translational feedback loops within each cellular oscillator, in which a heterodimer of the transcription factors CLOCK and BMAL1 bind to the promoters of *Period* (Per1–3) and *Cryptochrome* (Cry1/2) genes and drive production of the PER and CRY proteins, which return to the nucleus following phosphorylation in the cytoplasm to inhibit their own transcription (Reppert and Weaver, 2002; Ko and Takahashi, 2006). Each cellular oscillator is then synchronized within a tissue by currently uncharacterized factors, and expression patterns within this tissue-level oscillator can be adjusted to changes in the ambient light environment via signals from the hypothalamic suprachiasmatic nuclei (SCN), which receives direct photic input from the retina (Yoo et al., 2004). Since the relatively recent elucidation of many of the genes involved in the core clock mechanism, several genetic models of molecular circadian clock disruption have been generated with varying results on reproduction and puberty (Boden and Kennaway, 2006). Mice harboring a somatic knockout of the core clock gene *Bmal1* exhibit significantly delayed puberty in addition to a loss of other activity and hormone secretion rhythms (Boden and Kennaway, 2004, 2005), while mutation of its heterodimeric partner *Clock*, which also disrupts free-running locomotor rhythms, appears to have little effect on pubertal progression, although adult females are subfertile (Chappell et al., 2003; Kennaway et al., 2004; Miller et al., 2004). Interestingly, lesions of the SCN disrupt adult estrous cyclicity, but appear to have no effect on rates of pubertal progression, at least in rats (Mosko and Moore, 1979). It is unknown, however, if pre-pubertal rhythms of hormone secretion persist in SCN-ablated animals. If so, this could indicate that the SCN may not be required for these reproductive rhythms, instead implicating autonomous cell-specific oscillators within the reproductive axis itself, evidence for which has been growing.

Awareness of the development and participation of cellular circadian oscillators is of particular interest to studies of pubertal initiation, given the rhythmic nature of reproductive hormone secretion prior to and during puberty. These molecular oscillators appear to function in multiple reproductive axis components, including GnRH neurons (Chappell et al., 2003; Gillespie et al., 2003; Olcese et al., 2003; Hickok and Tischkau, 2009), anterior pituitary cells including gonadotropes (Yoo et al., 2004; Resuehr et al., 2007), adrenal cortical cells (Fahrenkrug et al., 2008; Son et al., 2008), and within steroidogenic cells in the gonads (Fahrenkrug et al., 2006; Karman and Tischkau, 2006; He et al., 2007; Nakao et al., 2007; Alvarez et al., 2008; Bebas et al., 2008; Sellix and Menaker, 2010). This suggests that development of all or some of these tissue-specific oscillators may contribute to the observed reproductive hormonal rhythms. In developing rodents, changes in hormone secretory rhythms are often mirrored by alterations in locomotor activity patterns, in which both males and females elicit bimodal bouts of activity pre-pubertally that consolidate into a single period of activity in the dark phase shortly after puberty, as measured by preputial separation in males and vaginal opening (VO) in females (Hagenauer et al., 2011). In both sexes, the later bout of activity phase advances over several days/weeks to eventually merge with the activity peak observed at lights off. Interestingly, this activity consolidation appears to be mediated by the actions of gonadal steroids, since GDX males and females exhibited bimodal activity patterns into adulthood. Additionally, testosterone administration to GDX males can reverse this pattern (Daan et al., 1975; Morin and Cummings, 1981). These patterns, at least in rodents, are circadian in nature and are not merely sleep-associated, as the above effects persist in peri-pubertal animals raised in constant darkness (Hagenauer et al., 2011). What portion of trigger for pubertal initiation, then, is mediated by these endogenous timing mechanisms is unclear; however, exogenous signals that can entrain these cellular oscillators can exert robust effects on the timing of puberty.

## HOUSE OF THE RISING SUN: PHOTOPERIODIC CUES AND PUBERTAL PROGRESSION

Over the last several decades, it has become clear that photic cues, indicating not only the present point within the light/dark phase, but also communicating day length, play a large part in determining rhythms of hormone synthesis and secretion, and are clearly involved in timing sexual maturity in both opportunistic and seasonal breeders. Light stimulates specific melanopsin-expressing ganglia within the retina that signal to the SCN via a monosynaptic pathway (Ebling, 2010). SCN entrainment, in turn, uses sympathetic connections to the pineal gland, which then inhibits or stimulates melatonin production in the light or dark phase, respectively (Ebling, 2010). Melatonin production therefore acts also to reflect changes in day length, the evaluation of which is crucial to short-lived seasonal breeders. Recent evidence indicates that while many brain regions and peripheral tissues express melatonin receptors MT1 and MT2, the pars tuberalis of the pituitary is an important melatonin-binding region, acting as an interval timer to communicate the progression of shorter or longer day lengths to the reproductive axis and other physiological axes (Lincoln et al., 2002; Hazlerigg and Wagner, 2006).

If the possibility exists, then, that circadian clocks may be involved in the development of hormonal rhythms within the reproductive axis, is there evidence that pubertal changes become programmed during or after the development of circadian physiology? Recent studies in rats indicate that although molecular rhythms in the SCN and periphery develop quite early, during fetal development, and are "ticking" even in neuronal precursor cells (Kowalska et al., 2010), the ability to entrain and couple oscillators to behaviors develops later, temporally associated with the critical window of organizational steroid exposure. Orbital enucleation of juvenile rats prior to postnatal day 11 results in diurnal (in contrast to nocturnal) activity in these animals, whereas the same procedure performed later has no effect on the consolidation of activity in the dark phase (Gall et al., 2008). Unfortunately, it was not determined whether early enucleation permanently affects or shifts the timing of pubertal progression.

Evidence also exists that circadian patterns of neuronal activity are associated with normal reproductive function, and additionally that appropriate phase relationships among neurotransmitter release may be required. In a recent study, it was found that pharmacologically administered serotonin and dopamine precursors were able to increase testicular development in mice when given in a phase relationship 12 h apart, while a more "dissonant" phase relationship of 8 h between treatments impaired testicular growth, testosterone production, and spermatogenesis (Sethi and Chaturvedi, 2011). These data suggest that even in mice, which are continuous and not seasonal breeders, a mechanism of an internal coincidence timer exists, in which phase relationships of neuroendocrine activation typically induced by changes in photoperiod can profoundly affect the development of the reproductive axis. Seasonal breeders typically use this "internal coincidence model" to time pubertal progression to coincide with an optimal photoperiod for reproduction. In these species, including sheep and Siberian hamsters, photoperiodic control exerts considerable influence on pubertal progression (Foster et al., 1985). In fact, in seasonal breeders, achievement of a particular body size and nutritional state is clearly not the only component required for pubertal progression. Early studies revealed that lambs born under decreasing light conditions (i.e., in the autumn) reach pubertal size in the spring, yet delay puberty until the subsequent autumn, which suggests that photoperiodic cues in this species can override metabolic signals for progression (Foster and Ryan, 1979; Foster et al., 1986). Intriguingly, this observed delay is not a result of a decreased ability to secrete GnRH/LH, as gonadectomy of autumn-born lambs increases LH dramatically, demonstrating an intact negative feedback response to steroids (Foster and Ryan, 1981). Instead, this mechanism appears to represent an inability of the GnRH secretory system to respond to E2 with *positive* feedback-style surges, as E2 implantation in these animals continues to exert only negative feedback, profoundly inhibiting LH release (Foster et al., 1985). In contrast, lambs born in the spring ovulate normally in the fall, even as day length is decreasing; however experimental exposure to artificially shortened days can delay ovulation for up to a year. Under artificial short-day photoperiods, however, 1 week of long-day light exposure is sufficient to initiate ovulation following a return to a short-day photoperiod (Foster and Ryan, 1981), demonstrating a high sensitivity to changes in day length even during this refractory period. A similar regulatory mechanism exists in Siberian hamster females, who can either advance or delay puberty depending on when they are born in relation to the summer solstice (Butler et al., 2007). Females born into an environment of decreasing day lengths will delay puberty until the following spring, while females born a few weeks earlier will in many instances advance puberty, illustrating the importance of photoperiodic control over pubertal initiation (Butler et al., 2007). These findings also demonstrate that puberty can begin over a broad range of body mass, as pups born after the solstice attain the same weight as their counterparts when they begin pubertal progression. The above study used simulated natural photoperiods to mimic natural progression of day lengths; however, in a static photoperiodic setting, only Siberian hamster females exposed to light/dark conditions attain puberty, exhibiting commensurate increases in body weight, as well as increases in hypothalamic leptin receptor, and pro-opiomelanocortin (POMC) expression (Adam et al., 2000). Taken together, these findings indicate that even once a target body weight has been reached, photoperiodic cues exert profound effects on the timing of pubertal initiation.

There exist further variations in the timing of puberty relative to circadian activity, as illustrated by the *Octodon degus*, a diurnal Chilean rodent that exhibits a much longer estrous cycle than rats, mice, and hamsters. These animals begin to exhibit a sexual dimorphism in free-running locomotor activity long *after* puberty has concluded, a phenomenon dependent on gonadal steroids (Lee et al., 2004). In this model, circadian period is influenced after puberty, most likely by the development of sex steroid receptors in the SCN. Gonadectomy following puberty results in no period differences from what would be expected in intact animals, however, suggesting that while the actions of gonadal steroids are considered "organizational," effects exerted by this exposure are delayed until well past puberty. Much of the communication of photoperiodic encoding to the reproductive axis is performed by increased melatonin release from the pineal gland during the dark phase, as is evidenced by the absence of pubertal progression in pinealectomized ewes (Yellon and Foster, 1986). In conflict with this, however, are data showing that short-day patterns of melatonin secretion alone are not sufficient to initiate puberty (Yellon and Foster, 1986). While cues communicating metabolic status in seasonal breeders are insufficient to induce puberty, nutritional status may still play a role in allowing the development of the transition from a negative- to a positive feedback response to E2 in females. In peri-pubertal ewes, undernutrition potentiated the negative feedback effects of E2 on LH secretion, while increasing nutrition resulted in the ability of E2 to accelerate LH pulse frequency and amplitude (Foster et al., 1985). The interaction of the developing circadian system, as well as changes in neuroendocrine gene expression, occur against a backdrop of alterations in exposure to steroid hormone levels beginning *in utero*, adding a further layer of complexity.

## MAMA, YOU BEEN ON MY MIND: ORGANIZATIONAL EFFECTS OF PRE-PUBERTAL STEROID EXPOSURE ON PUBERTAL TIMING

There is a considerable amount of evidence pointing to the role of perinatal steroid hormone exposure in programming sexual differentiation and later pubertal development, although it remains

unclear what role this exposure may play in setting the timing of pubertal progression. In particular, several studies demonstrate that perinatal exposure of females to testosterone can masculinize portions of reproductive axis function, and is correlated with early puberty, altered linear growth rate, and the development of PCOS (Ibanez et al., 1996, 1998b, 2006; McCartney et al., 2007). This is of particular significance, given that elevated testosterone has been found to correlate with the insulin resistance that accompanies childhood obesity in pre-pubertal girls (Ibanez et al., 2009). Testosterone, likely of adrenal origin, may thus exacerbate problems associated with metabolic dysfunction, and may impact the reproductive axis by accelerating puberty. This rise in testosterone may be dissociated from gonadotropin stimulation, however, since earlier studies in juveniles suggest that testosterone production in males is not responsive to LH stimulation. Indeed, in hypophysectomized juvenile male rats, LH administration has little to no effect on testosterone production, in contrast to the marked response observed in adults (Odell and Swerdloff, 1976).

In female juvenile rats, circulating E2 remains relatively high until weaning, after which levels severely diminish until gradual diurnal increases in LH stimulate ovulation (Ojeda et al., 1976), and yet it remains unclear precisely when pituitary responsiveness to either GnRH or sex steroids becomes fully developed. In juvenile rats, gonadotropes in the anterior pituitary appear to respond to GnRH administration by preferentially increasing FSH over LH as early as 15 days of age (Debeljuk et al., 1972). Interestingly, this preference for FSH secretion appears to be dependent on E2, as it is reversed by ovariectomy (Ojeda et al., 1976), suggesting that ovarian E2 even in juveniles may play a role in determining gonadotropin levels. Even though the gonads may be unresponsive to gonadotropins in early juvenile development, the hypothalamic–pituitary components of the reproductive axis appear to still be responsive to the *negative* feedback effects of steroids: some early studies suggest that even under the conditions of low circulating sex steroids found in pre-pubertal animals, negative feedback responses to steroids are functional. Testosterone was found to be equally effective at decreasing basal LH levels in GDX male rats at 10, 21, or 70 days of age (Odell et al., 1974), and E2 was found to effectively suppress gonadotropin levels in juvenile female rats, but unable to elicit preovulatory LH surges until later development (Andrews et al., 1981). These results are inconsistent with the previously posited "gonadostat" theory of puberty, in which sensitivity to the negative feedback effects of steroids is altered throughout development. This hypothesis states that the juvenile pause results from an increased sensitivity to sex steroids during this developmental window (applying the "brake"), and also that free steroids are relatively plentiful due to a drop in serum hormone binding globulin (SHBG) observed during this period. However, studies in primates have found a robust sexual dimorphism in GnRH/LH responsiveness to pre-pubertal gonadectomy, resulting in dramatic increases in secretion only in females (Plant, 1985, 1986), suggesting that the development of classical feedback responses of the brain to sex steroids prior to puberty is not universal among species or between sexes.

The most intriguing and perplexing results regarding the timing of pubertal progression are found in exploration of the origins of *positive* feedback in females, particularly in rodents. Even though VO in rats does not occur until ∼day 35, a bolus of E2 can elicit an LH surge as early as 21 days, and progesterone injection in E2-primed juvenile females can elicit surges as early as 14 days of age (Puig-Duran and MacKinnon, 1978). These data align with the recent work in transgenic mouse females in which estrogen receptor alpha (ERα) was selectively deleted from *Kiss1*-expressing neurons (discussed in detail below), which display VO at 10–15 days (Mayer et al., 2010). If the capacity to produce preovulatory LH surges in response to ovarian steroids is present at this stage of development, then why is there the observed delay? Additionally, studies in primates suggest that the inhibition of GnRH/LH secretion during the juvenile pause and the initiation of puberty are regulated by timed changes in the CNS and triggered not by gonadal steroids but by other peripheral factors indicating that a threshold metabolic state has been achieved. If this were the case, why then would an ERα-dependent phenomenon be required for the juvenile pause? Perhaps pre-weaning levels of circulating E2 are required to keep the reproductive axis at bay until central inhibitory mechanisms are fully developed, or until the development of hypothalamic circuitry capable of reacting to E2 with both negative *and* positive feedback responses. The most compelling hypothalamic neuropeptidergic candidate modulating pubertal initiation, kisspeptin, is discussed in detail below. Ultimately, although it is becoming clear that maternal steroid hormone exposure and levels of circulating steroids in early infancy play a role in determining pubertal progression rate, precisely how and through what neuroendocrine substrates remains unclear. Perinatal steroid release from mothers, as well as reception of these signals in developing animals is also likely influenced by nutritional state even at this early stage. The role of metabolic signaling during embryonic development in establishing expression patterns required for pubertal progression will be explored below.

## LIKE A ROLLING STONE: KISSPEPTIN AS A STEROID HORMONE-SENSITIVE DRIVER OF GnRH SECRETION

Research into the molecular mechanisms underlying puberty has enjoyed a renaissance over the last decade, particularly since the discovery of kisspeptin (Kiss1) and neuronal populations that express and release this peptide. While its importance to reproduction was initially unknown (Lee et al., 1996), beginning in 1999 several groups successively published findings that kisspeptin is the ligand for the orphan G protein-coupled membrane receptor GPR54 in both rodents and humans (Lee et al., 1999; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001), which is now also known as the kisspeptin receptor (KISS1R). In 2003 two labs demonstrated cases of humans with idiopathic hypogonadotropic hypogonadism (IHH) who had mutations in KISS1R (de Roux et al., 2003; Seminara et al., 2003) that resulted in primary dysfunction of the reproductive axis. Mutations in *Kiss1* and *Kiss1r* were recapitulated in mice, which also displayed abnormal sexual maturation as well as decreased levels of gonadotropins and sex steroids (d'Anglemont de Tassigny et al., 2007; Dungan et al., 2007; Lapatto et al., 2007; Clarkson et al., 2008). Notably, a large majority of GnRH neurons express *Kiss1R* and are stimulated by kisspeptin (Irwig et al., 2004; Messager et al., 2005; Gottsch et al., 2006; Liu et al., 2008), providing initial evidence that kisspeptin directly regulates these neurons. Additionally kisspeptin can induce rapid

LH secretion via GnRH neuronal secretion in multiple species (Gottsch et al., 2004; Dhillo et al., 2005; Messager et al., 2005; Plant, 2006; Smith et al., 2006b), further demonstrating that *Kiss1* neurons directly signal to GnRH neurons (Clarkson and Herbison, 2006b). Interestingly, both *Kiss1* expression and GnRH neuronal sensitivity to kisspeptin appear to be developmentally regulated, in that electrophysiological responses of adult GnRH neurons to kisspeptin differ from those recorded from juvenile, pre-pubertal neurons (Han et al., 2005).

Kisspeptin neurons are also sexually dimorphic (Kauffman et al., 2007), express sex steroid receptors such as ERα (Gottsch et al., 2006; Smith et al., 2006b), and are differentially responsive to sex steroid feedback (Smith et al., 2005, 2007; Gottsch et al., 2009). Two main subpopulations of Kiss1 neurons have been found in rodents: the bilateral anteroventral periventricular (AVPV) and arcuate nuclei (ARC) of the hypothalamus. In female mice *Kiss1* expression is modulated by E2 levels: high levels of AVPV kisspeptin expression correspond with elevated ovarian E2 levels, while the opposite effect is observed in the ARC, which leads to the hypothesis that the ARC and AVPV are responsible for negative and positive feedback effects of E2, respectively (Smith et al., 2005; Dungan et al., 2006; Herbison, 2008; Popa et al., 2008; Gottsch et al., 2009). Evidence in ewes also identifies the preoptic area (POA) as a critical region for *Kiss1* regulation of the LH surge (Hoffman et al., 2011). Altered steroid hormone exposure during the developmental critical period leads to masculinization/defeminization (Kauffman et al., 2007; Gonzalez-Martinez et al., 2008) or feminization (Homma et al., 2009) of *Kiss1* neurons.

## WHEN YOU GONNA WAKE UP: POTENTIAL ROLE OF KISSPEPTIN IN THE TRANSITION TO PUBERTY FROM THE JUVENILE PAUSE

In addressing the role of kisspeptin in the previously posited "brake/accelerator" models of pubertal initiation, a recent report suggests that, at least in mice, kisspeptin neurons may be involved in both mechanisms (Mayer et al., 2010). During puberty, while GnRH content within the POA remains relatively constant, hypothalamic kisspeptin levels begin to rise, yet the cause of this increase is still unknown. Aside from observed disruption of kisspeptin signaling and a lack of pubertal progression seen in humans and mice with KISS1 and KISS1R mutations, further evidence for a functional link is evidenced by the temporal advancement of puberty that occurs in mice when treated with exogenous kisspeptin before puberty onset (Matsui et al., 2004; Navarro et al., 2004). Additionally, puberty can be delayed with Kiss1R antagonist treatment (Pineda et al., 2009). In revisiting earlier observations on GnRH/LH activity during puberty, the question arises if Kiss1 neurons might be mediating the gradually increasing daily patterns of hormone secretion observed. Indeed, recent data suggest that in adult female mice, only on proestrus or following E2-priming in ovariectomized (OVX) animals, expression levels of *Kiss1* are rhythmic with peaks occurring parallel with the GnRH surge (Khan and Kauffman, 2011). While this has not yet been investigated throughout development, it is tempting to speculate that rhythmic *Kiss1* expression and release may be responsible for the increasing diurnal rhythms of LH secretion observed in females. However, since this rhythm of expression is only evident in the presence of elevated E2 in adults, it remains unclear whether rhythmic production of *Kiss1* would occur peri-pubertally.

Another very recent *in vitro* study suggests that a complementary rhythm of *Kiss1R* expression in GnRH neurons, which is also evident only during E2 positive feedback, may also exist (Tonsfeldt et al., 2011), such that incremental timed increases in *Kiss1* expression and release corresponding with timed sensitivity of GnRH neurons to Kiss1 would act synergistically to drive GnRH and LH secretion in early puberty, supported by increasing ovarian E2. These daily "mini-surges" of gonadotropin secretion may then be sufficient to stimulate further follicular development and E2 production, which would then increase the amplitude of the neuroendocrine rhythms further to produce E2-stimulated positive feedback responses observed in adult females. The role of E2 in determining the effects of kisspeptin on pubertal progression appears to be significant, as highlighted by a recent study examining transgenic mice harboring a deletion of ERα only in Kiss1-expressing cells (Mayer et al., 2010). To achieve deletion of ERα specifically in kisspeptin neurons, a newly generated kisspeptin-IRES-Cre (KissIC) transgenic mouse line was crossed with ERα floxed mice ($ER\alpha^{lox/lox}$), and the double transgenic conditional knockout progeny were termed "KERKO" mice (Mayer et al., 2010). When these mice were analyzed for onset of puberty, as assessed by VO, they displayed a remarkable advancement of puberty. While their wild type littermates displayed VO on average at ∼29 days, the average for KERKO mice was ∼13 days. This was particularly unexpected since kisspeptin treatment in immature female rats only advanced VO by ∼5 days (Navarro et al., 2004), and it suggests that the kisspeptin neurons may be controlling puberty through other signals than kisspeptin itself. It is also possible that the response to administered kisspeptin varies from KERKO mice and other genetic models due to variance in the ability of the administered peptide to reach the critical neurons or synapses, or by activating opposing systems.

Indeed, recent studies indicate that kisspeptin neurons are actually multiphenotypic, expressing a host of other neuropeptides such as galanin, met-enkephalin, neurokinin B (NKB), and dynorphin (Goodman et al., 2007; Porteous et al., 2011). In addition to early VO, KERKO mice display abnormal estrous cyclicity with anovulation, and kisspeptin expression is differentially affected in the two main sites of expression, with AVPV levels diminished but ARC levels increased (Mayer et al., 2010), in agreement with previously observed activational effects of E2 on *Kiss1* expression (Kauffman et al., 2007; Kauffman, 2010a,b). As previously mentioned, data has shown that the AVPV is involved in positive feedback and it is postulated that the ARC is involved in negative feedback (Smith et al., 2005; Dungan et al., 2006; Herbison, 2008; Popa et al., 2008; Gottsch et al., 2009); these data confirm that ERα serves multiple purposes in these nuclei, acting as an inhibitor of kisspeptin expression in ARC neurons (the brake) and a stimulator in AVPV neurons (the accelerator).

## FOREVER YOUNG: COMPLEX MECHANISMS UNDERLYING PUBERTY

Discovery of a universal initiator of puberty has proved difficult possibly due to the importance of reproduction in the survival of species. GnRH neurons are especially known for their

heterogeneity and there are subpopulations that are still being characterized. Very few GnRH neurons are needed to successfully initiate puberty and maintain cyclicity (Gibson et al., 1984; Herbison et al., 2008), and once the system is initiated it may be able to maintain itself. Indeed, one group reported that *Kiss1R*-knockout female mice are still able to produce an E2-primed LH surge, suggesting additional pathways mediating E2 positive feedback on GnRH neurons (Dungan et al., 2007). Further evidence supporting redundancy in these circuits as well as calling into question the importance of the *Kiss1* neurons (versus kisspeptin itself) was demonstrated in a series of neuron ablation experiments (Mayer and Boehm, 2011). The aforementioned KissIC (Mayer et al., 2010) and a GPR54-IRES-Cre (GPIC) mouse lines were used to induce cell death with diphtheria toxin A (DTA) at different ages, with surprising and somewhat difficult-to-interpret results. When the KissIC line was crossed with ROSA26-DTA mice, over 90% of kisspeptin expressing cells were removed from the hypothalamus, and presumably from peripheral tissues such as the ovaries and pituitary (where there was some reported recombination) and possibly the placenta as well. However, cell death was not confirmed by looking at other markers known to be in kisspeptin neurons, such as NKB, and controls for damage to neurons adjacent to the DTA-affected kisspeptin cells were not assessed. While these mice displayed reduced ovarian mass and some minor aberrant estrus cycles, overall they did not differ substantially from controls, displaying normal development of ovarian follicles, similar average of VO, and normal fertility and fecundity. As the expression of DTA in these mice occurs when kisspeptin in expressed developmentally, an inducible diphtheria toxin receptor (iDTR) line was also used to examine ablation at different ages. Injections of diphtheria toxin post-pubertally at 20 weeks of age revealed that adult female mice require kisspeptin neurons for normal estrous cyclicity and the ability to produce offspring. Similar results were found when injection occurred at postnatal day 20. Such phenomena are not without precedent: it appears that in both reproduction and feeding, neuronal ablation during development can be compensated for by as yet unknown mechanisms, while removing the same neurons in adulthood has severe consequences (Luquet et al., 2005). A similar set of experiments were carried out with the GPIC mice crossed to the DTA and iDTR mice. Many, but not all, GnRH neurons express GPR54, and it was reported that in the DTA paradigm GnRH neurons numbers were reduced by about 90% with an accompanying decrease of *GnRH* mRNA levels by about 93%, while the iDTR in the 20 week adult ablated approximately 97% of GnRH neurons. GPIC/R26-DTA females did have reduced ovarian mass, but whether this is attributable to the reduction in GnRH neurons or other sites of GPR54 expression is not known. Strikingly, these mice all bred successfully and had mostly normal estrous cyclicity with no differences in time of VO, suggesting either developmental compensation or the ability of the remaining 10% of GnRH neurons to maintain reproduction. Remarkably, the diphtheria toxin treated adult mice remained cyclic, although subfertile, with only 50% producing offspring. When contrasted with findings that kisspeptin neuron ablation in adults causes reproductive malfunction, this suggests that, while kisspeptin signaling represents one significant pathway of reproductive patency, other signals from phenotypic kisspeptin neurons may also be important.

## THE LEVEE'S GONNA BREAK: THE ROLE OF METABOLIC CUES AS INITIATORS OF PUBERTY

While some studies in humans suggest correlations between the advance of pubertal maturation and the risk of reproductive cancers, type 2 diabetes, and metabolic syndrome, many of these studies were not corrected for childhood BMI and thus the data remain controversial and inconsistent (Gail et al., 1989; Petridou et al., 1996; Titus-Ernstoff et al., 2001; Frontini et al., 2003; Ahlgren et al., 2004; Riman et al., 2004; Vo and Carney, 2007; Lacey et al., 2009; Moorman et al., 2009). Indeed, these earlier results have been called into question by more current studies that controlled for BMI in childhood (Freedman et al., 2003; Must et al., 2005; Lakshman et al., 2008). However, a recent report on a large population of Norwegian women found that, even when data were adjusted for BMI, there was a strong inverse relationship between mortality and age at menarche (Jacobsen et al., 2007), which suggests that other factors may contribute to an increased risk of mortality with low age at menarche. Beyond possible physiological abnormalities, early puberty is also associated with significant psychosocial issues. In addition to increased rates of depression and anxiety in girls with early onset puberty (Siegel et al., 1999; Stice et al., 2001; Ge et al., 2003; Kaltiala-Heino et al., 2003; Blumenthal et al., 2009; Conley and Rudolph, 2009; Reardon et al., 2009), increases in delinquent behavior, smoking, and early sexual experiences have been reported in both girls and boys (Johansson and Ritzen, 2005; Ostovich and Sabini, 2005; van Jaarsveld et al., 2007). Some studies point to these problems persisting into adulthood, along with lower quality of life, higher rates of eating disorders, lower academic achievement, and higher rates of substance abuse (Graber et al., 2004; Johansson and Ritzen, 2005; Michaud et al., 2006; Zehr et al., 2007). In light of the established and potential detrimental effects of both early puberty and disruption of fertility, understanding the metabolic factors controlling puberty and reproductive maturation and function remains crucial.

Due in part to the connection of early puberty onset and overweight/obesity, considerable research over the last 5 years investigated the link between metabolism, kisspeptin, and reproduction. One such potential connection is leptin, a circulating hormone secreted by adipocytes in proportion to body fat stores thereby acting as an adiposity signal (Cummings and Schwartz, 2003; Farooqi and O'Rahilly, 2005). In addition to the exciting results of leptin replacement therapy on obesity in people with congenital leptin deficiency (Schwartz et al., 2000; Farooqi and O'Rahilly, 2005), leptin therapy can also be effective at treating some types of infertility. While many patients reporting congenital leptin deficiency began leptin treatment as pre-pubertal children, one study reported on adult subjects who all displayed hypogonadotropic hypogonadism, although there was one case of possible spontaneous pubertal development after an approximate 20 year delay (Ozata et al., 1999). This holds true for mice with congenital leptin deficiency (*ob/ob*) or leptin receptor mutations (*db/db*). These mice remain perpetually pre-pubertal with incomplete reproductive organ development and low LH levels (Swerdloff et al., 1976; Coleman, 1978; Zhang et al., 1994; Tartaglia et al., 1995), and leptin treatment in *ob/ob* mice restores the maturation of reproductive organs and fertility while weight loss alone cannot induce these changes (Barash et al., 1996; Chehab et al., 1996, 1997; Mounzih

et al., 1997; Cunningham et al., 1999; Chehab, 2000). In younger children, leptin therapy has enabled normal pubertal progression without causing early onset puberty (Farooqi et al., 1999, 2002), suggesting that while leptin is permissive for pubertal progression, it does not serve as a lone initiation signal for puberty. Leptin has also been used to restore menstruation in women with hypothalamic amenorrhea and lipodystrophy (Welt et al., 2004; Musso et al., 2005; Chou et al., 2011). Other studies in mice demonstrated that supraphysiological leptin levels can accelerate the onset of puberty to a limited extent (Ahima et al., 1997; Yura et al., 2000).

Leptin binds to receptors in multiple areas of the brain (Balthasar, 2006), including the ARC, which also houses Kiss1 neurons. It has been demonstrated that the active form of the leptin receptor (*LepRb*, also known as *Ob-Rb*) is not expressed in GnRH neurons themselves (Finn et al., 1998; Quennell et al., 2009), but is present in a subpopulation of Kiss1 neurons in the ARC, although the reported extent of this co-expression varies widely (Smith et al., 2006a; Cravo et al., 2011; Louis et al., 2011; True et al., 2011). Additionally, hypothalamic *Kiss1* mRNA levels are reduced in *ob/ob* mice (Smith et al., 2006a; Quennell et al., 2011) and streptozotocin-induced diabetic rats (Castellano et al., 2006, 2009). Leptin was able to partially restore Kiss1 levels in *ob/ob* mice (Smith et al., 2006a) and fully in diabetic rats (Castellano et al., 2006). Fasting and undernutrition reduce *Kiss1* expression and alter reproductive function, and these defects can be rescued by exogenous kisspeptin administration (Castellano et al., 2005; Roa et al., 2008). A recent study investigating the effects of overnutrition and undernutrition on kisspeptin expression and pubertal onset used female rats in varying litter sizes to achieve postnatal underfed, normal fed, and overfed models (Castellano et al., 2011). Overfeeding resulted in increased body weight, earlier onset of puberty (as measured by VO), and increased leptin and hypothalamic *Kiss1* expression, while opposite effects were seen in the underfed rats, along with lower weight ovaries and uteri. Immunohistochemistry for *Kiss1* showed lower kisspeptin in the ARC in the underfed rats and increased kisspeptin in the AVPV of overfed rats, but when peri-pubertal rats were challenged with central administration of Kp-10, all groups responded similarly. As noted in the study, litter size manipulations may have changed additional signals (Knox et al., 2009; Kinsey-Jones et al., 2010) and alterations of other peripheral hormones may have also affected *Kiss1* expression and pubertal timing (Tena-Sempere, 2008; Forbes et al., 2009). Whether this demonstrates a causative relationship or only the ability of kisspeptin to both be affected by and bypass the underlying causes of reproductive defects remains a key question.

It was previously reported using double-label *in situ* hybridization (ISH) that about 40% of the *Kiss1* expressing neurons in the ARC also expressed *LepRb* (Smith et al., 2006a), but three recent studies have not found such high levels of co-localization. Citing issues with sensitivity of *LepRb* mRNA, the first study used LepRb-eGFP mice to examine co-localization of LepRb with GnRH and Kiss1 in female mice (Louis et al., 2011). In agreement with earlier reports (Finn et al., 1998; Quennell et al., 2009), no co-localization of LepRb and GnRH was observed, but using a trans-synaptic tracer (wheat germ agglutinin, WGA), experimenters determined that GnRH neurons receive input from nearby LepRb-eGFP neurons. However, they did not observe high levels of Kiss1/LepRb co-localization. Kisspeptin immunoreactivity (Kp-ir) did not co-localize with LepRb-eGFP in the AVPV, and no co-localization of leptin-induced STAT3 phosphorylation (pSTAT3-ir) with Kp-ir in the POA of sheep was noted. Due to difficulties using Kp-ir in the ARC, even with colchicine treatment, NKB was used as a marker. Rather than the 40% co-localization in the ARC seen by ISH, only 5–6% NKB/LepRb-eGFP co-localization was observed. Many other cell types in the ARC robustly express LepRb, and using WGA as a tracer it was observed that at least some of these LepRb neurons lay in close contact with Kiss1 neurons also in the ARC. The second study used a new line of *Kiss1*-Cre mice crossed with GFP and βGal reporter lines to mark kisspeptin neurons (Cravo et al., 2011). Using pSTAT3-ir to mark leptin responsive cells, no co-localization was reported in the AVPV of these mice. pSTAT3-ir/*Kiss1*-Cre co-localization in the ARC was seen in ∼10–15% of *Kiss1*-Cre neurons (depending on rostral-to-caudal level), which translated to between 2 and 10% of the pSTAT3-ir neurons (depending again on level), as there were more pSTAT3-ir positive cells in the ARC. A third study explored activation of three markers of leptin signaling: pSTAT3 and 5 as well as pS6 as a marker of mTORC1 pathway signaling in kisspeptin neurons, using Kp-ir (Quennell et al., 2011). While leptin injections in fasted mice activated all of these pathways in the AVPV, ARC, and other areas, experimenters observed no co-localization of Kp-ir with leptin-induced signals in the AVPV and were unable to determine co-localization in the ARC due to the dense kisspeptin fiber network. The disparity between these studies may be due to differing techniques, but the results of the newer studies bring into question how much of a direct effect leptin has on kisspeptin neurons, and how much of the effect of leptin on reproduction must be afferent to the kisspeptin neurons or through different cell pathways altogether.

This question is currently being examined. Now that multiple Kiss1-Cre mouse lines have been created (Mayer et al., 2010; Cravo et al., 2011; Gottsch et al., 2011), they can be used as powerful tools to dissect the importance of gene pathways in these neurons. A recent study used one of these lines to delete LepRb from the kisspeptin neurons (Donato et al., 2011). After gonadectomy to intensify the expression of *Kiss1* mRNA in the ARC, leptin treatment was used in conjunction with pSTAT3-ir to observe leptin signaling. Using this technique, approximately 20% co-localization was seen in the ARC of wild type males and 13% in wild type females, and this was reduced to 5% in the conditional homozygous knockouts (Kiss1-Cre/LepRb$^{flox/flox}$), which suggests significant but incomplete deletion of LepRb from Kiss1 neurons. These mice exhibited no defects in age at VO, days to pregnancy, or fecundity, and they conclude that leptin signaling in these neurons is unnecessary for reproduction. Due to the fact that partial leptin signaling was still present in these mice, perhaps a stronger demonstration of this could have been achieved by reactivating the LepRb in Kiss1 neurons, as was done in the ventral premammillary nucleus (PMV) later in the same publication. Notably, bilateral lesions of the PMV did not prevent normal food intake and body weight responses to leptin injections, but sexual maturation was disrupted by these lesions. Restoration of LepRb in the PMV rescued puberty, improved fertility and normalized GnRH content, although it did not affect body weight and food intake.

PMV neurons with LepRbs innervate both AVPV Kiss1 neurons and GnRH terminals, thus providing a potential pathway for leptin to regulate both Kiss1 and GnRH. An earlier study in juvenile primates demonstrated that lesions proximal to this area resulted in precocious puberty, implicating this hypothalamic region not only in metabolic sensing, but also in the regulation of pubertal timing (Windsor-Engnell et al., 2007). Another group examined the interactions of kisspeptin and leptin using a long-term calorie restriction model in OVX female rats with or without low levels of E2 replacement (True et al., 2011). Forty percent of caloric restriction (CR) decreased leptin levels in both calorie restricted groups, with lower leptin levels observed in OVX animals. Surprisingly, LH levels were significantly decreased with CR only in E2-treated rats, suggesting that LH inhibition by CR requires the actions of E2. *Kiss1* mRNA in the ARC was decreased with calorie restriction regardless of E2 treatment, but AVPV *Kiss1* was unchanged in both groups. However, physiological doses of leptin had no effect on ARC *Kiss1* levels and, strangely, AVPV *Kiss1* actually decreased. Due to considerable variability in LH levels in rats with 40% CR, treatments were repeated with 50% CR, but only in OVX + E2 animals this time. Greater loss of body weight and reduced LH variability were observed, suggesting that a certain amount of weight loss is necessary to inhibit LH. In this paradigm both ARC and AVPV *Kiss1* levels were decreased and were not restored by 72 h leptin treatment. While these data conflict with previous studies showing the ability of leptin to rescue *Kiss1* levels (Castellano et al., 2006; Smith et al., 2006a), the authors argue that this is due to a difference of pharmacological versus physiological dosing. The same group examined whether there was significant leptin signaling in the Kiss1 neurons in the ARC, and while they too saw high levels of pSTAT3-ir in the ARC of leptin treated animals, there were very few co-localized Kp-ir/pSTAT3-ir cells, in agreement with previously discussed recent results (Cravo et al., 2011; Louis et al., 2011). While the results from Donato et al. (2011) imply an indirect mechanism for the involvement of leptin with kisspeptin signaling and reproduction, the study by True et al. (2011) suggests that leptin may not be the metabolic signal integrating reproduction and nutrition, rather, another candidate or candidates such as glucose, insulin, ghrelin, and neuropeptide Y (NPY) might instead represent the elusive missing link. Regardless, future studies teasing apart the neuronal circuitry will be aided by new transgenic mouse lines such as the recently created Kiss1-Cre (Mayer et al., 2010; Cravo et al., 2011).

## TANGLED UP IN CUE: ENDOGENOUS CENTRAL AND PERIPHERAL CLOCKS AS POSSIBLE LINKS BETWEEN METABOLISM AND REPRODUCTION

Although many studies in human and animal models suggest that achievement of a threshold body mass is important for pubertal initiation, much of these data are correlative, and studies in rodents investigating this "body fat hypothesis" have found only limited evidence of such a link (Bronson and Manning, 1991). Due to this, some investigators have proposed that the neuroendocrine control of reproduction converging at the level of GnRH neurons is instead responsive to rapid nutritional changes, using multiple metabolic cues to communicate changes to the hypothalamus (Wade and Schneider, 1992; Wade et al., 1996). This "metabolic fuels hypothesis" posits that availability of oxidizable substrates may represent the crucial signal to the hypothalamic circuitry regulating reproduction, and that even obesity-related infertility may result from a lack of usable calories, as hyperphagic adipocytes may sequester oxidizable fuels required for fertility. A number of experimental models resulting in glucoprivation result in rapid declines in LH secretion, suggesting that hypothalamic reproductive circuits are extremely sensitive to hypoglycemia, such that both estrous cyclicity in adults as well as timing of pubertal initiation would be altered by nutritional state (Schneider et al., 1993, 2000). The neuronal circuitry responsible for communicating these metabolic changes to GnRH neurons is incompletely characterized, but studies implicate a role for hepatic signals transmitted via vagal innervation of the area postrema/nucleus tractus solitaries (Wade et al., 1996). It is currently unclear if these signals are transduced to communicate with GnRH neurons via *Kiss1*-expressing populations.

While recent studies suggest that kisspeptin is a required player in pubertal initiation, and that leptin may also contribute to this process, it still remains unclear what other peripheral factors may play a role in conferring the signal of metabolic readiness to Kiss1 neurons and/or to the rest of the neuroendocrine reproductive axis. Furthermore, the observation of daily leptin (Sinha et al., 1996; Palmert et al., 1998; Kasa-Vubu et al., 2002) and adiponectin (Shea et al., 2005; Gomez-Abellan et al., 2010; Scheer et al., 2010) rhythms in peri-pubertal animals and humans in conjunction with a growing awareness of the contribution of circadian oscillators in both metabolic control and reproduction suggest that endogenous clocks may play a role in the initiation of puberty. If, indeed, developing circadian oscillators functioning at single or multiple points in the reproductive axis are required for eventual maturation of the positive feedback effects of E2 in females, it is important to examine, then, how human lifestyle patterns of nutrition both perinatally and during juvenile development might affect these central and peripheral oscillators. Due to the recursive relationship between cellular and organismal metabolic regulators and the circadian clock (reviewed in detail in Asher and Schibler, 2011), a recent study used a model of aberrant metabolic state to determine what effects metabolic abnormalities may exert on core clock and clock-controlled gene expression patterns. Ando et al. (2011) found that *ob/ob* mice lacking leptin production exhibited long-lasting alterations in clock gene expression patterns that began early in development and persisted into adulthood. Interestingly, clock gene expression patterns in the SCN were unaltered in *ob/ob* mice, but expression patterns in the liver and adipose tissue were severely blunted, even in adults raised on a low-calorie diet (Ando et al., 2011). Short-term (1 week) administration of leptin was able to rescue clock gene cycling in the liver, but not as dramatically as in adipose tissue (Ando et al., 2011), which implies that exposure to this adipose-derived factor throughout development is required for function of peripheral clocks. Reproductive hormone secretion patterns were not examined in this study; however, previous work has established that *ob/ob* mice exhibit delayed puberty and diminished reproductive capacity, both of which can be reversed by leptin administration. Leptin itself is secreted from adipose cells with a circadian rhythm (Sinha et al., 1996; Palmert et al., 1998; Kasa-Vubu et al., 2002;

Wilson et al., 2003), suggesting that this adipokine may act as a permissive factor for pubertal development by maintaining oscillators throughout the reproductive axis. It is unknown if molecular clock oscillations in either Kiss1 or GnRH neurons are affected in leptin-deficient mice, but studies described below demonstrate that metabolic signals can dramatically affect hypothalamic gene expression patterns. Deviations from a normal pattern of leptin production, such as those encountered in obesity, may then lead to dysregulation of normal hypothalamic gene expression patterns required for timing puberty. It is clear from other work that restricted feeding paradigms, which have no effect on oscillators within the light-entrainable SCN, can drive circadian oscillations of gene expression in peripheral tissues, even in the absence of leptin signaling, as demonstrated by the reinstatement of clock cycling in the liver of *db/db* mice subjected to timed restricted feeding (Kudo et al., 2004).

There is considerable evidence in both human and rodent models that perinatal nutrition can also exert later effects on both metabolism and reproduction. Human mothers exposed to low-protein, high-carbohydrate diets often have low birthweight babies that exhibit accelerated growth in the juvenile period compared to children with proper perinatal nutrition (Ibanez et al., 1998a, 2001). This is mimicked in rats: low-protein fed dams give birth to pups with lower weight, which soon overtake their normally fed counterparts in food intake (Coupe et al., 2009; Tarry-Adkins et al., 2009). Additionally, the effects of maternal undernutrition have profound effects on gene expression rhythms in the hypothalamus, many of which persist into adulthood. A recent study found that these core clock gene expression patterns were blunted, and many other clock-controlled genes important for metabolic regulation, including orexigenic and anorexigenic peptides such as NPY, POMC, agouti-related peptide (AgRP), and cocaine and amphetamine-regulated transcript (CART), were also altered (Orozco-Solis et al., 2011). Additionally, maternal undernutrition resulted in profound alterations in expression patterns of fatty acid synthase, PGC1α, and glucokinase in the liver of pups (Orozco-Solis et al., 2011), demonstrating that perinatal nutrition is important for clock gene cycling in both central and peripheral clocks. Regarding human health, a combination of poor perinatal nutrition and childhood obesity would appear to have profound effects on gene expression patterning in both the neuroendocrine hypothalamus and peripheral organs, which could in turn impact timing mechanisms controlling duration of juvenile pause and pubertal initiation.

Enhancing the complex picture of metabolic regulation, more recent studies have added further links between cellular metabolism, circadian clock machinery, and neuroendocrine control of reproduction. It was recently found that the nutrient-sensitive adenosine monophosphate kinase (AMPK) likely plays a major role in both metabolic signaling in the liver and adipose tissues (Mor and Unnikrishnan, 2011), as well as a role in controlling GnRH secretion in response to adipocyte-derived factors such as adiponectin (Cheng et al., 2011). Additionally, AMPK affects endogenous circadian clocks by phosphorylation of negative feedback components PER and CRY, leading to the rapid degradation of these proteins in the clock loop (Lamia et al., 2009). AMPK nuclear accumulation and activity is circadian, and is likely an important regulator integrating cellular metabolism with the clock (Um et al., 2011). Interestingly, GnRH neurons and immortalized GT1–7 cells express AMPK subunits, and the AMPK activators metformin and AICAR have direct effects on GnRH secretory rate (Coyral-Castel et al., 2008; Roland and Moenter, 2011). Indeed, adiponectin inhibits GnRH secretion through an AMPK-mediated pathway (Cheng et al., 2011), and activation of this kinase decreases GnRH secretion associated with perinatal androgen exposure (Roland and Moenter, 2011), which induces a PCOS-like phenotype. Prenatal testosterone exposure results in an overactive GnRH pulse generator, with isolated GnRH neurons displaying an increased basal firing rate, an effect reversed by AMPK activation (Roland and Moenter, 2011).

## SLOW TRAIN: CONCLUSIONS AND FUTURE CONSIDERATIONS

If, indeed, appropriate timing of puberty and regulation of the reproductive axis in adults requires functioning circadian oscillators at multiple points, a model of metabolic signaling can be envisioned to integrate these disparate players. Under normal conditions of perinatal steroid and nutrition exposure, the neuroendocrine hypothalamus of juvenile animals would experience antiphasic rhythms of leptin and adiponectin, both of which serve to signal metabolic status in a rhythmic pattern, with leptin acting as a permissive cue and adiponectin potentially preventing over-activation of the GnRH pulse generator. Priming of GnRH and Kisspeptin neurons with these rhythmic signals, along with the development of oscillators within these neuronal populations, could then lead to gradual increases in GnRH/LH secretion also with a circadian rhythmicity, as has been observed. Increases in neuroendocrine output would begin to prime the gonads, and in females, ovarian estrogen could potentially drive these hypothalamic rhythms toward positive feedback responsiveness, resulting in ovulation. In adulthood, these rhythms would then only be evident under elevated E2 exposure, driving ovulation with an infradian (>24 h) rhythm appropriate with follicular development and sexual behavior. Conversely, metabolic disruptions resulting from prenatal undernutrition and childhood obesity would alter this process at multiple points. Levels of leptin and adiponectin would increase concomitant with increased fat mass, and metabolic syndrome associated with obesity and sedentary behavior would decrease activation of AMPK in multiple cell types, thus blunting core clock and clock-controlled gene expression patterns throughout the reproductive axis. This model remains purely conjectural, however, and will require extensive studies that examine gene expression oscillations in reproductive cell types throughout the juvenile pause and puberty, and investigation into how these patterns are altered by obesity and other metabolic dysfunction.

While endogenous clocks likely play a role in physiological processes governing complex behaviors and higher cognitive function, as well as in fundamental molecular mechanisms controlling cellular respiration, chromatin remodeling, and oxidative damage and repair, precisely how these clocks exert their effects by controlling rhythms of transcription, translation, and protein abundance remains unclear, and efforts remain ongoing to include them in

existing conceptual models. Future work will be necessary to determine how peripheral metabolic signals, gonadal steroid hormones, and genetically programmed developmental processes converge to precipitate one of the most crucial processes in mammals – that of sexual maturation. More basic research in this area will be better able to inform studies of how trends in human behavior governing nutrition and activity may exert broader impacts on our developmental physiology as a whole.

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# Gliotransmission by prostaglandin $E_2$: a prerequisite for GnRH neuronal function?

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Over the past four decades it has become clear that prostaglandin $E_2$ ($PGE_2$), a phospholipid-derived signaling molecule, plays a fundamental role in modulating the gonadotropin-releasing hormone (GnRH) neuroendocrine system and in shaping the hypothalamus. In this review, after a brief historical overview, we highlight studies revealing that $PGE_2$ released by glial cells such as astrocytes and tanycytes is intimately involved in the active control of GnRH neuronal activity and neurosecretion. Recent evidence suggests that hypothalamic astrocytes surrounding GnRH neuronal cell bodies may respond to neuronal activity with an activation of the erbB receptor tyrosine kinase signaling, triggering the release of $PGE_2$ as a chemical transmitter from the glia themselves, and, in turn, leading to the feedback regulation of GnRH neuronal activity. At the GnRH neurohemal junction, in the median eminence of the hypothalamus, $PGE_2$ is released by tanycytes in response to cell–cell signaling initiated by glial cells and vascular endothelial cells. Upon its release, $PGE_2$ causes the retraction of the tanycyte end-feet enwrapping the GnRH nerve terminals, enabling them to approach the adjacent pericapillary space and thus likely facilitating neurohormone diffusion from these nerve terminals into the pituitary portal blood. In view of these new insights, we suggest that synaptically associated astrocytes and perijunctional tanycytes are integral modulatory elements of GnRH neuronal function at the cell soma/dendrite and nerve terminal levels, respectively.

**Keywords: gliotransmitter, cyclooxygenase, nitric oxide, hypothalamus, reproduction**

## INTRODUCTION

Sexual development, puberty, and adult fertility are achieved by events that are initiated within the central nervous system and require the maturation and function of a neural network that transmits both homeostatic and external cues to the discrete hypothalamic neuronal population that releases gonadotropin-releasing hormone (GnRH) from neuroendocrine terminals within the median eminence into the pituitary portal vessels to control gonadotropins (luteinizing hormone, LH and follicle stimulating hormone, FSH) secretion (Terasawa and Fernandez, 2001; Herbison and Neill, 2006; Malpaux, 2006; Ojeda and Skinner, 2006; Plant, 2006; Donato et al., 2011). In turn, these gonadotropins act on the ovaries and testis to regulate the secretion of sex steroids and the production of eggs and sperm.

In addition to neurons, accumulating evidence over the past two decades indicates that glial cells, and in particular astrocytes and tanycytes, also contribute to the neural network that converges onto GnRH neurons to control reproduction. Both the neuronal and glial elements of this GnRH neural network are subject to the direct modulatory influence of gonadal steroids (Garcia-Segura and McCarthy, 2004; Ronnekleiv and Kelly, 2005; Mong and Blutstein, 2006; Wintermantel et al., 2006; Christian and Moenter, 2010; Prevot et al., 2010a; Bellefontaine et al., 2011). Although neuronal elements regulate the activity of GnRH neurons through a complex array of excitatory and inhibitory synaptic inputs, glial cells communicate with GnRH neurons via the activation of specific growth-factor-dependent signaling pathways (reviewed in Melcangi et al., 2002; Herbison and Neill, 2006; Mahesh et al., 2006; Ojeda and Skinner, 2006; Sharif and Prevot, 2010).

The main glial population in the brain consists of astrocytes that ensheathe synapses and are in contact with blood vessels. They regulate blood flow, provide much-needed energy to neurons, and supply the building blocks for neurotransmitters at the synapses, in addition to dynamically contributing to information processing within the central nervous system (Haydon and Carmignoto, 2006; Martineau et al., 2006; Iadecola and Nedergaard, 2007; Eroglu and Barres, 2010; Halassa and Haydon, 2010; Pfrieger, 2010; Di Castro et al., 2011; Panatier et al., 2011), including the hypothalamus (Hatton and Wang, 2008; Theodosis et al., 2008; Gordon et al., 2009; Panatier, 2009; Oliet and Bonfardin, 2010). Tanycytes are elongated radial glial cells that have many features in common with astrocytes and are closely associated with neuroendocrine terminals in the median eminence of the hypothalamus (Theodosis et al., 2008; Prevot et al., 2010a,b; Dale, 2011; Sild and Ruthazer, 2011). As integrative hubs, astrocytes and tanycytes likely play a fundamental role in shaping and regulating the GnRH system.

Here, we will review recent findings that illustrate the remarkable interplay between glia and neurons within the hypothalamo-hypophyseal–gonadal axis, and show that different glial cell types regulate different aspects of the architecture, function, and plasticity of the GnRH system through dynamic and often multidirectional interactions with specialized neuronal junctions, such as synapses and neurohemal junctions. We will mainly restrict our focus to the roles of hypothalamic astrocytes and tanycytes subserved by the release of prostaglandin $E_2$ ($PGE_2$), a molecule that has long been known to regulate GnRH neuronal function and has recently been identified as a gliotransmitter.

## PROSTAGLANDIN $E_2$ AND THE CENTRAL CONTROL OF REPRODUCTION

Prostaglandin $E_2$ is one of a number of prostanoids synthesized from arachidonic acid, which is produced from membrane phospholipids by a phospholipase $A_2$. Arachidonic acid is converted to bioactive prostanoids by the cyclooxygenases (COX-1 and COX-2) and a class of terminal synthases (see for review Bosetti, 2007; **Figure 1**). Several studies suggest that $PGE_2$ is mainly derived from the COX-2 pathway (Brock et al., 1999; Vidensky et al., 2003; Sang et al., 2005). $PGE_2$ signaling is propagated by four G-protein-coupled receptors, EP1–EP4 (see for review Coleman et al., 1994; **Figure 1**).

Prostaglandin $E_2$ has been known to play a role in the central control of reproduction for more than 35 years. The first indication that $PGE_2$ was involved in the process of GnRH secretion was provided by experiments showing that $PGE_2$ injected into the third ventricle of the rat brain induced the release of LH into the general circulation (Harms et al., 1973) and of GnRH into the pituitary portal blood vessels (Eskay et al., 1975; Ojeda et al., 1975b). A similar stimulatory effect of $PGE_2$ on GnRH release has also been documented in monkeys using push–pull perfusion in conscious animals (Gearing and Terasawa, 1991). To bring about the activation of the GnRH axis, $PGE_2$ acts at two main hypothalamic sites: the preoptic-anterior hypothalamic region in which GnRH cell bodies reside, and the tuberal region of the hypothalamus, which contains the median eminence and GnRH-releasing neuroendocrine terminals (Ojeda et al., 1977). The use of COX inhibitors such as indomethacin has provided further support for a physiological role of the prostaglandins in the control of GnRH release. Indomethacin administration suppresses the LH surge induced by estradiol during anestrus in ewes (Carlson et al., 1974) and during the early follicular phase in rhesus monkeys (Carlson et al., 1977). In rats, the intraventricular or intrahypothalamic administration of indomethacin inhibits both pulsatile LH release and the LH discharge induced by ovarian steroids (Ojeda et al., 1975a). Other studies have demonstrated that the microinjection of either aspirin, a non-steroidal COX inhibitor, or N-0164, a prostaglandin and thromboxane antagonist, into the tuberal region of the rat hypothalamus results in the suppression of ovulation (Labhsetwar and Zolovick, 1973; Botting et al., 1977). Finally, experiments conducted using hypothalamic explants *in vitro* have revealed that $PGE_2$ is an effective stimulator of GnRH release from median eminence nerve terminals (Gallardo and Ramirez, 1977; Ojeda et al., 1979, 1986b).

A sizable body of evidence also implicates $PGE_2$ as a physiological component of the GnRH system during postnatal development. For instance, $PGE_2$ can induce the release of GnRH long before puberty in both mice and rats (Ojeda et al., 1986a; Prevot et al., 2003b). As puberty approaches, the increasing output of estradiol from the developing ovaries induces a preovulatory surge

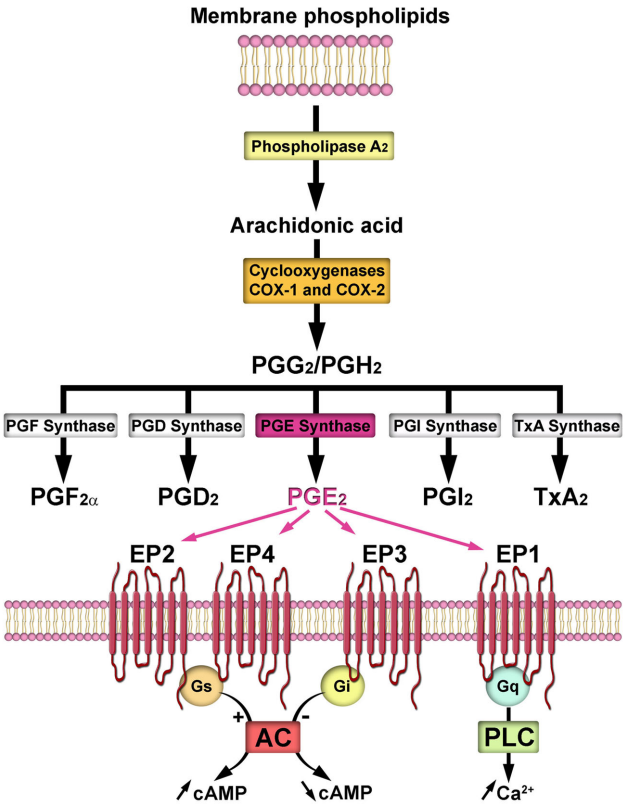


**FIGURE 1 | Prostaglandin $E_2$ ($PGE_2$) biosynthesis and signaling.** Upon its release from plasma membrane phospholipids by phospholipase $A_2$, arachidonic acid is converted to the unstable endoperoxide intermediates, prostaglandin $G_2$ ($PGG_2$), and prostaglandin $H_2$ ($PGH_2$) by the cyclooxygenases (COX-1 and COX-2, encoded by separate genes). Both COX isoforms catalyze the same reactions, but while COX-1 is constitutively expressed, COX-2 is rapidly and transiently upregulated by cytokines and growth factors. Terminal synthases convert both $PGG_2$ and $PGH_2$ into prostaglandins [$PGE_2$, $PGD_2$, $PGF_2\alpha$, prostacyclin ($PGI_2$)], and thromboxane ($TxA_2$). Once synthesized, $PGE_2$ immediately diffuses away and activates its specific E-prostanoid receptors (EP1–4), which belong to the family of seven-transmembrane G-protein-coupled receptors. EP2 and EP4 are coupled to Gs and stimulate the adenylyl cyclase (AC)–cyclic adenosine monophosphate (cAMP)–protein kinase A (PKA) pathway. In contrast, EP3 is coupled to Gi and inhibits AC activation, resulting in decreased cAMP concentrations. EP1 is thought to be coupled to the Gq-phospholipase C (PLC) pathway, leading to an elevation of free cytosolic calcium concentrations (Milatovic et al., 2011). Notably, an examination of the capacity of the hypothalamus to metabolize arachidonic acid through the COX pathway has revealed a pubertal increase in the formation of $PGE_2$, particularly during the first proestrus (Ojeda and Campbell, 1982). Intriguingly, the increase in $PGE_2$ synthesis is not associated with changes in the formation of $PGF_2\alpha$, $PGI_2$, $PGD_2$, or thromboxane $A_2$ from exogenous arachidonic acid, suggesting that it is a specific event directly associated with the peripubertal activation of the reproductive hypothalamus (Ojeda and Campbell, 1982). Such a selective synthesis of $PGE_2$ has also been shown to be triggered by estrogens during early postnatal development (Amateau and McCarthy, 2002).

of GnRH/LH. Biochemical analyses at this last phase of sexual maturation have demonstrated that the capacity of the reproductive hypothalamus to metabolize arachidonic acid through the COX pathway leads to a specific increase in $PGE_2$ synthesis (**Figure 1**), particularly during the first proestrus (Ojeda and Campbell, 1982). This effect appears to be estrogen-dependent since it is mimicked by the treatment of juvenile animals (early post-weaning period) with estradiol at doses capable of inducing a preovulatory surge of LH (Ojeda and Campbell, 1982). More recent studies have shown that an estradiol-induced increase in hypothalamic $PGE_2$ levels can be seen even in newborn rats (Amateau and McCarthy, 2002). Intriguingly, experiments showing that estradiol treatment upregulates both COX-2 mRNA and protein synthesis in the hypothalamus of female rats during postnatal development (Amateau and McCarthy, 2004) raise the possibility that estrogens may act on COX-2 expression to promote $PGE_2$ synthesis at puberty.

Since the levels of arachidonic acid in tissue phospholipids are to some degree influenced by the dietary intake of different polyunsaturated fatty acids (e.g., linoleic acid), malnutrition during early life could cause deficits in arachidonic acid synthesis (de Souza et al., 2011; Lauritzen and Carlson, 2011), which in turn could lead to changes in the production of prostaglandins and thus interfere with the maturation of the GnRH system. Indeed, a diet-related deficiency of essential fatty acids initiated before fertilization in female rats has been shown to significantly delay the onset of reproductive capacity in female offspring without affecting the progression of gestation or the delivery of healthy litters by the dams (Smith et al., 1989). In this study, delayed puberty was associated with reduced $PGE_2$ synthesis within the hypothalamus. The deficit did not appear to be due to the impairment of COX activity, but to diminished arachidonic acid bioavailability (Smith et al., 1989).

## GLIA, THE MAIN SOURCE OF PROSTAGLANDIN $E_2$ WITHIN THE GnRH NEUROSECRETORY SYSTEM

Although $PGE_2$ was initially postulated to be an intracellular messenger produced by the binding of neurotransmitters to receptors located on GnRH neurons and acting within these neurons (Ojeda et al., 1982; Gearing and Terasawa, 1991; Rettori et al., 1992), this concept has been revisited following studies showing that the actions of $PGE_2$ on GnRH release are initiated by its binding to specific membrane receptors (Coleman et al., 1994) expressed by GnRH neurons (Rage et al., 1997) and the recognition that astrocytes represent a major source of $PGE_2$ in the brain (Ma et al., 1997; Bezzi et al., 1998; Hirst et al., 1999). Two decades ago, seminal studies by Ojeda and colleagues revealed that the $PGE_2$-mediated activation of GnRH neuronal secretory activity triggered by estrogen at the time of puberty required the activation of growth-factor-dependent glial signaling pathways involving receptor tyrosine kinases of the erbB family (Ojeda et al., 1990; Junier et al., 1991; Ma et al., 1992).

Of the four known members of the erbB family (**Figure 2**), three of them, erbB1, erbB3, and erbB4, bind and are activated by cognate ligands. In contrast, erbB2 has no known ligand, and functions primarily as a modulator of the other members of the family (Hynes and Lane, 2005). While erbB receptors do not appear to be expressed in GnRH neurons (Ma et al., 1994b, 1999; Voigt et al., 1996; Prevot et al., 2003b), erbB1, erbB2, and erbB4, but not erbB3, are expressed in hypothalamic astrocytes, known to morphologically and physically interact with GnRH cell bodies (Witkin et al., 1995; Cashion et al., 2003; Baroncini et al., 2007; Sandau et al., 2011a) both in rodents and humans (**Figures 3** and **4**; Ma et al., 1999; Prevot et al., 2003b; Sharif et al., 2009). In addition, hypothalamic astrocytes express the erbB1 ligand, transforming growth factor alpha (TGFα; **Figure 4**), and several forms of the erbB4 ligand, neuregulin (Ma et al., 1992, 1994a, 1999; Sharif et al., 2009). Importantly, gonadal steroids have been found to induce dramatic increases in the expression levels of the erbB receptors and their ligands within the hypothalamus at puberty; no such changes are seen in the cortex or other brain regions unrelated to reproductive control (Ma et al., 1992, 1994a, 1999).

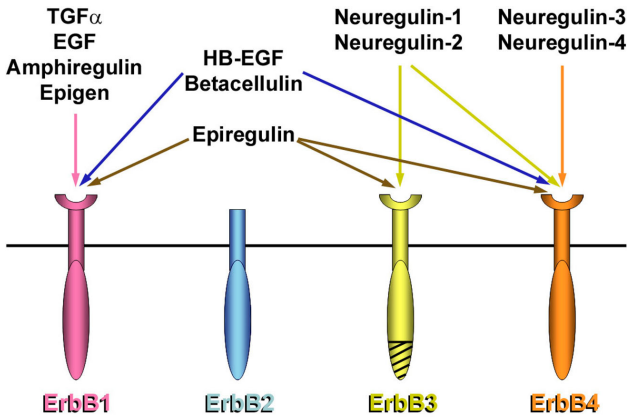


**FIGURE 2 | The erbB family of receptors and their ligands.** ErbB1 (or EGFR, epidermal growth factor receptor) and erbB4 are fully functional receptors that possess an extracellular ligand-binding domain and a cytoplasmic protein tyrosine kinase domain and can function as homo- or heterodimers. In contrast, erbB2 (or neu), which lacks a ligand-binding domain, and erbB3, which is defective in its intrinsic tyrosine kinase activity (dashed lines), must heterodimerize with another member of the erbB family for signal transduction. The different EGF-like growth factors exhibit different binding specificities for the erbB receptors. While TGFα, EGF, amphiregulin, epigen, neuregulin-3, and neuregulin-4 are specific for a single member of the receptor family, the five other EGF-like ligands can bind two or three receptors each. EGF, epidermal growth factor; HB-EGF, heparin binding-EGF; TGFα, transforming growth factor α.

The pharmacological or genetic inhibition of erbB1, erbB2, and/or erbB4 receptors delays the onset of puberty (Ma et al., 1992; Apostolakis et al., 2000; Prevot et al., 2003b, 2005) and alters adult reproductive function in rodents (Prevot et al., 2005). *In vitro* studies using either hypothalamic explants or primary cultures of hypothalamic astrocytes with a GnRH-producing neuronal cell line have shown that erbB receptor ligands can stimulate GnRH release from the explants or neuronal cells, but do so indirectly, by inducing astrocytes to secrete $PGE_2$ (Ojeda et al., 1990; Ma et al., 1997, 1999; Prevot et al., 2003b, 2005). In addition, ligand activation of erbB receptors has been shown to promote morphological rearrangements in hypothalamic astrocytes (**Figure 4G–I**; Sharif et al., 2009) thus raising the possibility that erbB signaling may also influence the astrocytic coverage of GnRH neurons *in vivo* (see for review Prevot et al., 2010b).

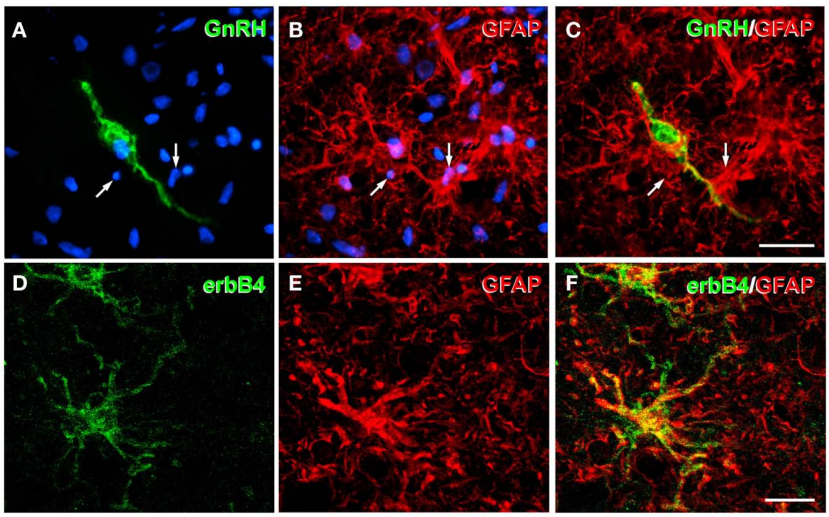


**FIGURE 3 | Astrocytes morphologically interact with GnRH neurons and express erbB4 receptors in the tuberal region of the human hypothalamus. (A–C)** Photomicrographs showing a GnRH neuronal cell body (green) to which the processes of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes (red, arrows) are abundantly apposed. Cell nuclei are stained with Hoechst (blue). Adapted from (Baroncini et al., 2007) with permission. **(D–F)** GFAP-immunoreactive astrocytes (red) of the tuberal region of the human hypothalamus express erbB4 receptors (green; M. Baroncini and V. Prevot, unpublished data). Scale bars = 20 μm **(C)**, 10 μm **(F)**.

*In vitro* experiments suggest that erbB signaling in hypothalamic astrocytes is functionally connected to the neuronal glutamatergic system, the primary mode of excitatory transsynaptic communication used by hypothalamic neurons (van den Pol and Trombley, 1993), and one that is known to increase GnRH secretion (Donoso et al., 1990; Claypool et al., 2000) and accelerate the initiation of puberty in both rodents and primates (Urbanski and Ojeda, 1987, 1990; Plant et al., 1989). In hypothalamic and non-hypothalamic astrocytes alike (Bezzi et al., 1998; Zonta et al., 2003a,b), transmitter spillover from nearby synaptic activity results in an elevation of $PGE_2$ release (McCarthy et al., 2008; Glanowska and Moenter, 2011). For example, neuronally released glutamate can engage biochemical signaling in astrocytes through the co-activation of AMPA and metabotropic glutamate receptors to cause a ligand-dependent increase in astrocytic erbB signaling and $PGE_2$ release (Dziedzic et al., 2003). This in turn signals back to GnRH neurons (**Figure 5**) facilitating neuroendocrine development and adult reproductive function (Prevot et al., 2003b, 2005).

## PROSTAGLANDIN $E_2$, A NEWLY UNCOVERED GLIOTRANSMITTER WITHIN THE GnRH NEUROSECRETORY SYSTEM

Even though $PGE_2$ has been known to trigger GnRH release from the hypothalamic neurons controlling reproduction for almost 40 years, it is only very recently that it has been identified as a potent excitatory regulator of GnRH neuronal activity, both in male and female mice (Clasadonte et al., 2011). Using patch-clamp recordings in brain slices from transgenic mice expressing green fluorescent protein (GFP) under the control of the GnRH promoter, we showed that $PGE_2$ induced a reversible membrane depolarization of GnRH neurons leading to the initiation of spike firing via the postsynaptic effect involving activation of a non-selective cation current (**Figure 5**; Clasadonte et al., 2011) reminiscent of the ones recently described in GnRH neurons by other groups (Zhang et al., 2008; Roland and Moenter, 2011). Although GnRH neurons are known to express both the EP1 and EP2 subtypes of prostaglandin receptors *in vivo* (Rage et al., 1997; Jasoni et al., 2005), the excitatory effect of $PGE_2$ on GnRH neuronal activity was selectively mimicked by the EP2 receptor agonist butaprost (Clasadonte et al., 2011) previously shown to promote GnRH release in the GnRH-producing neuronal cell line, GT1–7 (Rage et al., 1997). The $PGE_2$-mediated membrane depolarization of GnRH neurons was also shown to require the cAMP/protein kinase A (PKA) pathway (Clasadonte et al., 2011), which is known to be coupled to the EP2 receptor (**Figure 1**; Coleman et al., 1994; Sang et al., 2005) and to underlie the stimulatory effect of $PGE_2$ on GnRH secretion (**Figure 5**; Ojeda et al., 1985).

As alluded to above, the selective disruption of erbB4 signaling in astrocytes by the overexpression of a dominant-negative erbB4 receptor under the control of the human GFAP promoter leads to diminished $PGE_2$ release in response to ligand-dependent erbB4 activation, leading in turn to reduced GnRH release, delayed puberty, and disrupted adult reproductive function (Prevot et al., 2003b, 2005). Intriguingly, electrophysiological analyses have shown that the spontaneous activity of GnRH neurons in these animals is decreased and that this deficiency is mimicked by the bath application of either fluoroacetate, an inhibitor of astrocyte metabolism (Fonnum et al., 1997; Henneberger et al., 2010), or the COX blocker indomethacin, to slices of the preoptic region from wild-type animals (Clasadonte et al., 2011). The fact that GnRH neuronal activity in all these conditions can be rescued by exogenous $PGE_2$ (Clasadonte et al., 2011) strongly suggests that glial $PGE_2$ is an important component of the homeostatic mechanism

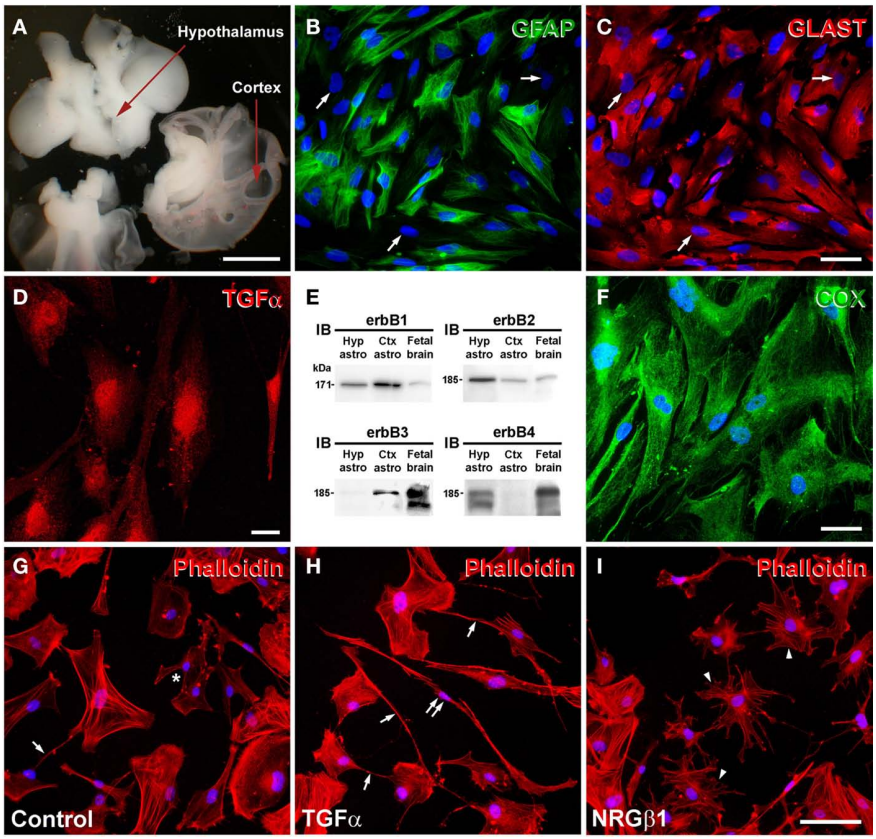


**FIGURE 4 | Human hypothalamic astrocytes express the molecular components required for a glia-to-neuron communication through the erbB-prostaglandin signaling system.** Primary cultures of human hypothalamic astrocytes have been prepared from 9 to 12-week-old human fetuses **(A)**. The cultures are composed of 98% of cells immunopositive for the astrocytic markers GFAP [**(B)** green] and the glutamate–aspartate transporter GLAST [**(C)** red]. Note that cells that express GFAP at low to undetectable levels are nevertheless strongly immunopositive for GLAST (arrows). **(D)** Human astrocytes in culture express TGFα protein (red). **(E)** Western blot analysis of erbB receptor expression in primary cultures of human cortical and hypothalamic astrocytes. While all four erbB receptors are expressed in the fetal brain, hypothalamic astrocytes (Hyp astro) express erbB1, erbB2, and erbB4, but not erbB3, and cortical astrocytes (Ctx astro) express erbB1, erbB2, and erbB3 but not erbB4 receptors. IB, immunoblot. **(F)** Human hypothalamic astrocytes in culture are immunopositive for COX (green). **(G–I)** EGF ligands induce profound morphological rearrangements of human hypothalamic astrocytes *in vitro*. Cell morphology was examined by visualization of the actin cytoskeleton using Alexa Fluor 568-conjugated phalloidin (red). Hypothalamic astrocytes exhibit heterogeneous shapes under control conditions, i.e., polygonal cells, cells with short and thick extensions (asterisk) or long and thin processes (arrow) **(G)**. TGFα (50 ng/mL for 3 days) stimulates the extension of long and thin processes (arrows) and the apparition of bipolar cells (double arrows) **(H)** while treatment with neuregulin-1 NRGβ1 (50 ng/mL for 3 days) increases the number of multipolar cells with thick processes (arrowheads) **(I)**. Nuclei are counter-stained with Hoechst [**(B,C,F–I)**, blue]. Scale bars = 3 mm **(A)**, 50 μm **(B,C,F)**, 20 μm **(D)**, 100 μm **(G–I)**. Adapted from (Sharif et al., 2009) with permission.

controlling GnRH neuronal excitability. The role of glia in the control of GnRH neuronal activity is further supported by a recent study demonstrating that glial prostaglandins may regulate the efficacy of GABAergic inputs to GnRH neurons in ovariectomized mice (Glanowska and Moenter, 2011). Using GnRH–GFP transgenic mice and patch-clamp recordings in brain slices, the authors demonstrated that the repeated action-potential-like depolarization of a GnRH neuron caused a short-term reduction in the frequency of spontaneous GABAergic postsynaptic currents in the same neuron, suggesting the presence of local circuit interactions between GnRH neurons and their GABAergic afferents (Chu and Moenter, 2005; Glanowska and Moenter, 2011). It is important to note that in this local circuit, the activation of $GABA_A$ receptors exerts a depolarizing action that can trigger action potential firing due to the elevated chloride levels maintained in adult GnRH neurons (DeFazio et al., 2002; Han et al., 2002; Herbison and Moenter, 2011). Consequently, this represents a negative feedback loop in which depolarized GnRH neurons reduce the activity of their own excitatory GABAergic afferents. In addition to being steroid-dependent and under the influence of both glutamatergic and endocannabinoid signaling mechanisms via the activation of presynaptic metabotropic glutamate receptors and cannabinoid CB1 receptors respectively (Chu and Moenter, 2005; Glanowska and Moenter, 2011) this local negative feedback loop also requires the action of glial-derived prostaglandins (Glanowska and Moenter, 2011). Indeed, the incubation of brain slices with

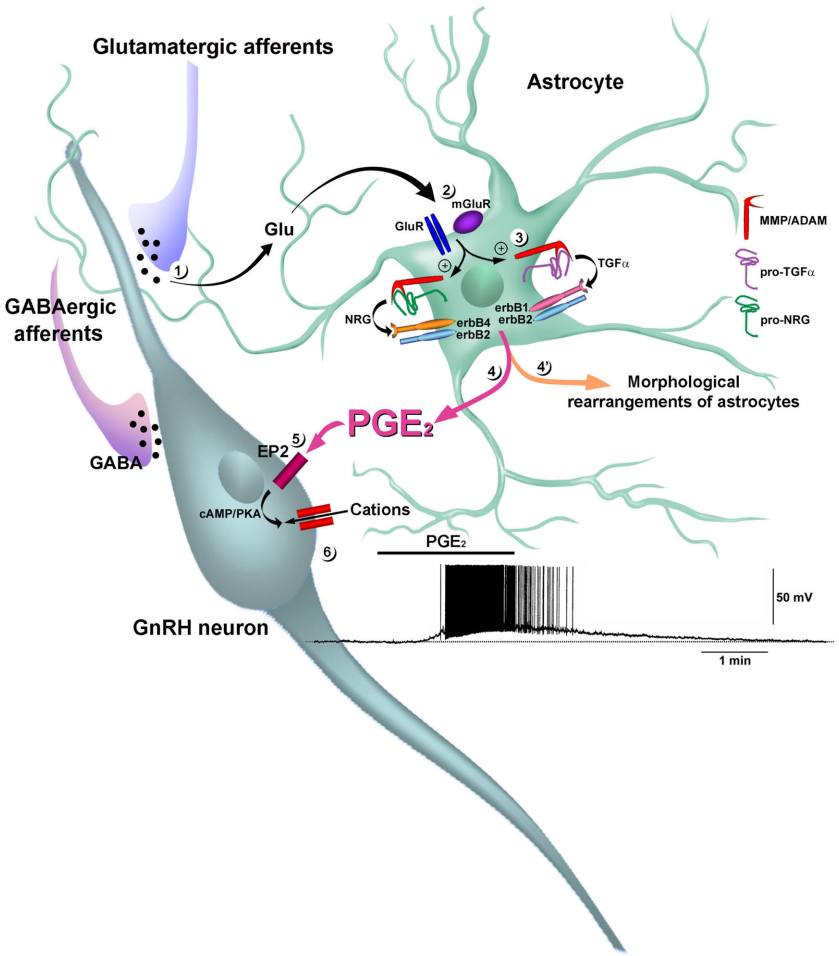


**FIGURE 5 | Prostaglandin $E_2$ acts as a gliotransmitter to stimulate GnRH neuron electrical activity.** Neuronally released glutamate (Glu) (1) co-activates metabotropic glutamatergic (mGluR) and AMPA glutamatergic receptors (GluR) in astrocytes (2), stimulating the activity of zinc-dependent matrix metalloproteinases (MMPs) of the ADAM (a disintegrin and metalloproteinase) family (3). The MMPs catalyze ectodomain shedding of the pro-EGF ligands pro-TGFα and pro-NRG (pro-neuregulin). In particular, the processing of pro-TGFα has been shown to involve the metalloproteinase ADAM17, also known as tumor necrosis factor α converting enzyme (TACE). The subsequently released mature TGFα and NRG activate erbB1/erbB2 and erbB4/erbB2 heterodimers, respectively (Dziedzic et al., 2003). The co-activation of glutamatergic receptors induces the recruitment of erbB1, erbB4, and their pro-ligands to the cell membrane, where multiprotein complexes form, as demonstrated by the direct physical association of glutamatergic and erbB receptors (not shown). The activation of erbB receptors in hypothalamic astrocytes promotes profound morphological changes, including the retraction of cytoplasm, stellation of cells and the elongation of processes (see **Figure 4G–I**) (4′). The activation of erbB receptors also promotes the release of $PGE_2$ (Ma et al., 1997, 1999; Dziedzic et al., 2003) (4), which stimulates a cAMP/protein kinase A (PKA) pathway in GnRH neurons through the mobilization of EP2 receptors (EP2; Clasadonte et al., 2011) (5). Activation of this signaling pathway induces a reversible membrane depolarization of GnRH neurons leading to the initiation of spike firing via a postsynaptic effect involving the activation of a non-selective cation current (Clasadonte et al., 2011) (6).

indomethacin, the broad-spectrum prostaglandin receptor antagonist AH 6809, or fluorocitrate, which like fluoroacetate, is a specific blocker of astrocyte metabolism, prevents the depolarization-induced suppression of GABAergic transmission in GnRH neurons (Glanowska and Moenter, 2011). Since GABA exerts a depolarizing action in this local circuit, we could envisage that glial prostaglandins, by suppressing excitatory drive, would reduce GnRH neuronal activity. Estradiol could also differentially influence this local inhibitory feedback to exert its positive or negative feedback effects (Glanowska and Moenter, 2011). Thus, in addition to exerting a direct postsynaptic excitatory action on the cell body of GnRH neurons, prostaglandins released from astrocytes can also participate in mechanisms that regulate the activity of their GABAergic presynaptic inputs (**Figure 5**). In the GnRH system, thus, $PGE_2$ fulfills all the criteria that qualify a compound as a "gliotransmitter" (Parpura and Zorec, 2010): (i) it is synthesized by astrocytes, (ii) its regulated release is triggered by physiological stimuli, (iii) it acutely activates the firing of GnRH neurons and modulates the activity of their GABAergic afferents, and (iv) it plays a role in an important physiological function, i.e., the neuroendocrine control of reproduction, which is vital to species' survival.

### A ROLE FOR ASTROGLIAL PROSTAGLANDIN $E_2$ IN DENDRITIC SPINE PLASTICITY IN GnRH NEURONS?

Gonadotropin-releasing hormone neurons exhibit a simple bipolar morphology (**Figure 3A**) with one or two very long dendritic processes that can extend up to 1 mm (Campbell et al., 2005, 2009). Intriguingly, recent studies have demonstrated that the density of spines along these dendrites is subject to robust increases not only during sexual development in immature animals (Cottrell et al., 2006), but also at the onset of the GnRH/LH surge induced by gonadal steroids in ovariectomized adult mice (Chan et al., 2011). Although sexual maturation and the surge mechanism have been shown to require the neuronal expression of sex-steroid receptors (Wintermantel et al., 2006; Raskin et al., 2009; Mayer et al., 2010), studies suggesting that astrocytic mechanisms might control the stabilization of individual dendritic processes and their subsequent maturation into spines (Nishida and Okabe, 2007), together with the demonstration that specific juxtacrine signaling pathways are involved in sculpting astrocyte–dendritic spine interactions (Murai et al., 2003), raise the possibility that astrocytes play a role in the physiological changes of synaptic structure underlying GnRH neuronal maturation and function. $PGE_2$ release by astrocytes could be central in this process and $PGE_2$ has in fact been shown to mediate the dramatic neuronal spine plasticity induced by estrogens in the developing preoptic region (Amateau and McCarthy, 2002, 2004; Wright and McCarthy, 2009). This effect involves the activation of AMPA and metabotropic glutamate receptors (Amateau and McCarthy, 2002; Wright and McCarthy, 2009), known to promote erbB-dependent $PGE_2$ release in hypothalamic astrocytes (Dziedzic et al., 2003), as well as the EP2/PKA signaling pathway (Amateau and McCarthy, 2002), recently found to be functional in native GnRH neurons (Clasadonte et al., 2011; **Figure 5**). Importantly, estrogens, which have long been known to regulate neuronal spine plasticity in the adult hippocampus (Woolley and McEwen, 1992, 1994), have also been shown to promote comparable changes in the immature hippocampus (Amateau and McCarthy, 2002). However, in the hippocampus, the underlying mechanisms do not appear to require $PGE_2$ synthesis (Amateau and McCarthy, 2002), suggesting that increases in $PGE_2$ synthesis are selectively used by estrogens to promote dendritic spine plasticity in the developing preoptic region. Further studies are required to determine whether estrogenic effects on the plasticity of hypothalamic neurons such as those seen in newborn rodents can also occur later in postnatal life and/or in adulthood.

### $PROSTAGLANDIN E_2$ IS A KEY MEDIATOR OF GnRH RELEASE, NEURONAL–GLIAL–ENDOTHELIAL INTERACTIONS, AND CELL PLASTICITY AT THE GnRH NEUROHEMAL JUNCTION

Neuroendocrine GnRH neurons send axons to the median eminence, where they release their neurohormone into the pituitary portal blood vessels for delivery to the anterior pituitary. The median eminence, which lies ventral to the third ventricle in the tuberal region of the hypothalamus, constitutes one of the key sites for the regulation of GnRH release (see for review Hrabovszky and Liposits, 2008; Ojeda et al., 2008; Prevot et al., 2010a; Yin and Gore, 2010). The modulation of GnRH release by $PGE_2$ within the median eminence was suggested as soon as *in vitro* systems to statically incubate median eminence nerve terminals were developed, i.e., in the late 70s (Negro-Vilar et al., 1979; Ojeda et al., 1979). Experiments showing that the $PGE_2$-induced GnRH release from median eminence explants requires the mobilization of intracellular calcium stores (Ojeda and Negro-Vilar, 1985; Ojeda et al., 1988) have suggested a role for the EP1 receptor in this process (**Figure 1**). In line with this assumption are other findings demonstrating that GnRH neurons express EP1 receptors *in vivo* and that the EP1 agonist 17-phenyl trinor $PGE_2$ promotes

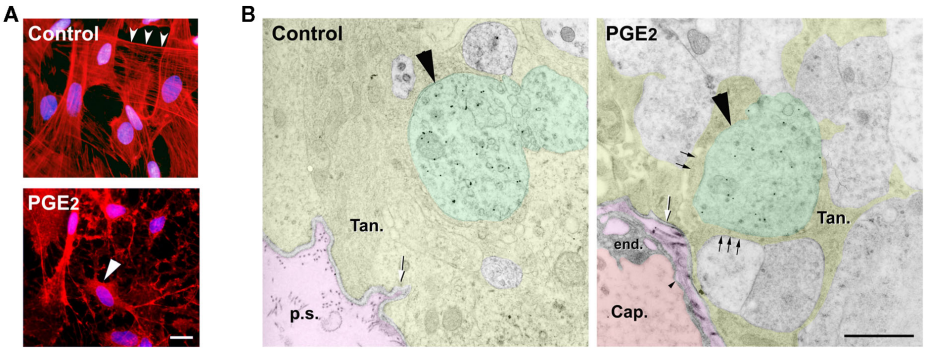


**FIGURE 6 | Prostaglandin $E_2$ promotes the retraction of tanycytic processes *in vitro* and induces neuroglial plasticity causing GnRH neurosecretory terminals to advance toward the pericapillary space in isolated median eminence explants. (A)** Tanycytes in culture were stained with Alexa Fluor 568-conjugated phalloidin to visualize filamentous actin (red) and with Hoechst to stain nuclei (blue). In control unstimulated tanycyte cultures, actin was localized adjacent to the cell membrane (top panel, cortical actin, arrowheads) and was also diffused throughout the cytoplasm. $PGE_2$ treatment (280 nM, 30 min) promoted tanycyte retraction (bottom panel, long arrowhead). **(B)** Electron micrographs of GnRH immunoreactive axon terminals (big arrowhead, green) from female rat median eminence explants incubated for 30 min in the presence or absence of $PGE_2$ (1 μM). Under basal unstimulated conditions (Control), GnRH nerve endings (big arrowhead, green) were maintained at a distance from the brain basal lamina (white arrow) delineating the pericapillary space (p.s., pink) by thick enclosing tanycyte end-feet (Tan., yellow). $PGE_2$ treatment caused the advancement of GnRH axon terminals (big arrowhead, green) toward the brain basal lamina (white arrow) and the apparent retraction of most of the astroglial sheath (black arrows, yellow) from those neurosecretory terminals that were separated from the fenestrated (small arrowhead) portal capillaries (Cap., red) by only a few nanometers. end., endothelium. Scale bars = 10 μm **(A)**, 1 μm **(B)**. Reproduced from (de Seranno et al., 2010) with permission.

GnRH release from GT1–1 cells *in vitro* (Rage et al., 1997) without affecting GnRH neuronal firing in brain slices (Clasadonte et al., 2011).

Within the median eminence, GnRH axon terminals are intimately associated with cell processes belonging to specialized unciliated ependymal cells named tanycytes. Tanycyte cell bodies are attached together at the apex by tight junctions (Mullier et al., 2010) and line the floor of the third ventricle. They send out long slender processes that eventually contact the pial surface of the brain where the fenestrated pituitary portal vessels reside, via end-feet (Page, 1994; Ciofi et al., 2009; Mullier et al., 2010). These tanycyte end-feet not only enclose the GnRH nerve terminals, possibly providing a diffusion barrier (Kozlowski and Coates, 1985; Meister et al., 1988; Ugrumov et al., 1989; King and Letourneau, 1994), but also display a high degree of structural plasticity across the ovarian cycle in rats (Prevot et al., 1998, 1999). During the estrous cycle, under basal conditions, e.g., in diestrus, GnRH nerve terminals are completely wrapped up in tanycyte end-feet (Prevot et al., 1998, 1999). In proestrus, following the activation of the reproductive axis, the end-feet are retracted, presumably due to increasing levels of gonadal steroids (King and Letourneau, 1994), thus allowing GnRH neurons to directly contact the pericapillary space (Prevot et al., 1998). By analogy with the function-related plasticity documented in the neural lobe of the pituitary (Hatton, 1997), these data argue for the importance of tanycyte structural rearrangement in delivering peak levels of GnRH to the pituitary during the preovulatory surge. The intriguing possibility that $PGE_2$ could be involved in the control of these

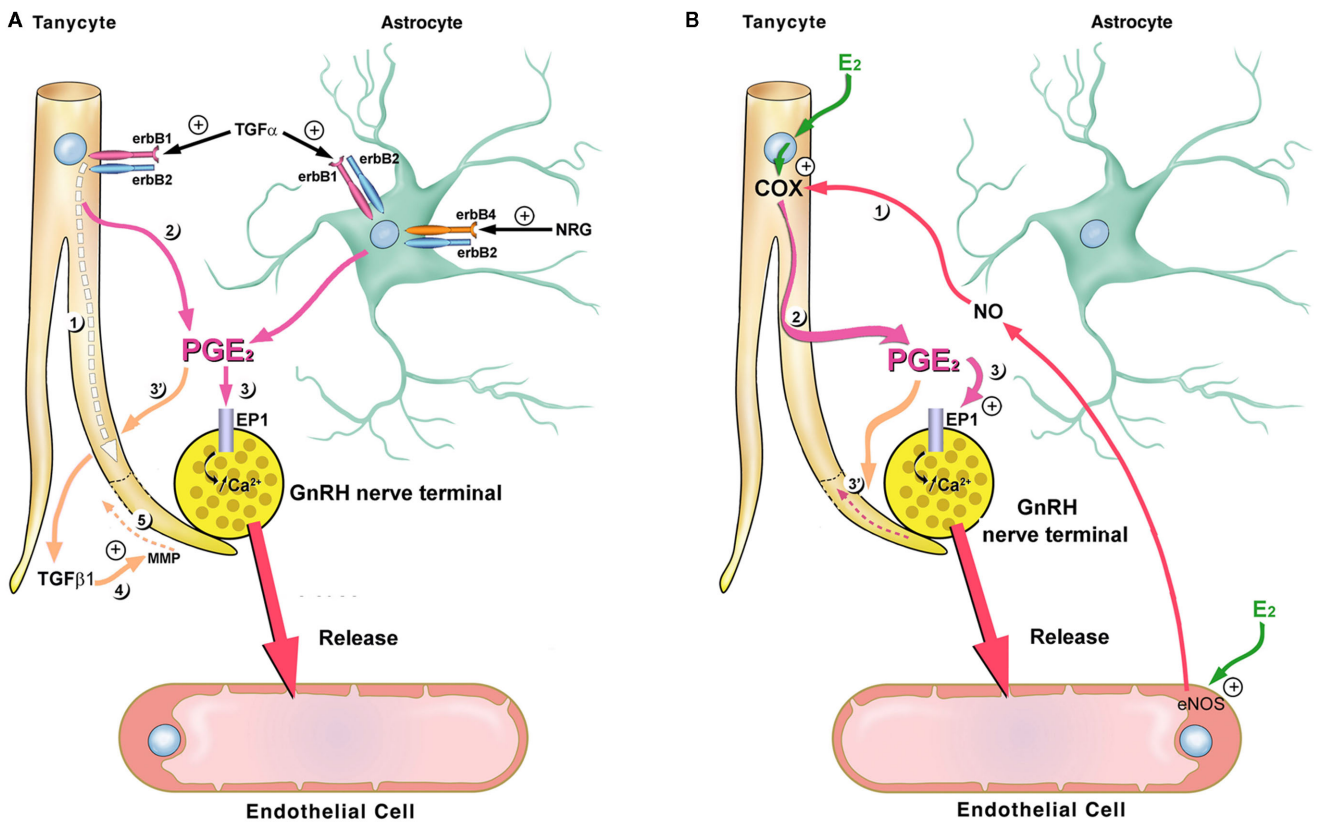


**FIGURE 7 | Schematic representation of neural–glial–endothelial interactions involved in the control of GnRH neurosecretion in the median eminence. (A)** Glial–neuronal interactions in the median eminence involve the production of epidermal growth factor (EGF)-related peptides by glial cells. Activation of erbB1/erbB2 and erbB4/erbB2 heterodimers by TGFα and NRG, respectively, promotes the release of $PGE_2$ from astrocytes. The binding of TGFα to tanycytic erbB1 receptors results in the recruitment of erbB2 co-receptors and signal transduction. The ligand-dependent activation of erbB1 receptors in tanycytes results in biphasic plastic changes characterized by an initial phase of tanycyte outgrowth (1) and a secondary phase of retraction (5). Although the initial outgrowth (1) is independent of the TGFβ1 system, the subsequent retraction requires $PGE_2$ synthesis (2), a $PGE_2$-dependent increase in the production of TGFβ1 (3′) and matrix metalloproteinase (MMP) activity (4). In addition to promoting TGFβ1 synthesis by tanycytes (3′), $PGE_2$ released by tanycytes (2) and astrocytes is able to directly stimulate GnRH release at nerve endings through the EP1 receptor (EP1)-mediated mobilization of intracellular calcium stores (3). **(B)** Endothelial–neuronal interactions at the level of the median eminence involve the production of nitric oxide (NO) by the endothelial cells of fenestrated capillaries of the portal blood vessels. Upon its secretion, NO diffuses from its source and stimulates the production of $PGE_2$ from tanycytes. $PGE_2$ promotes the release of GnRH into the blood stream by the direct stimulation of nerve endings (3) and by promoting their access to the pericapillary space by inducing cytoarchitectural changes in tanycyte end-feet (1–3′). Estrogens are likely to be the key humoral factors involved in the orchestration of the endothelia-to-glia communication that allows GnRH neurons to directly contact the pituitary portal blood vessels on the day of proestrus. Estrogen treatment upregulates COX expression in tanycytes and stimulates endothelial nitric oxide synthase (eNOS) expression in median eminence endothelial cells. Adapted from (Prevot, 2002) with permission.

plastic phenomena arises from recent studies using either median eminence explants (de Seranno et al., 2010) or primary cultures of tanycytes isolated from the median eminence (Prevot et al., 2003a; De Seranno et al., 2004; de Seranno et al., 2010; **Figure 6**). When $PGE_2$ is applied to median eminence explants at concentrations known to stimulate GnRH release, structural remodeling occurs at the neurohemal junction in a matter of minutes causing GnRH neurosecretory terminals to advance toward the pericapillary space (de Seranno et al., 2010), a phenomenon that probably results from the retraction of tanycyte end-feet (**Figure 6B**), as suggested by the $PGE_2$-promoted tanycyte retraction seen *in vitro* (**Figure 6A**). As extensively reviewed elsewhere (Prevot et al., 2010a,b; Bellefontaine et al., 2011), $PGE_2$ synthesis in tanycytes of the median eminence could be prompted by two independent but complimentary cell-based mechanisms, one involving glial–glial interactions set in motion by the paracrine activation of TGFα/erbB1 signaling pathway in tanycytes, as depicted in **Figure 7A** (Prevot et al., 2003a), and another involving endothelial–tanycyte interactions and the release of nitric oxide (NO) by vascular endothelial cells, which in turn directly modulates COX activity in tanycytes, as described in **Figure 7B** (De Seranno et al., 2004; de Seranno et al., 2010). Both pathways could be subject to the modulatory influence of gonadal steroids, as estrogens are known to upregulate both TGFα expression in astroglial cells (Ma et al., 1992, 1994a) and COX expression in tanycytes (de Seranno et al., 2010). Finally, the physiological importance of $PGE_2$ in the cell–cell communication processes regulating GnRH release has been highlighted by experiments in which the COX inhibitor indomethacin is infused directly into the median eminence, resulting in the marked impairment of the rat ovarian cycle, which requires the coordinated delivery of GnRH into the hypothalamo-hypophyseal portal system (de Seranno et al., 2010). Indeed, the local inhibition of prostaglandin synthesis has been shown to arrest the ovarian cycle in either diestrus or estrus when GnRH release is low (Levine and Ramirez, 1982) and GnRH neuroendocrine terminals are enclosed by tanycyte end-feet (Prevot et al., 1998, 1999).

## CONCLUSION

Several observations made over the last two decades have demonstrated that $PGE_2$ known for almost 40 years to play an important role in the regulation of the hypothalamic–pituitary–gonadal axis, is a transmitter released by astrocytes and tanycytes, and intimately linked with GnRH neuronal function in both the preoptic region and the median eminence of the hypothalamus, where the cell bodies and the neuroendocrine terminals of GnRH neurons in rodents are respectively located. However, many mysteries regarding the underlying mechanisms remain unsolved. For example, even though recent studies suggest that GnRH neurons can directly communicate with neighboring astrocytes via juxtacrine signaling pathways (Sandau et al., 2011a,b), a true understanding of how these GnRH neurons interact with hypothalamic astrocytes to modulate $PGE_2$ gliotransmission is missing. Are these communication processes involved in sculpting astrocyte–dendritic spine interactions and in promoting the physiological changes in synaptic structure that underlie GnRH neuronal maturation and function? How is $PGE_2$ released from hypothalamic astrocytes?

Now that a general strategy for the application of molecular genetics to the study of neuron–glia interactions and gliotransmission has been elucidated, the next several years should provide an opportunity to begin to address these questions.

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# Food restriction-induced changes in gonadotropin-inhibiting hormone cells are associated with changes in sexual motivation and food hoarding, but not sexual performance and food intake

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We hypothesized that putative anorectic and orexigenic peptides control the motivation to engage in either ingestive or sex behaviors, and these peptides function to optimize reproductive success in environments where energy fluctuates. Here, the putative orexigenic peptide, gonadotropin-inhibiting hormone (GnIH, also known as RFamide-related peptide-3), and the putative anorectic hormones leptin, insulin, and estradiol were examined during the course of food restriction. Groups of female Syrian hamsters were restricted to 75% of their *ad libitum* food intake or fed *ad libitum* for 4, 8, or 12 days. Two other groups were food-restricted for 12 days and then re-fed *ad libitum* for 4 or 8 days. After testing for sex and ingestive behavior, blood was sampled and assayed for peripheral hormones. Brains were immunohistochemically double-labeled for GnIH and the protein product of the immediate early gene, *c-fos*, a marker of cellular activation. Food hoarding, the number of double-labeled cells, and the percent of GnIH-Ir cells labeled with Fos-Ir were significantly increased at 8 and 12 days after the start of food restriction. Vaginal scent marking and GnIH-Ir cell number significantly decreased after the same duration of restriction. Food hoarding, but not food intake, was significantly positively correlated with cellular activation in GnIH-Ir cells. Vaginal scent marking was significantly negatively correlated with cellular activation in GnIH-Ir cells. There were no significant effects of food restriction on plasma insulin, leptin, estradiol, or progesterone concentrations. In the dorsomedial hypothalamus (DMH) of energetically challenged females, strong projections from NPY-Ir cells were found in close apposition to GnIH-Ir cells. Together these results are consistent with the idea that metabolic signals influence sexual and ingestive motivation via NPY fibers that project to GnIH cells in the DMH.

**Keywords: appetitive behavior, estradiol, ingestive behavior, leptin, neuropeptide Y, progesterone, RFamide-related peptide-3, sex behavior**

## INTRODUCTION

Metabolic control of the reproductive system has been demonstrated in every order of the class Mammalia (Bronson, 1989). Reproduction is inhibited when the availability of oxidizable fuels is scarce, and reproduction is rapidly stimulated when fuels become abundant (Bronson, 1986; Szymanski et al., 2007). Mechanisms that measure fuel availability and modulate reproductive processes serve to optimize reproductive success in environments where food availability and energy demands fluctuate (Bronson, 1989; Wade and Schneider, 1992; Schneider, 2006). The mechanisms that switch behavioral priorities from ingestive to reproductive behaviors might occur at multiple loci, including effects on behavioral motivation (the internal desire for food or sex), performance (mating and eating) and the hypothalamic–pituitary–gonadal (HPG) system, including the gonadotropin releasing-hormone (GnRH) pulse generator, pituitary gonadotropin secretion, and ovarian steroid secretion. Despite action at multiple loci, the majority of research has focused on metabolic challenges that induce anestrus, inhibit gonadotropin secretion, and stimulate food intake (Kalra et al., 1988; I'anson et al., 1991; McShane et al., 1992; Wade and Schneider, 1992; Foster et al., 1998; Henry et al., 1999; Cunningham, 2004; Schneider, 2004). Food deprivation and other metabolic challenges inhibit pulsatile GnRH secretion which, in turn, prevents pituitary luteinizing hormone (LH) secretion, ovarian steroid synthesis and secretion, and ovarian-steroid-dependent copulatory behavior in a wide variety of species, including Syrian hamsters (McClure, 1962; Morin, 1975; Ronnekleiv et al., 1978; Bronson and Marsteller, 1985; Foster and Olster, 1985; Armstrong and Britt, 1987; Bronson, 1988; Sprangers and Piacsek, 1988; Schneider and Wade, 1989; Thomas et al., 1990; Cameron, 1996; Shahab et al., 1997, 2006; Temple et al., 2002; Terry et al., 2005).

It is likely, however, that energy deficits influence behavioral motivation even before metabolic challenges become so severe that they induce anestrus. We have used female Syrian hamsters (*Mesocricetus auratus*). Lean Syrian hamsters become anestrus after a 48-h period of food deprivation, whereas pre-fattened Syrian hamsters, which do not become anestrus, show deficits in paracopulatory behaviors. Fattened Syrian hamsters food-deprived for 24–36 h show significantly decreased appetitive sex behaviors, such as decreased vaginal scent marking, and significantly increased appetitive ingestive behaviors, such as food hoarding (Schneider et al., 2007). Appetitive behaviors bring animals in contact with the goal object (mating partners or food), and often occur separated in time from mating and eating (Sherrington, 1906; Craig, 1917; Lorenz, 1950; Johnston, 1974, 1977; Lisk et al., 1983; Everitt, 1990). Syrian hamster appetitive sex behaviors include vaginal scent marking, an estradiol-dependent behavior that occurs with increasing frequency over days 1, 2, and 3 of the 4-day estrous cycle (with day 4 being proestrus; Johnston, 1977). In addition, appetite for food and sex can be assessed in this species by measuring the preference for males vs. food (the time spent with the male minus the time spent with food divided by the total time). Consummatory sex behavior is commonly measured in Syrian hamsters as the incidence of the lordosis reflex, a posture that allows male intromission on day 4 of the estrous cycle and requires proestrus concentrations of plasma estradiol and progesterone, tactile flank stimulation, and male olfactory cues (Lisk et al., 1983). Flank marking is yet another appetitive social behavior, more specifically, an agonistic behavior, that is higher in dominant female hamsters, increases with increases in plasma estradiol concentrations, and is inhibited at the time of estrus by the presence of adult male hamsters (Albers and Rawls, 1989; Albers and Rowland, 1989). With regard to ingestive behaviors, food hoarding is an example of appetitive behavior, whereas food intake is a consummatory behavior in Syrian hamsters (Smith and Ross, 1950; Waddell, 1951).

Consummatory sex and ingestive behavior can be simultaneously stimulated under special circumstances (Kaplan et al., 1992). Appetitive behaviors, however, are often in conflict, and females must choose between engaging in courtship or foraging for food. In nature, females typically have a choice between ingestive and sex behavior, and the decision can impact survival and reproductive success. Thus, we have included appetitive behaviors and the choice between food and males in our experiments. By attention to the decisions to engage in either reproductive or ingestive behavior, we hoped to gain insight into hormones and neuropeptides implicated in control of food intake and reproduction, such as gonadotropin-inhibiting hormone (GnIH), neuropeptide Y (NPY), leptin, insulin, estradiol, and progesterone.

In this experiment, appetitive and consummatory sex and ingestive behaviors were examined over the course of food restriction to test the following hypotheses: (1) Appetitive behaviors are more sensitive than consummatory behaviors to the effects of mild food restriction, (2) changes in appetitive behavior are correlated with increases in neural activation in cells that contain GnIH, and (3) cells that contain NPY project to the vicinity of GnIH cells in the dorsomedial hypothalamus (DMH). GnIH and NPY were examined for the following reasons.

Gonadotropin-inhibiting hormone has been implicated in environmental control of reproduction and food intake. GnIH was first identified from quail hypothalamus. Treatment with the newly identified peptide inhibited gonadotropin release from pituitary cells *in vitro* in a dose-dependent manner, and hence it was named GnIH (Tsutsui et al., 2000). Orthologous neuropeptides were subsequently discovered in a wide range of vertebrate species (reviewed in Bentley et al., 2010; Kriegsfeld et al., 2010; Smith and Clarke, 2010; Tsutsui et al., 2010). Evidence has accumulated that the mammalian homolog of GnIH, RFamide-related peptide-3 (RFRP-3, Arg-Phe-NH2 in the C terminus), acts as a negative regulator of gonadotropin secretion in all species investigated, including hamsters, mice, rats, cattle, sheep, non-human primates, and human beings (Kriegsfeld et al., 2006; Johnson et al., 2007; Clarke et al., 2008; Anderson et al., 2009; Smith and Clarke, 2010). In the past 5 years, the accumulated evidence across many mammalian species has revealed many similarities among mammals and birds in the function of the orthologous peptides, and there is general consensus that "GnIH" is the appropriate nomenclature for both peptides. It is unlikely, however, that inhibition of gonadotropin secretion is the only function of this peptide.

We hypothesize that GnIH is a modulator of sex and ingestive motivation in Syrian hamsters because intracerebroventricular treatment with GnIH disrupts sex behavior of female white-crowned sparrows and male rats (Bentley et al., 2006; Johnson et al., 2007), and increases food intake in male rats (Johnson et al., 2007), sheep, mice, and monkeys (Clarke, personal communication). GnIH cells in Syrian hamsters are restricted to the DMH, contain estradiol receptors (ER), and show neural activation in response to increased circulating concentrations of estradiol (Kriegsfeld et al., 2006). If GnIH is important for the effects of mild food restriction on motivation, increases in cellular activation of GnIH-immunoreactive (Ir) cells would be predicted to precede or coincide with increases in ingestive motivation and decreases in sexual motivation. Our hypothesis would be refuted if there were no increase in cellular activation in GnIH-Ir cells or if activation occurred too late to account for changes in behavior. Thus, the present experiments examined cellular activation in GnIH-Ir cells and appetitive sex and ingestive behavior after either 0, 4, 8, or 12 days of 25% food restriction or after 4 or 8 days of *ad libitum* feeding to females previously food-restricted for 12 days.

Neuropeptide Y is a hormone that has long been studied in relation to energy balance and reproduction, and more recently, NPY has been implicated in appetitive aspects of ingestion. NPY gene expression is increased in discrete nuclei of the hypothalamus, including the DMH, in response to energy restriction in rodents, including Syrian hamsters (Brady et al., 1990; Jones et al., 2004). Intracerebroventricular treatment with NPY rapidly increases food intake and suppresses mating behavior of male and female rodents, including Syrian hamsters (Clark et al., 1985; Stanley and Leibowitz, 1985; Kulkosky et al., 1988; Corp et al., 2001; Jones et al., 2004), and some data are consistent with the idea that appetitive aspects of behavior are more sensitive to NPY than consummatory aspects of behavior (Ammar et al., 2000). Most relevant to the present study, food hoarding is increased by treatment with NPY agonists and decreased by treatment with antagonists to specific NPY receptors in Siberian hamsters (Day et al., 2005).

Investigators interested in NPY effects on food hoarding have focused on the paraventricular nucleus (PVH) and arcuate nucleus (Arc) of the hypothalamus and the perifornical area, but not the DMH. Thus, we double-labeled for GnIH-Ir and NPY-Ir to determine whether there are NPY projections to the GnIH cells in the DMH. In addition, we measured plasma levels of progesterone, leptin, insulin, and estradiol because they are putative orexigenic agents and anorectic hormones implicated in control of energy balance and reproduction in a number of species including Syrian hamsters (Wade et al., 1991; Ahima et al., 1996; Schneider et al., 1998; Eckel, 2004; Klingerman et al., 2010). These hormones and neuropeptides had not been measured at different durations after *mild* food restriction and re-feeding and in relation to changes in GnIH prior to these experiments.

## MATERIALS AND METHODS

All subjects were adult (60–90 days of age), female Syrian hamsters obtained from Charles River Breeding Laboratories (Wilmington, MA, USA). Upon arrival, hamsters were housed singly in opaque, Nalgene cages (31 cm × 19 cm × 18 cm) in a room maintained at 23 ± 1˚C with a 14:10 light–dark cycle (lights on at 2200 h). Hamsters were fed Harlan Rodent Chow 2016 and water was available at all times. All procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the United States Department of Agriculture, and a protocol approved by the Lehigh University Institutional Animal Care and Use Committee.

### EXPERIMENT 1: EFFECTS OF ENERGY RESTRICTION ON BEHAVIOR, GnIH, AND CIRCULATING HORMONES

This experiment was designed to examine cellular activation in GnIH cells and circulating hormones after testing for appetitive and consummatory sex and ingestive behaviors in animals subject to mild food restriction for varying durations.

#### *Preference apparatus*

Hamsters were acclimated, trained, and tested in a preference apparatus designed to duplicate aspects of their native habitat, and to allow quantification of behaviors associated with the motivation to engage in either sex or ingestive behavior (Schneider et al., 2007). Hamsters in the wild live in isolation in underground burrows from which they emerge for only 90 min per day at dawn and dusk, and spend virtually every minute of this time foraging for and hoarding food (Gattermann et al., 2008). Matings have been observed only at the entrance to the female burrow. Together, these considerations suggest that decisions about whether to engage in ingestive or sex behaviors that occur near the burrow entrance during the 90-min above-ground foraging period are relevant to their reproductive success. Thus, each preference apparatus consisted of a home cage for the subject female connected via a vertical tube to two boxes: One with an adult male hamster (male box) and another box containing a food source (food box). Home cages were made from opaque, Nalgene cages (31 cm × 19 cm × 18 cm) lined with fine wood shavings with a door that was kept closed when the animals were not being trained or tested. The vertical tube was 134 cm in length and was connected to tubes in a T-configuration that were 40–50 cm in length. The food box contained a weighed amount (150 ± 5 g) of hoardable pellets made from standard laboratory chow (Harlan Rodent Chow 2016) that was broken into 2 cm pieces, a size that permits pouching and enables hamsters with full cheek pouches to fit readily through the tubes. The male boxes for the stimulus hamsters were made from clear, Plexiglas cages (27 cm × 20 cm × 15 cm) with wire barriers that allowed auditory, olfactory, and visual interaction, but prevented mating or fighting. The stimulus male boxes did not contain food or water.

Females were acclimated to the home cage for 24 h/day for at least 1 week prior to testing, which reduced any tendencies to sleep, move bedding, or hoard food into any other compartments during later preference testing. After acclimation to the home, females were trained to expect access to the food and male boxes at the onset of the dark period. Hamsters experienced training sessions with the food source box once a day for 2 days on days 1 and 2 of the estrous cycle, and training sessions with the male box once a day for 2 days on days 3 and 4 of the estrous cycle. Training is described in detail in two previous publications (Klingerman et al., 2010, 2011).

Females that showed at least two consecutive estrous cycles and had been acclimated and trained in the preference apparatus were first tested for baseline behaviors including food hoarding, vaginal scent marking, flank marking, and male preference calculated as (time spent with the male – time spent with food)/total time.

During baseline testing, 24 h food intake was measured for at least 4 days prior to the start of the experiment to obtain a 4-day average daily intake. The 25% food-restricted females were given 75% of their baseline daily intake by giving a pre-weighed food ration immediately after behavior testing, approximately 2 h after the onset of the dark phase of the light–dark cycle. For all food-restricted females, restriction started on day 4 of the estrous cycle, and all females were sacrificed on day 4 of a subsequent cycle. This level of food restriction was chosen because previous experiments showed that 25% food restriction for up to 16 days does not induce anestrus (Klingerman et al., 2010, 2011).

After baseline testing, the 48 hamsters were randomly placed into one of six groups that did not differ significantly in body weight (115–175 g). The groups included hamsters that were food-restricted by 25% (fed 75% of *ad libitum* food intake determined during baseline) for 4 days ($n = 6$), food-restricted for 8 days ($n = 6$), food-restricted for 12 days ($n = 12$), food-restricted for 12 days and re-fed *ad libitum* for 4 days ($n = 6$), food-restricted for 12 days and re-fed *ad libitum* for 8 days ($n = 6$), or fed *ad libitum* ($n = 12$).

Testing began at the onset of the dark phase of the photoperiod (1200 h) on day 3 and was conducted under dim, red illumination. The door to the home cage was opened and females were allowed access to both the male and food boxes for a total of 90 min. During the first 15 min, vaginal marking, flank marking, food hoarding and eating as well as location (male, food, or home cage) were recorded. After 15 min of observation, the experimenter stopped recording and the test continued for an additional 75 min (90 min total); i.e., the females continued to have access to both the male and food boxes. After the 90-min test was complete, the hamsters were returned to their respective cages and the doors to the home cages were closed. Weight of food in the home cage and

food box was measured and recorded to determine the amount of food hoarded and eaten during the 90-min test.

### Blood collection and perfusion

Female hamsters were tested in the preference apparatus on day 3 of the estrous cycle, and at the same time the next day, they were euthanized and a terminal blood sample was taken. Plasma was assayed for estradiol and progesterone concentrations to determine effects of food restriction, and to determine whether levels were below those that would induce lordosis. Plasma insulin and leptin concentrations were assayed to determine the effects of chronic restriction. In order to avoid the confounding effects of meals and cephalic phase hormone release, both food-restricted and *ad libitum*-fed animals were given access to the amount of food normally fed to the food-restricted females for 15 min 4 h before blood collection. This schedule was chosen because previous results showed that Syrian hamsters do not show post-fast hyperphagia, and plasma insulin and leptin concentrations are not significantly increased in Syrian hamsters until more than 4 h after a meal (Schneider et al., 2000). Thus, plasma hormone concentrations in our different groups of females would be expected to reflect length of food restriction rather than effects of meals. All hamsters were sacrificed before the onset of the dark phase of the photoperiod (1200 h) by an overdose of sodium pentobarbital (Ovation Pharmaceuticals, Inc., Deerfield, IL, USA). Blood was centrifuged at 3000 rpm and 5˚C for 20 min. Plasma was collected and frozen at −20˚C until analysis.

Animals were perfused intracardially with phosphate buffered saline (PBS, pH 7.4 at 4˚C) followed by 4% paraformaldehyde in PBS at the same temperature. Brains were removed, post-fixed for 24 h at 4˚C in 4% paraformaldehyde, and stored at 4˚C in 20% sucrose and 0.001% thimerosal until sectioning. All brains were sectioned within 30 days using a freezing microtome set at 40 μm. Hypothalamic brain sections were placed into polyvinyl pyrrolidone (PVP) and stored at −20˚C until immunohistochemical staining.

### Immunohistochemistry

Cellular activation in GnIH-containing cells was measured by double-labeling for intranuclear Fos, the product of the immediate-early gene, *c-fos*, a well established marker of changes in cellular activity in response to stimuli in rodents (Hoffman et al., 1993). Tissue was collected and every fourth 40 μm section was double-labeled using fluorescence immunohistochemistry. Fos (1:50,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was amplified with biotinylated tyramine (0.6%) for 30 min at room temperature prior to incubation in CY-2 conjugated streptavidin (1:200; Jackson ImmunoResearch Laboratories) for 1 h. Following labeling for Fos, sections were labeled using an antibody directed against GnIH specifically for Syrian hamsters (1:10,000; PAC 1365), with CY-3 donkey anti-rabbit (1:200) as the secondary antibody/fluorophore. The antibody has been extensively characterized in this species and has been shown to be specific to GnIH with no cross-reactivity with related RFamide peptides (Gibson et al., 2008).

The population of GnIH-expressing cells is restricted to the DMH in Syrian hamsters, unlike in sheep, birds, and other rodents (Kriegsfeld, 2006; Kriegsfeld et al., 2006, 2010). In addition, we typically find a few scattered peri-DMH cells in Syrian hamsters. We used standard procedures for dual-label immunofluorescence to count all double-labeled cells in Syrian hamster sections that contained the DMH (see below under Light and Confocal Microscopy).

The mean number of GnIH cells was obtained by counting the number of cells that were labeled by the GnIH antibody in each animal, taking the sum of all GnIH labeled cells for each experimental group, and dividing by the sample size of the group. The percent of GnIH cells that were also labeled for Fos was calculated for each animal by counting the number of double-labeled cells, dividing by the total number of GnIH cells (the sum of the Fos-labeled plus the non-labeled GnIH cells), and multiplying by 100.

### Light microscopy

Brain sections processed for immunocytochemistry were mounted and coverslipped and were investigated using a Zeiss Z1 microscope. Sections were examined using the standard wavelengths for CY-2 (488 nm) and CY-3 (568 nm). Every fourth section through the DMH was assessed, and those areas expressing GnIH-Ir were recorded for coexpression with Fos protein using confocal microscopy (see below). For light microscopy, areas identified as having double-labeled cells were digitally captured at 200× in 8 bit grayscale using a cooled CCD camera (Zeiss). The total number of GnIH cells and the percentage of cells expressing Fos were recorded by two independent observers blind to the experimental conditions.

### Confocal microscopy

Cells characterized as double-labeled with Fos/GnIH at the conventional microscopy level were confirmed with confocal microscopy to ensure that Fos was expressed within the cells rather than in overlapping cells in the same field of view. Likewise, cells classified as single-labeled were assessed to ensure that the conventional microscopy strategy did not result in false negatives. At least 10% of those cells quantified using conventional microscopy were assessed in confocal scans for Fos co-labeling. Regions of the brain with putative double-label identified at the light level were scanned at 400× using confocal microscopy. Cells were observed under a Zeiss Axiovert 100TV fluorescence microscope (Carl Zeiss, Thornwood, NY, USA) with a Zeiss LSM 510 laser scanning confocal attachment. The sections were excited with an Argon–Krypton laser using the standard excitation wavelengths for CY-2 and CY-3. Stacked images were collected as 1.0 μm multitract optical sections. Using the LSM 3.95 software (Zeiss), red and green images of the sections were superimposed. GnIH cells in the DMH were examined through their entirety in 1.0 μm steps. Each microscope channel (i.e., CY-2 and CY-3) was excited independently in the same focal plane, and the photographs were merged into a single red–green image (because in fluorescence confocal microscopy, two fluorescent channels cannot be viewed simultaneously). The software program, Adobe Photoshop, was used to turn individual channels on (illuminated) and off independently, in order to confirm double-labeling of individual cells. First, we identified cells with a visibly stained nucleus in the GnIH channel,

and then, when the other channel was illuminated, noted those in which Fos-staining cells filled the void. This procedure greatly reduced the potential for counting false positives compared to dual-label quantification performed using two chromogens.

To examine NPY contacts, GnIH-Ir cells with putative NPY contacts were scanned though the extent of each cell in 0.5 μm increments. Cells characterized as double-labeled with NPY/GnIH at the conventional microscopy level were confirmed with confocal microscopy to ensure that Fos was expressed within the cells rather than in overlapping cells in the same field of view. Likewise, cells classified as single-labeled were assessed to ensure that the conventional microscopy strategy did not result in false negatives. Only those cells in which the NPY- labeled fiber contacted a GnIH-Ir cell in the same 0.5 μm scan were counted as close contacts.

#### *Leptin and insulin radioimmunoassay*

Blood plasma was analyzed for leptin using the Multi-Species Leptin Radioimmunoassay (RIA) kit (Millipore, St. Charles, MO, USA). Samples were run in duplicate in the same assay with assay limits between 1.0 ng/ml and 50 ng/ml. Similarly, plasma insulin was measured in duplicate using a Rat Insulin RIA kit (Millipore, St. Charles, MO, USA) adjusted to use 50 μl of plasma with assay limits between 0.01 and 10.0 ng/ml. Insulin and leptin assays were performed by Millipore Biomarker Services (St. Charles, MO, USA).

#### *Estradiol and progesterone radioimmunoassay*

Blood plasma was analyzed for estradiol and progesterone using RIAs (TKE21 and TKPG2, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Assay limits were between 10.0 and 1035.4 pg/ml for the estradiol assay and 0.09 and 13.0 ng/ml for the progesterone assay. For progesterone values to fall within the acceptable range, blood plasma was diluted 1:10 prior to analysis. Estradiol and progesterone assays were conducted by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA, USA).

#### *Statistical analysis*

Behavioral, hormonal, and immunohistochemical data were analyzed using one-way analysis of variance (ANOVA) with different durations of food restriction as the main effect. In order to meet the assumption of homogeneity of variances, some behavioral scores were log $(x+1)$ transformed prior to the ANOVA (Sokal and Rohlf, 1969). This applied to food hoarding, male preference, and flank marking. $F\max_{(5,4)}$ for those variables after log transformation were 4.8, 11.72, and 12.9 respectively, showing that there were no significant differences among the variances of the transformed scores. Means and SE of the means of the raw, untransformed scores appear in all figures for ease of presentation. When main effects were significant, *post hoc* comparisons were made using Duncan's Multiple Range test. Correlation coefficients were calculated to determine whether there was a significant association between cellular activation in GnIH cells and each behavior variable, or between plasma hormone concentrations and each behavioral variable. Differences were considered statistically significant if $P < 0.05$.

### EXPERIMENT 2: EFFECTS OF FOOD DEPRIVATION AND BODY FAT CONTENT ON CELLULAR ACTIVATION IN GnIH CELLS AND NPY PROJECTIONS TO THE DMH

These two experiments examined cellular activation in GnIH cells in the DMH that were either susceptible to or buffered from severe metabolic challenges (food deprivation). Previous work determined that adult, estrous-cycling hamsters below 120 g in body weight were highly likely to show anestrus after 48 h or more of food deprivation, whereas those above 125 g were buffered from the effects of food deprivation due to their higher body fat content and the ability to oxidize free fatty acids from lipids stored in adipose tissue (Schneider and Wade, 1989).

Hamsters that were the same age, with the same diet composition, were created by feeding diets that differed in the energy required to ingest them. The low body weight group was fed four pellets (approximately 20 g) of standard rodent chow in the wire hopper that hangs into the ceiling of the cage. The high body weight group was fed powdered rodent chow *ad libitum* on the floor of the cage. The former group showed a high level of activity as they stood upright and gnawed at the pellets. The latter group, those fed the powdered chow, expended comparably less energy and gained body weight faster because they were not required to chew their food in order to consume it, and they slept in close proximity, if not right in the food.

In the first experiment, Experiment 2A, hamsters were either high ($n = 5$, $133.13 \pm 2.9$ g) or low body weight ($n = 6$, $113.6 \pm 3.5$ g) and half of each group was fed *ad libitum* or food-deprived for 72 h ending on day 4 of the estrous cycle, the day of the LH *surge* and ovulation. This experiment was designed to determine whether cellular activation in GnIH cells on the day of the LH surge would be affected by the severe energetic challenge known to induce anestrus, and whether having a high body fat content prior to deprivation would buffer this effect. LH assays were performed by The University of Virginia Ligand Assay and Analysis Core Laboratory Services using the Rat Sandwich-IRMA assay. Two-way ANOVA, with food availability and prior body weight as the two main factors, was used to analyze the data.

The second experiment, Experiment 2B, was designed to examine cellular activation in GnIH cells earlier, in the follicular phase, during the initiation of effects of food restriction on the GnRH *pulse generator*. Thus, 18 hamsters of a high ($n = 9$, $121.2 \pm 2$ g) or low body weight ($n = 9$, $104.2 \pm 3.1$ g) were food-deprived for either 36 h (euthanized on day 2 of the estrous cycle) or 50 h (euthanized on day 3 of the cycle). An additional group ($n = 6$, $131.4 \pm 2.5$) served as *ad libitum*-fed controls and data were analyzed with a one-way ANOVA.

In both experiments, the blood was sampled and assayed for LH, and hamsters perfused as described for Experiment 1. Animals were perfused intracardially with PBS (pH 7.4 at 4°C) followed by 4% paraformaldehyde in PBS at the same temperature. Brains were removed, post-fixed for 24 h at 4°C in 4% paraformaldehyde, and stored at 4°C in 20% sucrose and 0.001% thimerosal until sectioning. All brains were sectioned within 30 days using a freezing microtome set at 40 μm. Hypothalamic brain sections were placed into PVP and stored at −20°C until staining.

Double-labeling for Fos and GnIH and double-labeling for NPY and GnIH was carried out as described in Experiment 1

on every fourth section. NPY fibers were immunostained using an NPY antibody, rabbit polyclonal anti-NPY (1:10,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and was amplified with biotinylated tyramine (0.6%) for 30 min at room temperature prior to incubation in CY-2 conjugated streptavidin (1:200; Jackson ImmunoResearch Laboratories) for 1 h. Following labeling for NPY, sections were labeled using an antibody directed against GnIH specifically for Syrian hamsters (1:10,000; PAC 1365), with CY-3 donkey anti-rabbit (1:200) as the secondary antibody/fluorophore.

## RESULTS

### EXPERIMENT 1: DIFFERENT DURATIONS OF FOOD RESTRICTION, GnIH AND BEHAVIOR

#### *Ingestive behaviors*

One-way ANOVA showed a significant main effect of food availability on the amount of food hoarded [$F(5,42) = 2.64$, $P < 0.04$] as well as on the log $(x + 1)$ transform of the amount of food hoarded [$F(5,42) = 5.42$, $P < 0.02$; **Figure 1**, top]. Similarly, when the amount of food hoarded post-restriction was subtracted from baseline food hoarded, there was a significant effect of food restriction on the change in the amount of food hoarded [$F(5,42) = 2.75$, $P < 0.03$]. *Post hoc* tests showed that the amount of food hoarded was significantly higher in the 8-day and 12-day food-restricted groups compared to the *ad libitum*-fed group and the 4-day food-restricted group ($P < 0.05$).

There was a significant main effect of food availability on the amount of time spent eating during the preference test [$F(5,42) = 7.56$, $P < 0.0001$; **Table 1**]. Hamsters spent significantly more time eating after 4, 8, and 12 days of food restriction compared to hamsters fed *ad libitum* ($P < 0.05$; **Table 1**).

The amount of food eaten (g) during the 90-min test (**Figure 2C**) did not differ significantly among groups fed *ad libitum* or food-restricted for varying durations.

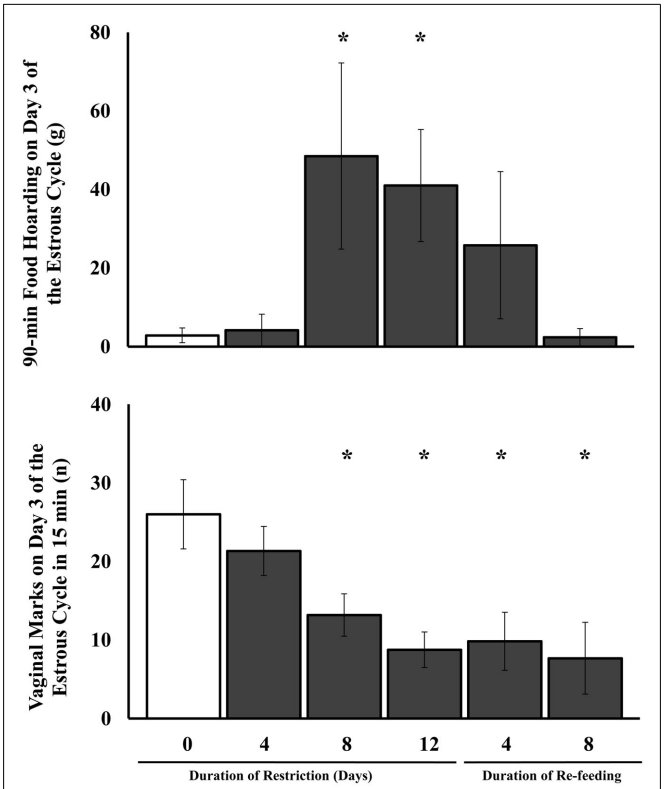


**FIGURE 1 | Mean and SE of the mean for the amount of food hoarded (top) and the number of vaginal marks produced in 15 min (bottom) on day 3 of the estrous cycle in Experiment 1.** Each group of food-restricted female Syrian hamsters were fed 75% of their *ad libitum* intake for different durations before testing. Re-fed hamsters were food-restricted for 12 days and then re-fed *ad libitum* for 4 or 8 days. *Significantly different from hamsters fed *ad libitum* at $P < 0.05$.

#### *Reproductive behaviors*

The effect of food availability on the number of vaginal scent marks per 15 min was significant [$F(5,42) = 4.66$, $P < 0.002$; **Figure 1**, bottom]. Hamsters food-restricted for 8 and 12 days showed significantly fewer vaginal scent marks than those fed *ad libitum* and those food-restricted for only 4 days ($P < 0.05$), but those re-fed for 4 and 8 days still showed significantly fewer vaginal scent marks than those fed *ad libitum* ($P < 0.05$).

The effect of food availability on the number of flank marks was significant [$F(5,42) = 3.48$, $P < 0.01$] as was the log $(x + 1)$ transform of flank marking [$F(5,42) = 4.7$, $P < 0.002$]. The number of flank marks in 4-day food-restricted females was significantly higher than that of females fed *ad libitum* ($P < 0.05$), but the flank marking scores of hamsters in the other groups were not significantly higher than those of hamsters fed *ad libitum* (**Table 1**).

Male preference was calculated as (the amount of time females spent with a male – the amount of time spent with food)/the total time in the preference apparatus (**Table 1**). There was a main effect of food restriction on male preference [$F(5,40) = 3.81$; $P < 0.007$]. There was a main effect of food restriction on the log $(x + 1)$ transform of male preference [$F(5,40) = 3.44$; $P < 0.01$]. *Post hoc* analysis showed that 12-day restricted hamsters had significantly lower male preference than *ad libitum*-fed female hamsters.

#### *Body weight*

When the hamsters' final body weights were subtracted from initial body weights, the groups differed significantly in the amount of body weight lost [$F(5,42) = 30.92$, $P < 0.0001$; **Figure 2A**].

Body weights among the groups were not significantly different at the start of the experiment. The effect of duration of food restriction on final body weight was significant [$F(5,42) = 4.37$, $P < 0.003$; **Figure 2B**]. Body weights were significantly decreased starting at 4 days after the start of food restriction ($P < 0.05$) compared to hamsters fed *ad libitum*. Hamsters fed *ad libitum* throughout the experiment were significantly heavier compared to all other groups except hamsters food-restricted for 12 days and re-fed *ad libitum* for 8 days (**Figure 2B**).

#### *GnIH immunoreactivity and cellular activation*

Cellular activation in GnIH-Ir cells was calculated as (the number of cells double-labeled for Fos-Ir and GnIH-Ir/the total number of GnIH-Ir cells) × 100. There was a significant main effect of food availability on cellular activation in GnIH-Ir cells

**Table 1 | Mean and SEM for appetitive behaviors (other than those shown in Figure 1) measured at different durations of 75% food restriction in female Syrian hamsters provided with a choice between food and a male hamster.**

| | Duration of food restriction (days) | | | | Duration of re-feeding | |
|---|---|---|---|---|---|---|
| | 0 (*Ad libitum*) | 4 | 8 | 12 | 4 | 8 |
| Time spent eating (s) | 24.6 ± 6.9 | 226.7 ± 59.4* | 225.8 ± 57.6* | 263.8 ± 48.7* | 79.2 ± 36.2 | 36.7 ± 20.9 |
| Time spent hoarding (s) | 0 ± 0 | 0 ± 0 | 35.0 ± 35.0 | 12.5 ± 6.2 | 72.5 ± 64.8 | 20.8 ± 20.8 |
| Flank marks (*n*) | 2.4 ± 1.5 | 7.5 ± 2.1* | 2.5 ± 1.3 | 0.3 ± 0.3 | 0 ± 0 | 0.2 ± 0.2 |
| Male preference (time with male-time with food)/total time | 0.31 ± 0.10 | 0.23 ± 0.13 | 0.04 ± 0.16 | −0.08 ± 0.10 | −0.12 ± 0.10 | 0.22 ± 0.21 |

**Significantly different from time 0 at $P < 0.05$.*

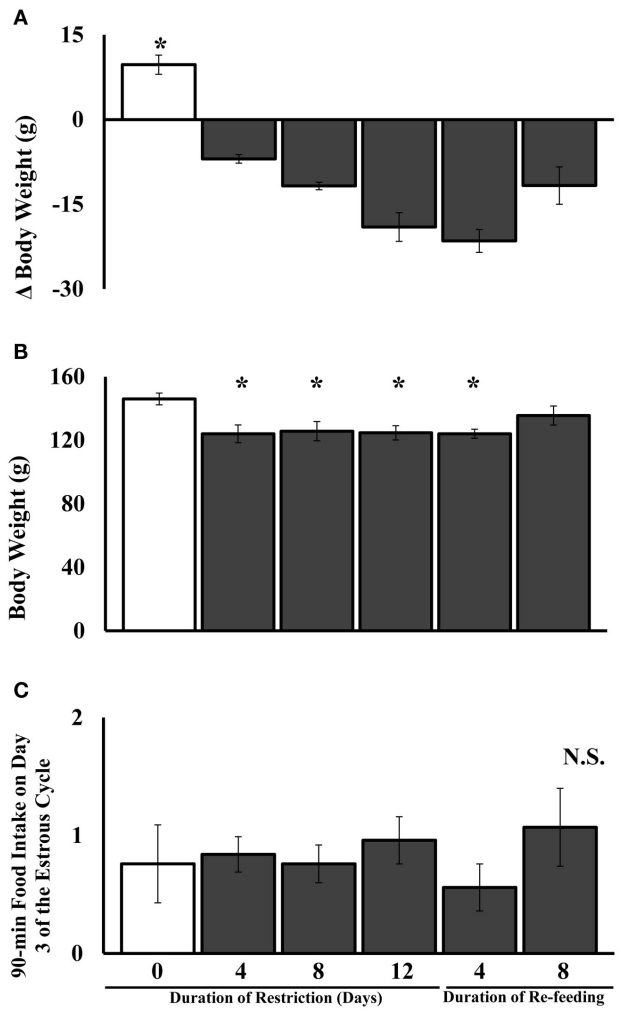


**FIGURE 2 | Mean and SE of the mean for (A)**. body weight change, **(B)** final body weight, and **(C)** 90-min food intake of female Syrian hamsters either fed *ad libitum* or food-restricted to 75% of their *ad libitum* intake for 4, 8, or 12 days or food-restricted for 12 days and then re-fed for 4 or 8 days in Experiment 1. *Significantly different from *ad libitum* at $P < 0.05$.

[$F(5,38) = 3.47$, $P < 0.01$; **Figure 3A**]. *Post hoc* analysis revealed a significant increase in cellular activation in GnIH-Ir cells at 8 and 12 days of food restriction compared to hamsters fed *ad libitum* ($P < 0.05$).

There was a significant effect of food restriction on the total number of GnIH-Ir cells [$F(5,38) = 2.88$, $P < 0.03$], with a significant decrease in the number of GnIH cells that were immunoreactive in the females food-restricted for 8 and 12 days compared to those fed *ad libitum* and those food-restricted for 4 days ($P < 0.05$; **Figure 3B**).

There was a significant main effect of food restriction on the absolute number of double-labeled Fos-Ir/GnIH-Ir cells [$F(5,38) = 2.457$, $P < 0.05$]. *Post hoc* analysis showed significant increases in the number of double-labeled cells 4 and 8 days after restriction ($P < 0.05$; **Figure 3C**).

### *Plasma leptin, insulin, estradiol, and progesterone concentrations*

There was a significant main effect of food treatment on plasma leptin concentrations [$F(5,41) = 2.50$, $P < 0.05$; **Figure 4**]. *Post hoc* comparisons revealed that plasma leptin concentrations did not differ between *ad libitum*-fed and food-restricted females after any level of food restriction. However, females food-restricted for 12 days and re-fed *ad libitum* for 4 days had significantly higher plasma leptin concentrations compared to females fed *ad libitum*. The effect of food restriction or re-feeding on plasma insulin, progesterone, or estradiol concentrations were not significant (**Figure 4**).

### *Correlations*

Changes in food hoarding during food restriction (**Figure 1**, top) showed a striking resemblance to changes in cellular activation in GnIH-Ir cells (**Figure 3A**). There was a significant positive correlation between the amount of food hoarded and percent of GnIH-Ir cells that were positive for Fos-Ir ($r = 0.585$; $P < 0.0001$), and a significant negative correlation between food hoarded and GnIH-Ir cell count ($r = 0.436$; $P < 0.003$). The amount of food hoarded was significantly correlated with body weight loss ($r = 0.368$; $P < 0.01$), but not with raw body weight. The correlations between food hoarding and other variables were not statistically significant (body weight, leptin, insulin, estradiol, or progesterone concentrations).

There was a significant negative correlation between time spent eating and body weight ($r = 0.437$; $P < 0.002$). The correlations between time spent eating and other variables were not statistically significant (change in body weight, number of GnIH-Ir cells, and percent of GnIH-Ir cells that were positive for Fos-Ir, plasma insulin, leptin, estradiol, and progesterone concentrations).

The correlations among 90-min food intake and the other variables were not statistically significant (body weight, change in body

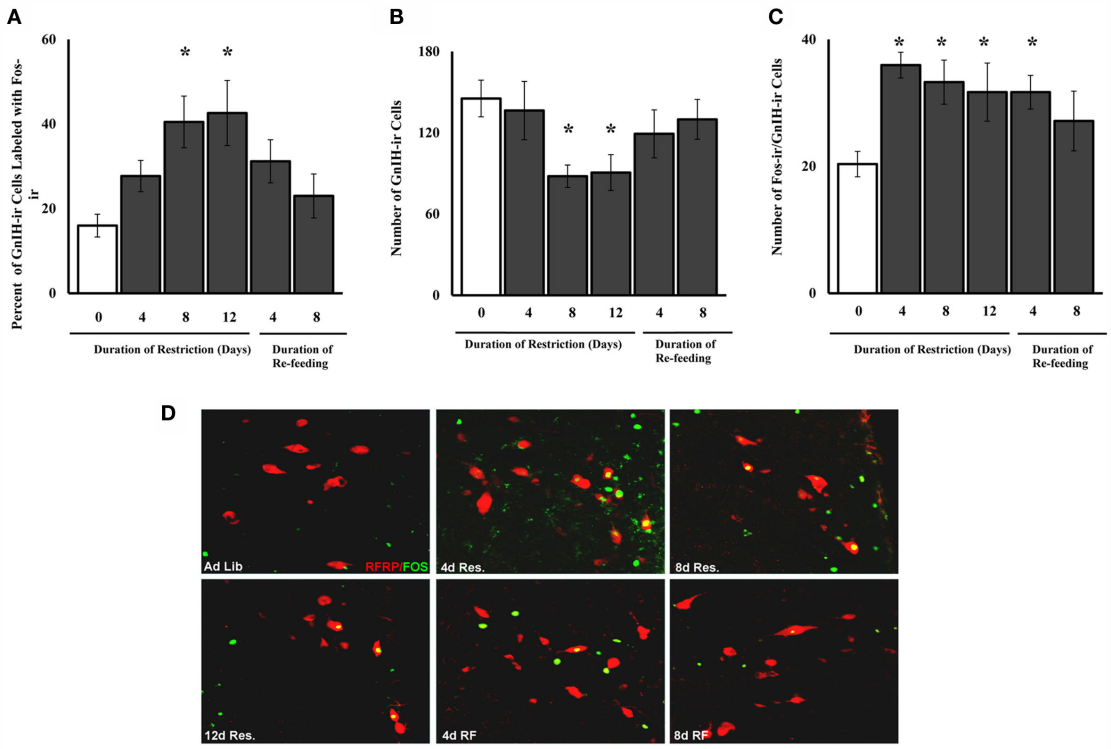


**FIGURE 3 | Mean and SE of the mean for (A)**. the percent of GnIH-Ir cells that showed Fos-Ir [(the number of double-labeled Fos-Ir and GnIH-Ir cells divided by the total number of GnIH-Ir cells) multiplied by 100], **(B)**. the number of GnIH-Ir cells per animal (both Fos-Ir-labeled and unlabeled), **(C)**. the absolute number of cells that were double-labeled for both Fos-Ir and GnIH-Ir in gonadally intact, female Syrian hamsters food-restricted for different durations, and **(D)**. photomicrographs of cells double-labeled for GnIH-Ir (red) and Fos-Ir (green) following food restriction and re-feeding. Food-restricted females were fed 75% of their *ad libitum* intake for 4, 8, or 12 days or were food-restricted for 12 days and then re-fed for 4 or 8 days in Experiment 1. *Significantly different from *ad libitum* at $P < 0.05$.

weight, number of GnIH-Ir cells, or percent of GnIH-Ir cells that were positive for Fos-Ir, plasma insulin, estradiol, and progesterone concentrations).

There was a significant negative correlation between vaginal scent marks and cellular activation in GnIH-Ir cells ($r = -0.314$; $P < 0.04$) and a positive correlation between the number of vaginal scent marks and the number of cells that showed GnIH-Ir ($r = 0.365$; $P < 0.02$). Vaginal scent marks were significantly negatively correlated with body weight loss; the more body weight lost, the fewer vaginal scent marks ($r = -0.619$; $P < 0.0001$), but vaginal scent marks were not significantly correlated with final body weight. Vaginal scent marks were also positively correlated with plasma progesterone concentrations ($r = 0.354$, $P < 0.02$), but not with leptin, insulin, or estradiol concentrations.

There were no significant correlations between flank marks and any other variables (number of GnIH-Ir cells, cellular activation in GnIH-Ir cells, change in body weight, insulin, estradiol, or progesterone concentrations).

There was a significant negative correlation between male preference and change in body weight ($r = 0.352$; $P < 0.01$), but the correlations between male preference and other variables were not statistically significant (body weight, number of GnIH-Ir cells, cellular activation in GnIH-Ir cells, plasma leptin, insulin, estradiol, or progesterone concentrations).

There was a significant positive correlation between body weight and plasma progesterone concentrations ($r = 0.302$; $P < 0.04$) and between body weight and plasma leptin concentrations ($r = 0.285$; $P < 0.05$), but not between body weight and plasma insulin or estradiol concentrations. Body weight was not significantly correlated with either the number of GnIH cells, or the percent of GnIH-Ir cells that were positive for Fos. Change in body weight was significantly positively correlated with plasma progesterone concentrations ($r = 0.451$; $P < 0.001$) and the number of GnIH-Ir cells ($r = 0.459$; $P < 0.002$) and significantly negatively correlated with the percent of GnIH-Ir cells that were positive for Fos-Ir ($r = 0.570$; $P < 0.0001$).

## EXPERIMENT 2: EFFECT OF METABOLIC CHALLENGES ON NPY FIBERS IN THE DMH

In Experiment 2A, females with either high or low body weight were either fed *ad libitum* or food-deprived for 72 h from day 1 to day 4 of the estrous cycle (**Figure 5**). Previous work showed that the lean, food-deprived females would become anestrus, whereas those that were fat at the start of deprivation would be buffered from the effects of deprivation (Schneider and Wade, 1989, 1990). Two-way ANOVA showed a significant main effect of food deprivation on final body weight, with lean females weighing less than fat [$F(1,6) = 9.78$, $P < 0.02$]. *Post hoc* analysis showed

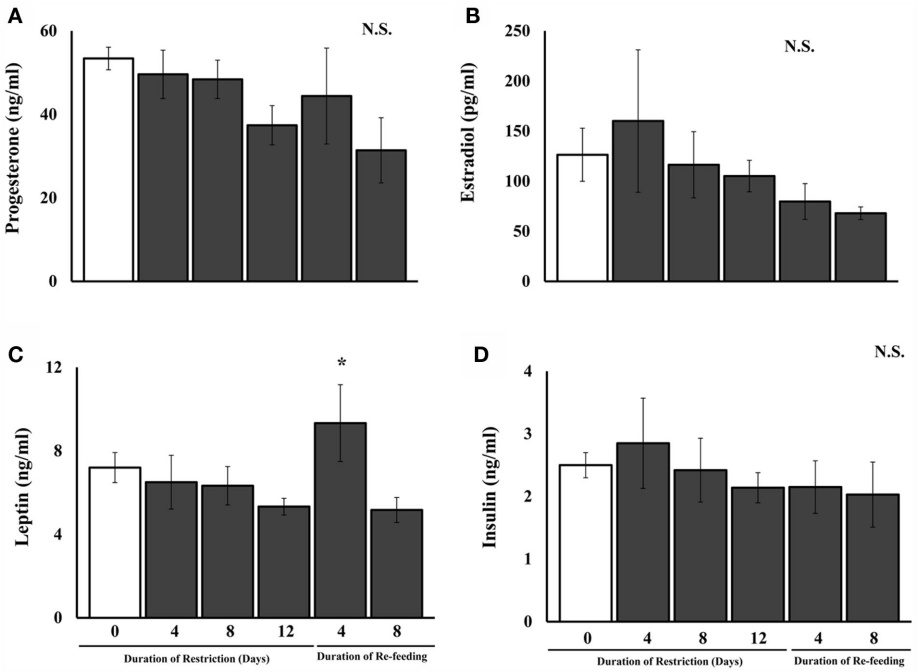


**FIGURE 4 | Mean and SE of the mean for plasma concentrations of (A)**. progesterone, **(B)**. estradiol, **(C)**. leptin, and **(D)**. insulin in female Syrian hamsters either fed *ad libitum* or food-restricted to 75% of their *ad libitum* intake at 4, 8, or 12 days after the start of food restriction or after 12 days of restriction and either 4 or 8 days of re-feeding in Experiment 1. *Significantly different from *ad libitum* at $P < 0.05$.

that food-deprived lean females weighed significantly less than food-deprived, fat females ($P < 0.05$). The same analysis of the change in body weight over the course of food deprivation showed a significant main effect of food deprivation [$F(1,6) = 49.51$, $P < 0.0004$]. Two-way ANOVA showed a significant main effect of food deprivation on the percent of GnIH-Ir cells that were positive for Fos-Ir [$F(1,6) = 7.69$, $P < 0.03$], no significant effect of body weight group, and no significant interaction. The more body weight lost, the higher the increase in percent of GnIH-Ir cells that were positive for Fos-Ir, and this correlation was significant ($r = 0.72$, $P < 0.02$). Body weight loss was significantly negatively correlated with the number of cells that were immunoreactive for GnIH ($r = 0.72$, $P < 0.02$). Neither the percent of GnIH-Ir cells positive for Fos-Ir nor the number of GnIH-Ir cells was significantly correlated with final body weight. There was a significant main effect of food deprivation or *ad libitum* feeding on plasma LH concentrations [$F(2,4) = 16.217$, $P < 0.01$]. As expected, the lean, food-deprived females had plasma LH concentrations significantly lower ($0.04 \pm 0.0001$ ng/ml) than the fat, food-deprived ($0.131 \pm 0.2$ ng/ml) and the *ad libitum*-fed control females (fat and lean combined $0.205 \pm 0.038$ ng/ml).

In Experiment 2B, females were sacrificed after either 1.5 or 2.5 days of food deprivation during the follicular phase of the estrous cycle (Days 1 and 2 of the estrous cycle) to determine whether there were changes in GnIH that occur in the early stages of metabolic challenge that would be expected to inhibit the GnRH pulse generator in lean, but not fat females (Morin, 1986). One-way ANOVA showed no significant effect of treatment group on the percent of GnIH-Ir cells that were positive for Fos-Ir, and a significant effect of treatment group on the number of GnIH-Ir cells [$F(2,16) = 27.95$, $P < 0.0001$; **Table 2**]. Both food-deprived groups (30 and 50 h of deprivation) had significantly fewer GnIH-Ir cells than did the *ad libitum*-fed controls ($P < 0.0001$). The percent of GnIH-Ir cells that were positive for Fos-Ir increased linearly with the amount of body weight loss and this correlation was significant ($r = 0.62$, $P < 0.004$). This variable was also significantly positively correlated with final body weight ($r = 0.576$, $P < 0.01$). The number of GnIH-Ir cells was also significantly negatively correlated with the amount of body weight lost ($r = -0.58$, $P < 0.01$) and with final body weight ($r = -0.59$, $P < 0.01$).

Double-labeling for GnIH-Ir and NPY-Ir revealed that NPY-Ir nerve fibers were densely packed in the DMH, and that putative NPY terminals can be observed in close proximity to GnIH cell bodies within this brain area (**Figure 7**) at low power light microscopy and confirmed at high power light microscopy and confocal microscopy. An average of 41.46% GnIH-Ir cell bodies per animal ($n = 6$) receive contacts from NPY-Ir fibers in the DMH.

### *Discussion*

These experiments showed the following: (1) There was a linear effect of energy availability (duration of food restriction) on ingestive and sex behavior in Syrian hamsters, (2) mechanisms that control motivation were more sensitive to energy availability than mechanisms that control the performance of eating and copulation, (3) there was a linear effect of energy availability on the number of GnIH-Ir cells and on cellular activation in GnIH-Ir cells in the DMH, (4) *cellular activation* in GnIH-Ir cells was positively correlated with food hoarding and negatively correlated with vaginal scent marking, (5) GnIH-Ir *cell number* was

**Table 2 | Mean and SEM for body weight, change in body weight, percent of GnIH-Ir cells double-labeled for Fos, and the number of GnIH cells in Syrian hamsters from Experiment 2B. Hamsters were sacrificed in the early follicular phase of the estrous cycle just 36 or 50 h after the start of food deprivation on the morning of day 1 of the estrous cycle to determine whether there might be changes in GnIH cells that would predict reproductive states at the time of the LH surge that would be expected to occur on day 4 of the estrous cycle. Anestrous was expected to occur only in lean food deprived females.**

| | *Ad libitum* | Fat, food-deprived | | Lean, food-deprived | |
|---|---|---|---|---|---|
| | | 36 h | 50 h | 36 h | 50 h |
| Body weight (g) | 131.4 ± 2.5 | 115.6 ± 3.0* | 104.7 ± 7.2* | 96.7 ± 0.5* | 85.9 ± 2.0* |
| Δ Body weight (g) | −0.03 ± 0.5 | −5.6 ± 0.6* | −12.2 ± 1.0* | −5.4 ± 0.2* | −14.6 ± 1.5* |
| %FOS + GnIH | 24.5 ± 3.3 | 32.3 ± 6.7 | 34.3 ± 4.2 | 35.3 ± 3.8 | 48.6 ± 8.9 |
| GnIH-ir | 122.6 ± 9.9 | 24.1 ± 5.1* | 38.0 ± 5.2* | 48.7 ± 11.6* | 36.8 ± 15.3* |

**Significantly different from ad libitum-fed at $P < 0.05$*

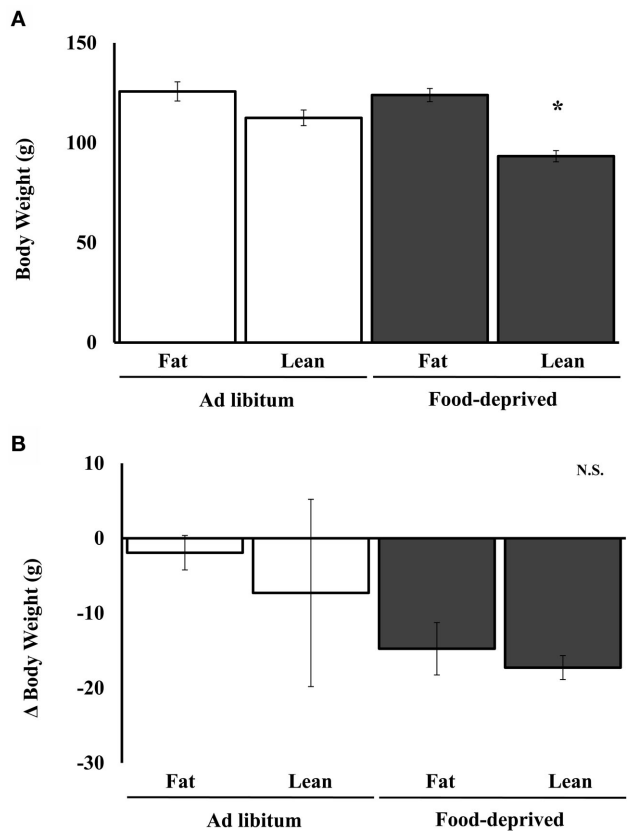


**FIGURE 5 | Mean and SE of the mean for (A)**. body weight of *ad libitum*-fed or food-deprived female hamsters that were lean or fat prior to the start of food deprivation and **(B)**. change in body weight of the same females after food deprivation in Experiment 2A. *Significantly different from *ad libitum* at $P < 0.05$.

negatively correlated with food hoarding and positively correlated with vaginal scent marking, (6) there was no significant effect of food restriction on plasma leptin, insulin, estradiol, or progesterone concentrations and no significant correlation among hormone concentrations and cellular activation of GnIH-Ir cells, and (7) strong projections of NPY-Ir fibers were found in close apposition to GnIH-containing cell bodies in the DMH. Together these results are consistent with the idea that a wide range of metabolic deficits have effects on the GnIH system that are significantly correlated with changes in motivation. These effects on cellular activation in GnIH-Ir cells occurred in females that showed normal circulating levels of ovarian steroids, i.e., females that were not hypogonadotropic. Furthermore, projections from NPY-Ir cells to GnIH cells in the DMH are one possible route of transmission of information about energy availability to the GnIH system.

By using mild levels of food restriction at different durations, we were able to dissociate appetitive from consummatory behaviors. Appetitive, but not consummatory behaviors were affected by shorter durations of food restriction. This, in turn, enabled the investigation of neuroanatomical correlates. Food hoarding and vaginal scent marking were significantly affected at 8 and 12 days of 25% food restriction, even though there was no effect on the consummatory behavior, food intake (**Figures 1** and **2**). Mild food restriction did not lead to significant decreases in circulating levels of estradiol and progesterone (**Figures 4A,B**), confirming that estrous cycles were not disrupted. The body weight, age and level of food restriction were chosen for these experiments because two previously published experiments showed that, under these conditions, estrous cycles were not disrupted and females showed no deficits in lordosis frequency or duration (Klingerman et al., 2010, 2011). Changes in cellular activation in GnIH-Ir cells in non-hypogonadotropic Syrian hamsters suggests that this peptide plays a role in control of behavioral motivation, rather than or in addition to control of the HPG system.

The most striking and unexpected observation was the close correlation between cellular activation in GnIH-Ir cells and the change in appetitive behaviors seen in mildly food-restricted females in Experiment 1 (**Figure 3**). Just as the appetitive behaviors (food hoarding and vaginal scent marking) showed significant changes at 8 and 12 days after restriction, cellular activation in GnIH-Ir cells also increased at these same time points. Restoration of food hoarding and Fos/GnIH-Ir to baseline levels occurred at the same time after re-feeding (**Figures 1** and **3**). In contrast to vaginal scent marking, flank marking was not significantly correlated with changes in cellular activation in GnIH-Ir cells or number of GnIH-Ir cells. Thus, the correlation was between neural activation in GnIH-Ir cells and appetitive ingestive and sex

behavior, rather than between GnIH and agonistic social behavior. There was also no correlation between activation in GnIH-Ir cells and consummatory behaviors.

An unexpected finding was that in Experiment 2A, lean and fat food-*deprived* (in contrast to mildly food-restricted) females differed significantly in their body weight and plasma LH concentrations, but did not differ in cellular activation in GnIH-Ir cells (**Figures 5A** and **6B**). Lean, but not fat, food-deprived females were expected to become anestrus based on previously published data (Schneider and Wade, 1989, 1990). Consistent with this prediction, the lean, food-deprived females had lower mean LH levels than fat, food-deprived females, yet both groups showed significant increases in cellular activation in GnIH-Ir (**Figures 6B,C**). Thus, it is possible that in Syrian hamsters, GnIH has important effects on behavioral motivation (**Figure 1**) without inhibition of LH secretion.

Food restriction significantly increased the number of Fos-positive GnIH-Ir cells as well as the percent of the total GnIH-Ir cells that were Fos-positive. Food restriction did not significantly increase the number of GnIH-Ir cells, and, in some groups, decreased the number GnIH-Ir cells (**Figures 3B** and **6A**). The increase in the number of activated GnIH-Ir cells coupled with a decreased number of GnIH-Ir cells might reflect increased GnIH release without a compensatory increase in GnIH synthesis. There is precedence in the literature for a decrease in cell number concomitant with an increase in cellular activation and release of peptide. For example, gonadatropin *releasing*-hormone (Gn*R*H)-Ir cells in rats also decrease in number during the latter part of the LH surge when the number and percent of Fos-Ir/GnRH-Ir double-labeled cells increases (Lee et al., 1990). In addition, a different environmental factor, short day length, also causes a decrease in GnIH-Ir cell number along with gonadal regression and inhibition of LH secretion (Kriegsfeld et al., 2010). Perhaps if we had sacrificed the hamsters earlier, the GnIH-Ir cell population might have remained stable in number. This could be confirmed by examination of GnIH-Ir at earlier time points, by examination of GnIH-Ir in hamsters treated with agents that block axon transport, and by measuring GnIH gene expression using *in situ* hybridization.

Another possibility is that increased cellular activation along with the observed decrease in the total number of GnIH-Ir cells represents inhibition of GnIH synthesis and secretion. This s unlikely because the decrease in cell number in this experiment did not occur in all experimental groups (for example the 4-day food-restricted group; **Figure 3B**), and there was a significant increase in double-labeled cells in all food-restricted females (**Figure 3C**; not just an increase in the percent of cells). As mentioned in an earlier paragraph, however, exposure of hamsters to short day length decreases GnIH-Ir cell number under circumstances in which increases in GnIH secretion appear to underlie inhibition of LH secretion and gonadal regression (Kriegsfeld et al., 2010).

The decrease in GnIH-Ir cell number, along with an increase in the percent of GnIH cells that were activated closely correlated with behavioral motivation might be a reflection of a subpopulation of cells that is particularly responsive to energy availability. Evidence in other species suggests that KiSS-1 and GnIH act together to coordinate the effects of day length and food availability. KiSS-1 expression increases with food restriction in Siberian hamsters (Paul et al., 2009), is associated with increased GnRH and LH secretion, and has been located in the DMH of rats (Brailoiu et al., 2005). It is not known whether there are KiSS-1-containing cells in the Syrian hamster DMH or sites that project to the DMH. Furthermore, GnIH and KiSS-1 might be implicated in both circadian and seasonal rhythms related to energy balance and reproduction in hamsters. For example, DMH lesions block the effects of short day length on the HPG system in Syrian hamsters. The suprachiasmatic nucleus (SCN) projects to a large proportion of GnIH cells in the DMH, and these project to the vicinity of GnRH cells. Thus, it is plausible that information from peripheral or central oscillators are influenced by metabolic fuel availability and project to the SCN, which, in turn, might influence GnIH, KiSS-1 or other cells in the DMH. Other investigators have suggested that the DMH itself receives information generated by the ingestion of meals in mice and rats (Gooley et al., 2006; Fuller et al., 2008), although other evidence contradicts the idea that the DMH is necessary for meal entrainment of circadian rhythms in rats and mice (Landry et al., 2006, 2007; Moriya et al., 2009).

Food restriction in Experiment 1 and food deprivation in Experiment 2A had significant effects on both cellular activation and on number of GnIH-Ir cells, but it is not clear how this information about food availability reaches the DMH. Food restriction, for example, had significant effects on appetitive behaviors without significant effect on plasma concentrations of ovarian steroids, insulin, or leptin, suggesting that information about fuel availability reaches GnIH cells via other means, e.g., via changes in plasma ghrelin or direct information about the availability of oxidizable metabolic fuels detected in periphery, brain stem or hypothalamic areas that project to GnIH cells.

One possibility is that GnIH cells in food-restricted females are more responsive to estradiol than those GnIH cells in females fed *ad libitum*. As expected, there were no significant food restriction-induced decreases in plasma estradiol concentrations, even in groups that showed food restriction-induced changes in sex and ingestive behavior (**Figure 4**). Thus, one possible explanation is up-regulation of ER on GnIH cells that project to areas involved in ingestive behavior and a down-regulation of ER in areas involved in sex behavior. A similar suggestion has been made regarding up-regulation of ER in other brain areas involved ingestive behavior (PVH) and a down-regulation of ER in brain areas involved in lordosis (VMH; Li et al., 1994; Panicker et al., 1998). The DMH and appetitive behaviors were not examined in these latter studies. However, ER-α co-localizes with GnIH cells in the Syrian hamsters DMH, and these cells respond to estradiol stimulation with significant increases in cellular activation (Kriegsfeld et al., 2006). Thus, future experiments will determine whether different levels of food restriction (mild to severe) down-regulates ER-α in GnIH cells in the DMH, or whether the effects of food restriction might occur downstream or independent from ER-α-containing GnIH cells.

One such downstream mediator might be GnRH. Midbrain GnRH and its metabolites have well-documented facilitatory effects on sex behavior that are unrelated to LH secretion (Moss and McCann, 1975; Moss and Foreman, 1976; Dudley et al., 1981; Dudley and Moss, 1988, 1991; Moss and Dudley, 1990; Wu et al., 2006). It is plausible that GnIH-mediated inhibition of GnRH

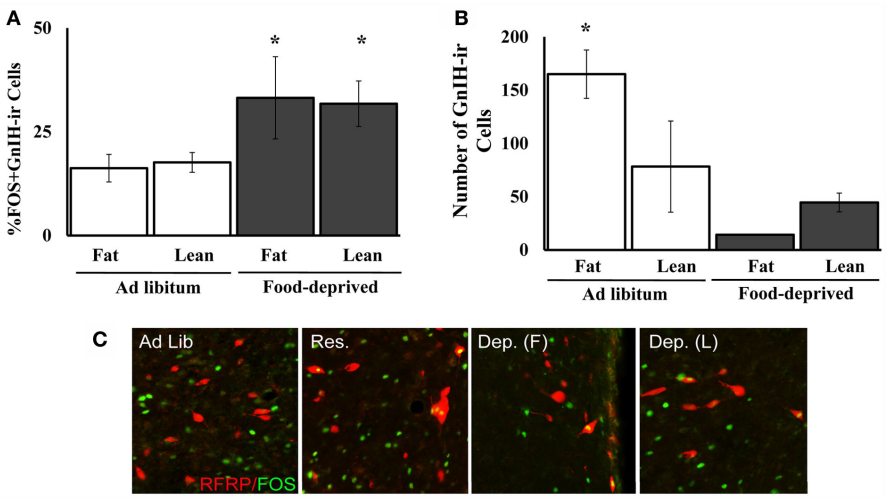


**FIGURE 6 | Mean and SE of the mean for (A)**. the percent of GnIH-Ir cells double-labeled for Fos-Ir in the DMH and **(B)**. the number of GnIH-Ir cells (both Fos-Ir-labeled and unlabeled) in the DMH of female Syrian hamsters that were food-deprived or fed *ad libitum*, and half of the food-deprived hamsters were lean and the other half were fattened prior to the start of food deprivation in Experiment 2A. **(C)**. Photomicrographs of GnIH/Fos-Ir in the groups described. *Significantly different from *ad libitum* at $P < 0.05$.

secretion accounts for inhibition of appetitive sex behavior in Syrian hamsters. Furthermore, the appetitive ingestive behavior, food hoarding, was significantly increased in the present experiment, consistent with mounting evidence that ingestive behaviors are increased by GnIH (Tachibana et al., 2005; Johnson et al., 2007). GnIH inhibits GnRH secretion in Syrian hamsters (Kriegsfeld et al., 2006), and at least one form of GnRH (GnRH-II) is inhibitory for ingestive behavior (Kauffman, 2004; Kauffman and Rissman, 2004a,b; Kauffman et al., 2005).

A large body of research implicates NPY in metabolic control of reproduction and ingestive behavior. NPY is a potent orexigenic peptide (Clark et al., 1984; Kulkosky et al., 1988; Corp et al., 2001; Clarke et al., 2005), inhibits sex behavior (Clark et al., 1985; Thornton et al., 1996), and inhibits LH secretion in the presence of low circulating levels of estradiol (Khorram et al., 1987; Sahu et al., 1987; Malven et al., 1992). NPY has greater effects on appetitive than consummatory behaviors (Ammar et al., 2000; Day et al., 2005; Keen-Rhinehart and Bartness, 2007). Furthermore, NPY cell bodies in the DMH and other brain areas have long been implicated in control of energy intake. NPY mRNA is overexpressed in the DMH during the hyperphagia of lactation (Smith, 1993) and in various models of obesity (Kesterson et al., 1997; Guan et al., 1998a,b; Tritos et al., 1998). Increases in NPY gene expression in the DMH of lean rats increases food intake and body weight, and accelerates the development of high-fat diet-induced obesity, and decreased NPY expression in the DMH prevents the hyperphagia, obesity and diabetes of Otsuka Long–Evans Tokushima Fatty rats (Yang et al., 2009). Thus, we were compelled to examine the proximity of NPY projections to GnIH cells in the DMH. Food-deprived females were used to maximize identification of NPY cells. NPY terminals showed strong projections to the DMH and were seen in close apposition to GnIH cells (**Figure 7**). It is possible that these NPY cells originate in the arcuate nucleus of the hypothalamus, the brain stem, or from within the DMH, all areas where NPY gene expression has been identified and from which

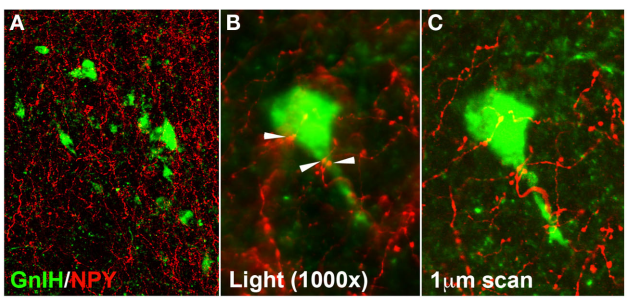


**FIGURE 7 | Representative photomicrographs of cells double-labeled for GnIH (red) and NPY (green).** A cluster of GnIH-r cells receive extensive NPY projections at 250× in this confocal scan **(A)** and a single GnIH-Ir cell with presumptive NPY boutons at 1000× at the conventional **(B)** and confocal **(C)** microscopic levels.

NPY cells project to the DMH in other rodents (Bai et al., 1985; Sahu et al., 1988; Bi et al., 2003).

In summary, these results show a clear correlation between cellular activation in GnIH-Ir cells and appetitive sex and ingestive behaviors. It is not known, however, whether GnIH secretion *causes* changes in motivation. GnIH might be a causal factor for increased hunger and food hoarding, decreased sexual motivation, or both, but it might be a non-functional correlate of other causal factors (metabolic events, other hormones, and other neuropeptides such as kisspeptin, NPY, alpha-melanocyte stimulating hormone or orexin). GnRH, for example, might influence ingestive and sex behavior by virtue of its direct link to metabolic cues, since recent evidence shows that GnRH neurons receive dendritic input from outside the blood–brain barrier (Herde et al., 2011). Further work is necessary to determine whether changes in GnIH cells are a fortuitous correlate or a causal factor in control of behavior. Nevertheless, these results are consistent with the idea that GnIH

in the DMH, and possibly NPY cells that project to the DMH are part of a system that prioritizes sex and ingestive behavior in order to optimize reproductive success in environments where energy availability fluctuates. Current experiments are underway to examine if central or systemic treatment with GnIH, NPY, and antagonists to their receptors have the expected influences on appetitive and consummatory sex and ingestive behavior in Syrian hamsters.

## ACKNOWLEDGMENTS

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# Neuroendocrine pathways mediating nutritional acceleration of puberty: insights from ruminant models

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The pubertal process is characterized by an activation of physiological events within the hypothalamic-adenohypophyseal–gonadal axis which culminate in reproductive competence. Excessive weight gain and adiposity during the juvenile period is associated with accelerated onset of puberty in females. The mechanisms and pathways by which excess energy balance advances puberty are unclear, but appear to involve an early escape from estradiol negative feedback and early initiation of high-frequency episodic gonadotropin-releasing hormone (GnRH) secretion. Hypothalamic neurons, particularly neuropeptide Y and proopiomelanocortin neurons are likely important components of the pathway sensing and transmitting metabolic information to the control of GnRH secretion. Kisspeptin neurons may also have a role as effector neurons integrating metabolic and gonadal steroid feedback effects on GnRH secretion at the time of puberty. Recent studies indicate that leptin-responsive neurons within the ventral premammillary nucleus play a critical role in pubertal progression and challenge the relevance of kisspeptin neurons in this process. Nevertheless, the nutritional control of puberty is likely to involve an integration of major sensor and effector pathways that interact with modulatory circuitries for a fine control of GnRH neuron function. In this review, observations made in ruminant species are emphasized for a comparative perspective.

**Keywords: GnRH, kisspeptin, leptin, NPY, POMC**

## INTRODUCTION

Pubertal development involves physical and behavioral changes that are linked to the activation of the hypothalamic-adenohypophyseal–gonadal axis (Sisk and Foster, 2004). The progression of events is controlled largely by genetic and environmental factors, among which nutrition has a major influence. Historically, most studies investigating the effects of nutrition on pubertal development have used models that represent states of nutrient or metabolic insufficiency (Foster and Olster, 1985; Manning and Bronson, 1989; Suttie et al., 1991). However, evidence that excessive weight gain and adiposity during childhood are associated with early onset of puberty in girls (Lee et al., 2007; Jasik and Lustig, 2008; Rosenfield et al., 2009), and indications that precocious puberty is associated with increased risks for development of polycystic ovarian syndrome, reproductive cancers, and psychological distress (Golub et al., 2008) has renewed interest in understanding the mechanisms by which nutrient sufficiency supports reproductive maturation.

Signals mediating nutritional and metabolic information are perceived largely at the level of the hypothalamus (Schneider, 2004) and are likely integrated in structural and cellular networks that control various neuroendocrine functions, including puberty. Although common mechanisms exist, functional differences among mammalian species add complexity to the ability to extrapolate observations made in distinct animal models. In the current review, we have focused the discussion on neuroendocrine pathways known to regulate the onset of puberty in ruminant species. Domestic ruminants have been used extensively as animal models in neuroendocrine research and the ability to effectively measure the temporal release of hypothalamic neuropeptides in these species is a particularly relevant feature. In this review, an overview of recent studies investigating the influence of elevated body weight gain during the juvenile period on timing the onset of puberty in ewe lambs and heifers is presented.

## PUBERTY AS A NEUROENDOCRINE EVENT

The onset of puberty in females is characterized by an activation of the hypothalamic-adenohypophyseal–gonadal axis that precedes the establishment of cyclic ovarian activity. The peripubertal increase in pulsatile release of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) supports final maturation of ovarian follicles and enhances ovarian steroidogenesis (Kinder et al., 1987). Elevated circulating concentrations of estradiol induce the preovulatory surge of GnRH/LH, which leads to first ovulation. During most of the juvenile period, the hypothalamic-adenohypophyseal–gonadal axis remains relatively quiescent, and the frequency of LH release is low (Foster and Jackson, 2006; Plant and Witchel, 2006). A major limiting factor for increased secretion of LH and development of preovulatory follicles during the juvenile period is the lack of appropriate stimulation of the gonadotropes by GnRH. This assertion is supported by the observation that ovarian function is stimulated in immature female monkeys treated with GnRH (Wildt et al., 1980), and in lambs treated with LH (Foster et al., 1984). Because estradiol can lead to a

surge-like release of LH in prepubertal females (Foster and Karsch, 1975; Andrews and Ojeda, 1977), it is believed that the estradiol positive feedback is functional before reproductive maturation is established. However, the lack of an appropriate stimulatory signal that sustains elevated GnRH neuronal activity necessary for continued ovarian function, or the presence of inhibitory signals that restrain GnRH neuronal activity, may explain the infrequent release of GnRH characteristic of the prepubertal period.

Increased sensitivity to estradiol negative feedback contributes to the inhibition of GnRH release in ewe lambs and heifers (Foster and Ryan, 1979; Day et al., 1984). The ability of low circulating concentrations of estradiol to inhibit the pulsatile release of LH is diminished during maturation, and frequency of LH pulses increases (Ebling et al., 1990). In primates, the frequency of episodic release of LH is low during a substantial portion of the juvenile period independent of gonadal influence (Pohl et al., 1995). However, estradiol-dependent maintenance of low gonadotropin secretion becomes relevant later during juvenile development (Pohl et al., 1995), and changes in estradiol negative feedback seem to also play a role in the establishment of heightened frequency of LH release in primates.

## METABOLIC-SENSING PATHWAYS MEDIATING THE NUTRITIONAL CONTROL OF PUBERTAL DEVELOPMENT

Adequate growth and adiposity are critical for normal progression of puberty in mammals. Growth restriction (Foster and Olster, 1985; Suttie et al., 1991) and excessive exercise (Manning and Bronson, 1989; Malina, 1994) during the juvenile period delay puberty, likely by decreasing the release of GnRH (I'Anson et al., 2000) in association with heightened negative feedback sensitivity to estradiol (Foster and Olster, 1985). In contrast, increased adiposity seems to facilitate reproductive maturation and advance the onset of puberty (Kaplowitz et al., 2001; Lee et al., 2007; Rosenfield et al., 2009). In cattle, a high proportion of heifers fed to gain weight at high rates during the juvenile period exhibit precocious puberty (Gasser et al., 2006a,b). This occurrence is associated with attenuation of estradiol negative feedback and increased pulsatile release of LH (Gasser et al., 2006a). Therefore, nutritional cues interact with gonadal steroid feedback to time the onset of puberty in females.

Studies investigating adiposity and adipocyte-derived hormones as essential factors for the initiation of puberty have revealed that leptin, a hormone secreted predominantly by adipocytes, has a critical role for the progression of puberty in various species, including ruminants (Zieba et al., 2005). Although leptin does not affect secretion of LH in adequately fed ewes (Henry et al., 1999) and cows (Amstalden et al., 2002), leptin prevents fasting-induced reduction in LH pulsatility in prepubertal heifers (Maciel et al., 2004). Because in mice GnRH neurons are not affected by leptin directly (Quennell et al., 2009), leptin's actions on GnRH/LH release in ruminants are likely mediated by intermediate pathways. In addition to leptin, information from other hormones (e.g., insulin and ghrelin) and nutrients (e.g., glucose, fatty acids, and amino acids) is also likely to be integrated in a complex neural network that perceive and signal availability of metabolic fuels to the control of reproductive function (Schneider, 2004).

Critical neuronal pathways mediating signals of nutrient sufficiency and insufficiency have been identified. Hypothalamic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons, and proopiomelanocortin (POMC) neurons are considered major pathways by which nutritional signals are effected (Crown et al., 2007). These populations of neurons in the arcuate nucleus express the leptin receptor and are responsive to changes in nutritional status (Kalra and Kalra, 2003). Specifically, NPY has been shown to mediate the inhibitory effects of undernutrition on reproductive function (Kalra and Crowley, 1984). Contrary to rats in which NPY has both stimulatory and inhibitory effects on LH release depending on gonadal steroid milieu (Sahu et al., 1987), NPY has a predominant inhibitory action on the release of LH in ruminants in the presence and absence of estradiol (Gazal et al., 1998; Estrada et al., 2003; Morrison et al., 2003). This effect of NPY has been shown to be largely due to inhibition of GnRH release (Gazal et al., 1998) and may be mediated by direct NPY actions on GnRH neurons (Klenke et al., 2010).

Intact juvenile female rats chronically exposed to NPY exhibit delayed sexual maturation (Catzeflies et al., 1993), and this effect may be mediated by the Y1 receptor (El Majdoubi et al., 2000; Pralong et al., 2000). Such observations indicate that a break in NPY inhibition may be critical for the peripubertal initiation of high-frequency, episodic release of GnRH. It is unclear, however, whether increased growth and adiposity during the juvenile period has an impact on NPY restraint of GnRH release. The number of NPY neurons and NPY content in the arcuate nucleus of male, juvenile rats reared in small litters to promote over nutrition does not differ from those of rats reared in normal-size litters (Plagemann et al., 1999). We recently began to investigate the role of hypothalamic NPY circuitry in controlling early onset of puberty in an animal model in which elevated body weight gain during the juvenile period accelerates puberty (Gasser et al., 2006a,b). In juvenile heifers that gained body weight at a high rate between 4 and 6.5 months of age, the expression of *NPY* in the arcuate nucleus was decreased compared to heifers that gained weight at lower rates (Allen et al., 2009). Using a similar dietary treatment, we also observed that the proportion of GnRH neurons in close proximity to NPY fibers in the preoptic area and hypothalamus was reduced in heifers gaining body weight at high rates (Alves et al., 2011). Interestingly, these structural changes in the NPY circuitry seem to be more evident in GnRH neurons located in the mediobasal hypothalamus. In this region, the proportion of GnRH neurons highly innervated by NPY fibers was reduced by ∼50% in heifers gaining weight at high rates (Alves et al., 2011). It is important to note that both groups of heifers were in positive nutrient balance and had nutrient requirements for growth met, except that they differed in the target rate of gain. In mice, there is evidence that neural projections originating in the arcuate nucleus are regulated by leptin during early postnatal development (Bouret et al., 2004a), and that changes observed in leptin-sensitive hypothalamic neurocircuitry may involve NPY neurons (Bouret et al., 2004b). Therefore, structural and functional changes involving hypothalamic NPY circuitry during the early juvenile period may be involved in the mechanisms by which excessive nutrition and adiposity support early onset of puberty.

The melanocortin system is also considered to have an important role in mediating the neuroendocrine control of metabolism and reproductive function (Schneider, 2004). Melanocyte-stimulating hormone alpha (α-MSH), one of the products of the proopiomelanocortin (*POMC*) gene in the hypothalamus, is considered a primary effector. Leptin stimulates the expression of *POMC* mRNA in mice, rats and sheep (Schwartz et al., 1997; Backholer et al., 2010), and a melanocortin receptor agonist (MTII) stimulates LH release in undernourished, ovariectomized, hypogonadotropic ewes (Backholer et al., 2010). The effects of melanocortins in stimulating hypothalamic–hypophyseal function seem to be mediated primarily by the melanocortin receptor type 4 (MC-4) because a MC-4 selective antagonist blocked the leptin-induced LH release in fasted rats (Watanobe et al., 1999). Although MC-4 is expressed in GT1-1 cells, a GnRH-secreting cell line (Khong et al., 2001), it is unclear whether GnRH neurons contain melanocortin receptor. Nevertheless, the endogenous antagonist of melanocortin receptors, AgRP, has also been demonstrated to alter gonadotropin release. In ovariectomized, estradiol, and progesterone-primed rats, AgRP abolishes the LH surge (Schioth et al., 2001), and in ovariectomized, adult rhesus monkeys, administration of AgRP suppresses episodic LH release (Vulliémoz et al., 2005).

Agouti-related protein and NPY are co-expressed in neurons within the arcuate nucleus (Broberger et al., 1998), and leptin treatment decreases expression of *NPY* and *AGRP* in the arcuate nucleus of adult rats (Ahima et al., 1999). Interestingly, leptin was ineffective in regulating *NPY* and *AGRP* expression in neonatal mice (Ahima and Hileman, 2000), indicating that leptin's effect on expression of those genes may be developmentally regulated. However, leptin appears to be critical for development of hypothalamic neuronal projections during the early postnatal period in mice and rats (Bouret et al., 2004a, 2008), and activates POMC neurons in the arcuate nucleus early during the postnatal period in mice (Bouret et al., 2004b). In our studies in prepubertal heifers, *AGRP* mRNA abundance in the hypothalamus was lower in heifers that gained body weight at high rates during the juvenile period (Allen et al., 2009). In contrast, *POMC* mRNA abundance in the arcuate nucleus was increased in heifers gaining weight at high rates (Allen et al., unpublished). Interestingly, these changes in gene expression were associated with an increase in circulating concentrations of leptin in heifers gaining weight at high rates (Allen et al., 2009; Alves et al., 2011).

In a study using hypothalamic tissue from non-human primates and rats, Roth et al. (2007) suggested that a network of genes involved in a range of cellular functions, including control of transcription and cellular metabolism, is activated at the time of puberty. In a recent study using microarray technology to investigate changes in gene expression in the arcuate nucleus of prepubertal heifers fed to gain weight at high or low rates, we observed that genes involved in a variety of biological functions are responsive to nutritional input during the juvenile period (Allen et al., unpublished). Differentially-regulated genes included those associated with regulation of cellular metabolic processes, receptor and intracellular signaling, and neuronal communication. Therefore, the prepubertal, growing female seems exquisitely sensitive to nutrient inputs because changes in the regulation of metabolic-sensing and effector pathways are in tune for the fine control of neuroendocrine functions. Mechanisms involved may include regulation of gene expression, control of cellular functions, and plasticity of functional structures within the hypothalamus.

## ROLE OF KISSPEPTIN IN MEDIATING NUTRITIONAL ACCELERATION OF ONSET OF PUBERTY

Observations that mutations in the kisspeptin receptor result in hypogonadotropic hypogonadism in humans (de Roux et al., 2003; Seminara et al., 2003) has implicated kisspeptin in the control of reproductive function. In mice, dysfunction of kisspeptin receptor leads to decreased gonadal development and impairment in timing of pubertal onset (de Roux et al., 2003; Seminara et al., 2003). Actions of kisspeptin on regulation of reproductive functions appear to be mainly by its direct effects on GnRH release (Caraty et al., 2007). Kisspeptin is a potent stimulator of LH secretion in mature (Caraty et al., 2007) and prepubertal females (Navarro et al., 2004; Kadokawa et al., 2008; Redmond et al., 2011a). In prepubertal rats, kisspeptin treatment induces early vaginal canalization (Navarro et al., 2004), indicating that puberty may be advanced by exogenous kisspeptin. In ewe lambs, intermittent injections of kisspeptin increase ovarian steroidogenesis and leads to a preovulatory surge of LH that is followed by ovulation/follicle luteinization (Redmond et al., 2011a).

Studies have implicated kisspeptin in mediating the nutritional control of reproduction. Feed restriction decreases *KISS1* mRNA in the hypothalamus of prepubertal rats and kisspeptin treatment alleviates undernutrition-induced delayed puberty in female rats (Castellano et al., 2005). In addition, rats reared in small litters to allow elevated body weight gain during the prepubertal period exhibit early onset of vaginal opening, increased *KISS1* expression, and greater number of kisspeptin neurons (Castellano et al., 2011). Although leptin signaling is considered important for pubertal development, the requirement for direct leptin signaling on kisspeptin neurons has been challenged recently. A study by Quennell et al. (2011) demonstrated that leptin induction of STAT3 phosphorylation, a major intracellular signaling mechanism induced by leptin, is absent in kisspeptin neurons. Furthermore, Donato et al. (2011) demonstrated that deletion of leptin receptor in kisspeptin neurons does not impair the onset of puberty in mice. Interestingly, the ventral premammillary nucleus appears to have a major role in mediating leptin's permissive effects for normal reproductive maturation in mice (Donato et al., 2011). Because the premammillary region has been involved in the seasonal control of reproduction in sheep (Malpaux et al., 1998), this hypothalamic region may serve to integrate metabolic and photoperiodic cues important for the onset of puberty in seasonal species.

It remains to be determined whether kisspeptin-independent actions of leptin on pubertal development observed in mice are conserved in other mammalian species. Nevertheless, intermediate pathways can be involved and the NPY system represents a potential candidate. Neuronal fibers containing NPY are observed in close proximity to kisspeptin neurons in sheep (Backholer et al., 2010). Recent studies in our laboratory indicated that this structural association between NPY and kisspeptin neurons may represent synaptic inputs (**Figure 1**; unpublished). However,

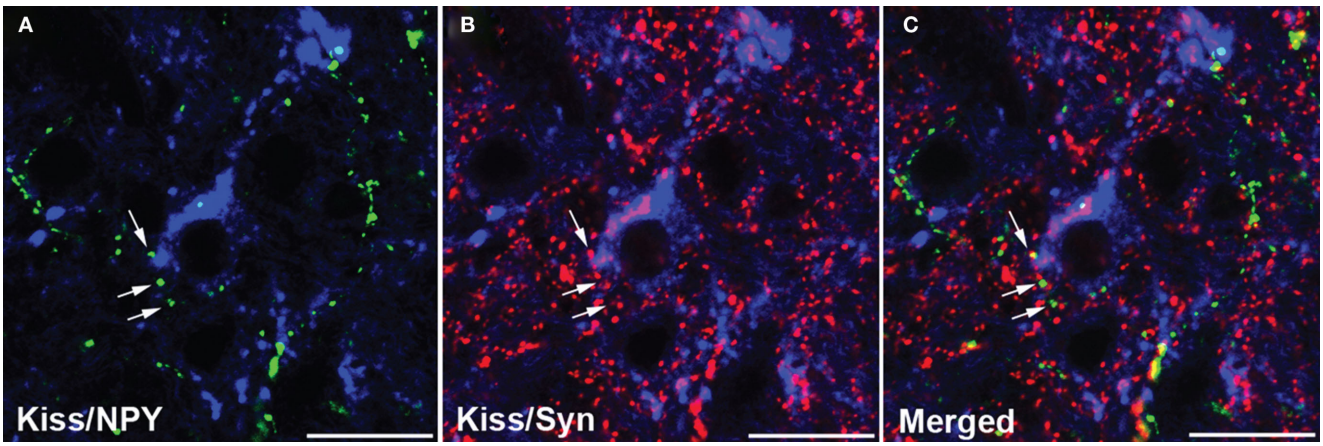


**FIGURE 1 | Confocal images (1-μm optical section) of a tissue section through the arcuate nucleus processed for triple-label immunofluorescent detection of kisspeptin (Kiss), neuropeptide Y (NPY), and synaptophysin (Syn).** Immunoreactive kisspeptin soma and dendrites (blue) are observed in close proximity to NPY- [gren; **(A)**] and synaptophysin- [red; **(B)**] positive axons. **(C)** Synaptophysin colocalization in NPY axons that are in close proximity to kisspeptin neurons. Arrows indicate examples of NPY/synaptophysin-positive terminals. Scale bar, 20 μm.

preliminary data indicated that the number of close contacts between NPY-containing fibers and kisspeptin neurons in the preoptic area and arcuate nucleus did not differ between ewe lambs fed to gain weight at high and moderate rates during the juvenile period (unpublished). Therefore, it is unclear whether regulation of the NPY-kisspeptin circuitry may contribute to mechanisms leading to the activation of kisspeptin neurons during pubertal development.

Expression of the *KISS1* gene increases during puberty in mice (Han et al., 2005), rats (Navarro et al., 2004), and monkeys (Shahab et al., 2005). In juvenile rats, the increase in *KISS1* expression was associated with increased frequency of LH pulses (Takase et al., 2009). In ovariectomized, estradiol-replaced ewe lambs, an increase in the number of *KISS1*-expressing cells is observed in the preoptic area early during the juvenile period (Redmond et al., 2011b), but these changes are unrelated to changes in the frequency of LH release. In contrast, the number of *KISS1*-expressing cells in the arcuate nucleus increases with acceleration of pulsatile LH release characteristic of pubertal development. A recent study has questioned the relevance of kisspeptin neurons for the establishment of reproductive function in mice (Mayer and Boehm, 2011). In that study, mice with genetic ablation of kisspeptin neurons, or ablation of kisspeptin receptor in neurons during fetal development exhibited normal fertility. In contrast, ablation of kisspeptin neurons in adult mice impaired normal cyclicity (Mayer and Boehm, 2011). Therefore, compensatory mechanisms and pathways may develop during fetal development in the absence of kisspeptin neurons. Further studies should determine if the pubertal onset of high-frequency pulsatile release of LH involves activation of kisspeptin neurons as downstream targets of pathways integrating estradiol negative feedback and nutritional information.

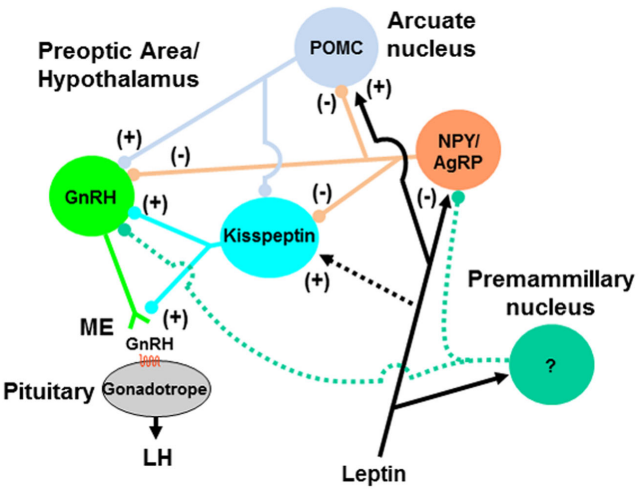


**FIGURE 2 | Model for pathways mediating the nutritional regulation of GnRH release during pubertal development.** Signals of nutrient sufficiency such as hormones (e.g., leptin) and metabolites are perceived by metabolic-sensing neurons in the hypothalamus (e.g., NPY/AgRP and POMC neurons) that project directly to GnRH neuron soma and dendrites, and/or terminals in the median eminence (ME; not represented). NPY/AgRP and POMC neurons may also regulate GnRH neurons indirectly via kisspeptin neurons. Neurons in the premammillary nucleus could also represent a leptin-sensitive pathway for regulation of GnRH neurons during pubertal transition, but neuronal phenotype and hypothetical projections (green dashed lines) are yet to be characterized. Direct action of leptin on kisspeptin neurons (black dashed line) is unlikely to represent a major pathway. Accelerated growth and adiposity during the juvenile period hastens the peripubertal activation of GnRH neurons by reducing inhibitory signals (e.g., NPY) and enhancing stimulatory signals (e.g., kisspeptin, POMC-derived peptides), and leads to increased frequency of episodic release of GnRH and early onset of puberty.

## CONCLUSION

The discovery of a link between excessive nutrient intake/weight gain during the infantile/juvenile period and early onset of puberty has exacerbated concerns of childhood obesity. Mechanisms mediating the nutritional acceleration of puberty involve an

integration of metabolic sensors and effectors, largely at the hypothalamic level. Leptin is likely to be involved in this process and may signal at multiple hypothalamic and cellular targets. The NPY system is a strong candidate for mediating leptin and other nutritional/metabolic signals that influence GnRH neurosecretion in ruminants. NPY may exert its effects through direct inputs on GnRH neurons and/or indirectly via intermediate pathways such as kisspeptin neurons (**Figure 2**). Other cells (e.g., POMC neurons) may also be involved. Whether the premammillary region of the hypothalamus has a role in mediating leptin's effects in ruminant species, as demonstrated in mice, remains to be determined. Ultimately, hypothalamic gene expression is affected by nutritional inputs, and the regulation of expression of a network of genes and their products affect cellular and structural functions that are critical for timing puberty in mammals.

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