



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

Cadence True, Kevin L. Grove and M. Susan Smith\*

Division of Neuroscience, Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR, USA

## Edited by:

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## \*Correspondence:

M. Susan Smith, Division of Neuroscience, Oregon National Primate Research Center, Oregon Health and Science University, 505 NW 185th Avenue, Beaverton, OR 97006, USA.  
e-mail: smithsu@ohsu.edu

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; Aloï 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; Quenell 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  $\alpha$ -melanocyte-stimulating hormone and  $\beta$ -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 led 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 effect 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 optic 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; Chiochio 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

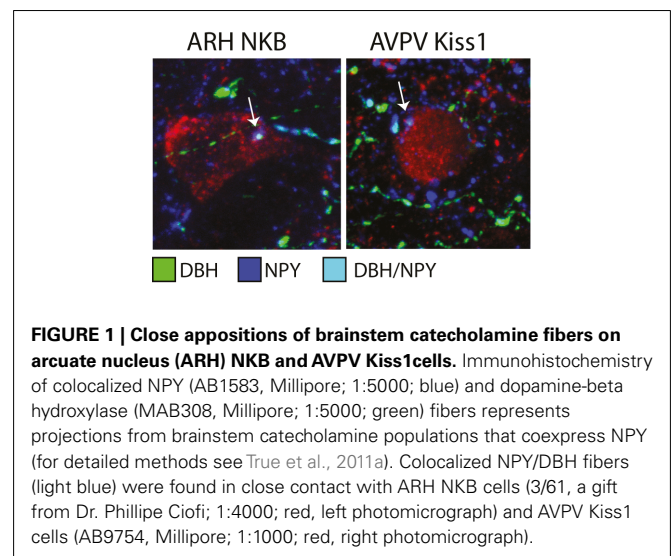
Certain neuronal populations are capable of sensing metabolic status through changes in circulating glucose levels. This characteristic offers an attractive and relatively simple mechanism through which metabolic status may be acutely sensed in the brain and potentially relayed to reproductive circuits. 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 (l'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

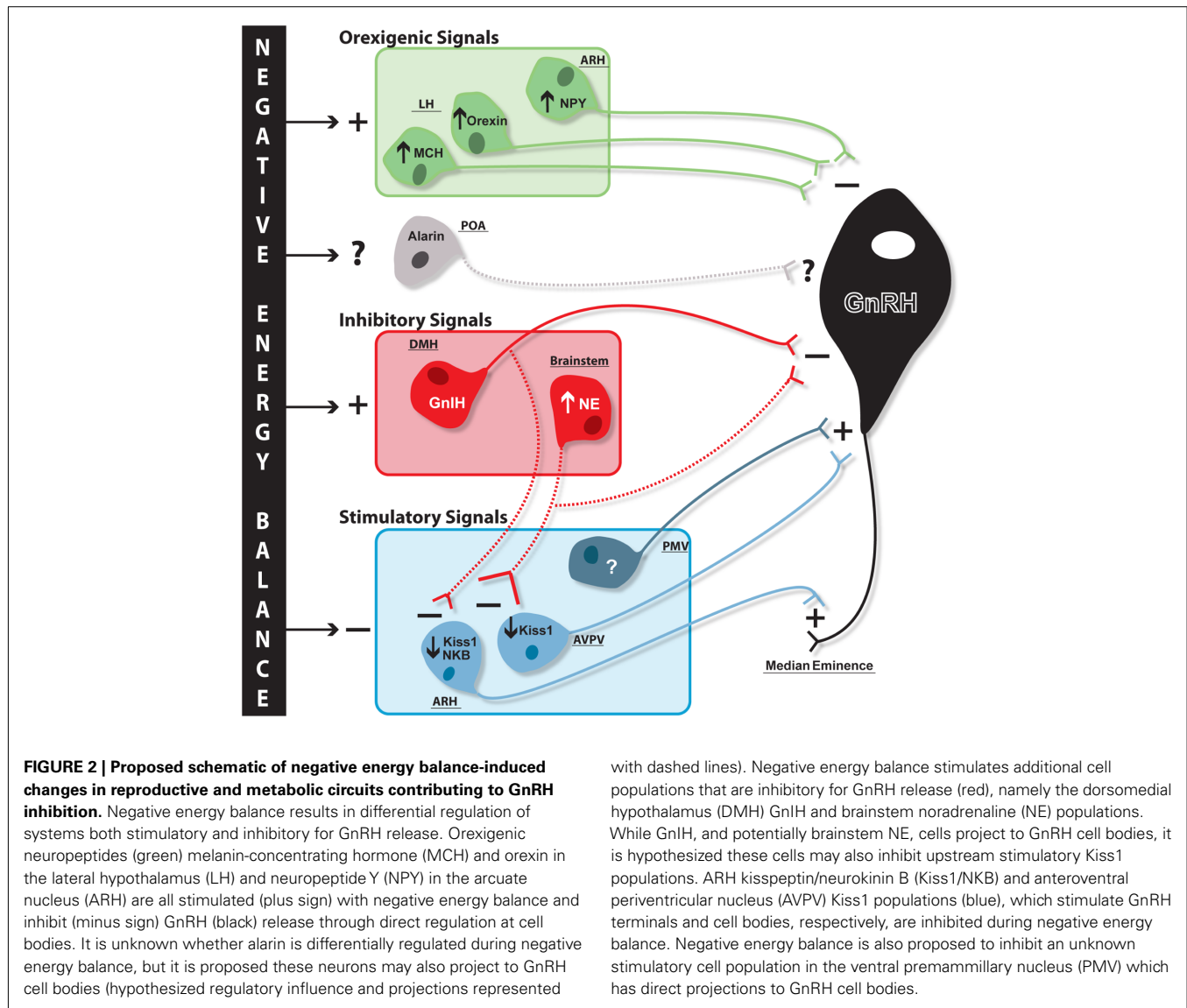
stimulatory for LH in the presence of high levels of estradiol and inhibitory in the presence of low levels of estradiol (Gallo and Drouva, 1979; Leung et al., 1982; Meyer and Goodman, 1985; Havern et al., 1991; Cagampang et al., 1992; Robinson and Kendrick, 1992; Herbison, 1997), as would be associated 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





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

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.

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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