Neuroendocrine integration of nutritional signals on reproduction

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Abstract

Reproductive function in mammals is energetically costly and therefore tightly regulated by nutritional status. To enable this integration of metabolic and reproductive function, information regarding peripheral nutritional status must be relayed centrally to the gonadotropin-releasing hormone (GNRH) neurons that drive reproductive function. The metabolically relevant hormones leptin, insulin and ghrelin have been identified as key mediators of this 'metabolic control of fertility'. However, the neural circuitry through which they act to exert their control over GNRH drive remains incompletely understood. With the advent of Cre-LoxP technology, it has become possible to perform targeted gene-deletion and gene-rescue experiments and thus test the functional requirement and sufficiency, respectively, of discrete hormone–neuron signaling pathways in the metabolic control of reproductive function. This review discusses the findings from these investigations, and attempts to put them in context with what is known from clinical situations and wild-type animal models. What emerges from this discussion is clear evidence that the integration of nutritional signals on reproduction is complex and highly redundant, and therefore, surprisingly difficult to perturb. Consequently, the deletion of individual hormone–neuron signaling pathways often fails to cause reproductive phenotypes, despite strong evidence that the targeted pathway plays a role under normal physiological conditions. Although transgenic studies rarely reveal a critical role for discrete signaling pathways, they nevertheless prove to be a good strategy for identifying whether a targeted pathway is absolutely required, critically involved, sufficient or dispensable in the metabolic control of fertility.

Key Words
- insulin
- leptin
- ghrelin
- GNRH
- fertility

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Introduction

Mammals, particularly females, must invest an enormous amount of energy into conceiving, carrying and rearing their progeny to preserve their genetic lineage. Therefore, to ensure adequate energy is available to support the high demands of pregnancy and successful weaning of offspring, reproductive function tends to become suppressed during times of low-energy availability. For this coordinated regulation of energy homeostasis and fertility to occur, the hypothalamic–pituitary–gonadal (HPG) axis governing reproductive function must receive and integrate information about peripheral metabolic status. Indeed, each level of the HPG axis has the capacity to respond to changing levels of nutritional cues (Comninos *et al*. 2014). However, the gonadotropin-releasing hormone (GNRH) neurons residing in the hypothalamus are the primary drivers of reproductive function, and impaired GNRH signaling is considered the underlying cause of reproductive suppression due to nutritional stress.

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Under normal conditions, GNRH is secreted in a pulsatile manner into the hypophyseal portal circulation and travels to the anterior pituitary gland where it stimulates the release of the gonadotropins (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) which then go on to drive gonadal function (Christian & Moenter 2010, Herbison 2016). However, during periods of energetic stress, GNRH pulses become suppressed, which in turn reduces pituitary secretion of the gonadotropins, ultimately resulting in hypothalamic hypogonadism (Compagnucci *et al*. 2002, Castellano *et al*. 2005, Huang *et al*. 2008). Although human obesity is unquestionably associated with infertility (Souter *et al*. 2011), suppression of neuroendocrine reproductive function due to nutritional stress can occur independently from long-term changes in adiposity and is largely due to immediate changes in energy availability (Cameron & Nosbisch 1991, Loucks *et al*. 1998, Wade & Jones 2004). Furthermore, the suppression of GNRH/LH pulsatility is related to the severity of the energetic stress (De Souza *et al*. 2007); yet, the dependence of LH pulsatility on energy availability appears to be nonlinear (Loucks & Thuma 2003). Although this delicate control of metabolic and reproductive function is known to occur primarily in the hypothalamus, unraveling the specific neural substrates and nutritional signals involved remains an active and challenging area of research. Not only are there numerous nutritional signals relaying information about peripheral energy status centrally but also the hypothalamic (and extra-hypothalamic) circuitry able to receive information from these signals and then go on to modulate HPG axis function is extensive.

In response to an energetic stress, such as an acute fast or chronic under-nutrition, a series of events involving a multitude of gut- and adipose-derived hormone responses (as well as activation of the stress axes and counter-regulatory response) is initiated, and it seems likely the consequent suppression of the reproductive neuroendocrine axis is due to the net effect of all of these. Trying to disentangle the specific pathways by which each factor independently exerts its impact on GNRH release proves extremely difficult and is arguably not an experimentally and physiologically meaningful approach, except perhaps in cases of specific genetic deficiencies, as no single pathway is ever operating in isolation *in vivo*. Nevertheless, identifying the discrete mechanisms underlying nutritional infertility provides important insight into how peripheral metabolic status is relayed centrally to the GNRH neurons and also highlights the complexity and inherent redundancies built in to safeguard reproductive function and thus species' survival.

This review will attempt to highlight some of the key neuroendocrine pathways whereby nutritional signals modulate reproduction, while also emphasizing the inherent redundancies of the system through discussions of whether identified pathways are absolutely required for, critically involved in and/or sufficient to permit the normal metabolic control of fertility.

Identifying the key players

As mentioned previously, the body's response to nutritional stress is multifaceted, so identifying the specific components critically involved in the suppression of reproductive function is challenging. Clinically, women presenting with nutritional infertility (i.e. functional hypothalamic amenorrhea (FHA)) exhibit a range of endocrine-metabolic aberrations, including (but not limited to) altered levels of gut and adipose hormones, reduced levels of plasma glucose, suppressed thyroid function, altered growth hormone secretion patterns and chronically elevated cortisol concentrations (Berga *et al*. 1989, Laughlin & Yen 1997, Laughlin *et al*. 1998, Schneider & Warren 2006, Scheid & De Souza 2010, Corr *et al*. 2011). Although it remains a possibility that each of these factors independently suppresses the neuroendocrine reproductive axis, we know from both animal and clinical studies that certain nutritional signals, such as leptin, insulin and ghrelin, exert a more critical influence over GNRH/LH pulsatility (and thus fertility) than others and that in at least some cases their effects are additive. This review will therefore focus on the discrete mechanisms whereby these key nutritional factors influence HPG function and will also briefly touch upon the role of stress axes activation in reproductive suppression due to low energy availability.

Leptin

Leptin, the hormone product of the obesity gene (*Ob*), appears to be a particularly important factor. Leptin is secreted from adipocytes into the bloodstream in proportion to body fatness, and signals energy sufficiency and acts on the hypothalamus to suppress feeding and increase energy expenditure. Serum leptin concentrations change gradually over time in response to changes in fat mass, but fall rapidly in response to complete fasting (Boden *et al*. 1996, Chan *et al*. 2003), and this fall in leptin concentration is thought to mediate reproductive suppression due to nutritional stress. For example, preventing the

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starvation-induced fall in leptin with exogenous leptin was shown to prevent the starvation-induced delay in ovulation in female mice (Ahima *et al*. 1996). Accordingly, women with infertility due to a negative energy balance exhibit reduced circulating leptin concentrations (Laughlin & Yen 1997, Corr *et al*. 2011), and administration of recombinant human leptin to these women not only increased their LH pulsatility and restored ovulation but also improved their thyroid and growth hormone axes, independent from dietary changes or weight gain (Welt *et al*. 2004). These data suggest that an acute decrease in circulating leptin plays a key role in mediating the effects of low energy availability on reproductive function.

In further support of leptin's role in the metabolic regulation of fertility, humans and mice with a congenital leptin deficiency (*ob/ob*), as well as leptin receptor (LepR)-deficient (*db/db*) mice, are infertile and obese despite being energy replete, and leptin treatment is sufficient to restore body weight control and reproductive function in leptin-deficient individuals (Ingalls *et al*. 1950, Hummel *et al*. 1966, Barash *et al*. 1996, Chehab *et al*. 1997, Mounzih *et al*. 1997, Farooqi *et al*. 1999, 2002). Like the global LepR-deficient mice, mice exhibiting forebrain neuron-specific deletion of LepR are also infertile and obese, highlighting that leptin's central actions, vs peripheral actions, are critically involved in regulating body weight homeostasis and fertility (Quennell *et al*. 2009). Lastly, central leptin immune neutralization using leptin antiserum caused a reduction in LH pulsatility in rats (Carro *et al*. 1997). These findings, among others, convincingly demonstrate that leptin plays an important physiological role in the regulation of metabolic and reproductive function.

Insulin

The pancreatic peptide hormone insulin (best known for regulating glucose homeostasis and body weight regulation (Belgardt & Bruning 2010)) has also emerged as a key modulator of reproductive function. Clinically, diabetic insulin insufficiency (i.e. type 1 diabetes) in humans is associated with delayed puberty, reduced LH pulsatility and hypothalamic hypogonadism, despite sufficient energy availability; yet, improved insulin therapy has ameliorated many of these issues (Codner *et al*. 2012). Additionally, reduced insulin levels, presumably due to energy deficiency, are likewise associated with suppression of neuroendocrine reproductive function in women with FHA (Laughlin & Yen 1997), which further suggests insulin promotes GNRH/LH secretion. Conversely, hyperinsulinemia and/or insulin resistance (i.e. type 2 diabetes), are often associated with elevated LH pulsatility and polycystic ovary syndrome (PCOS) (Laughlin *et al*. 1997), and insulin-sensitizing drugs (e.g. metformin) have successfully been used to induce ovulation and treat some of the pathophysiological features associated with PCOS (Katsiki & Hatzitolios 2010). Encouragingly, animal studies support these clinical findings. For example, lean and normoglycemic female mice exhibiting varying degrees of insulin resistance and hyperinsulinemia were shown to have subtle alterations in their HPG axis and also had polyovular follicles (Nandi *et al*. 2010). Although insulin can act both peripherally and centrally to modulate HPG axis function, we know that insulin's central actions play a role in mediating many of its reproductive effects as mice exhibiting brain-specific deletion of insulin receptors (InsR) exhibit subfertility due to mild hypothalamic hypogonadism (Bruning *et al*. 2000). However, in contrast to these brain-specific InsR-knockout mice, our group recently demonstrated that mice exhibiting forebrain neuron-specific deletion of InsR displayed normal HPG axis function (Evans *et al*. 2015), which challenges the hypothesis that neuronal insulin signaling is required. Nevertheless, many findings implicate abnormal insulin signaling in the pathogenesis of reproductive dysfunction. However, in contrast to leptin, central insulin signaling is not absolutely required for fertility.

Ghrelin

Unlike the anorexigenic peptides insulin and leptin, which promote GNRH/LH pulsatility and reproductive function, the orexigenic peptide ghrelin has been shown to consistently suppress GNRH pulsatility and gonadotropin release (Furuta *et al*. 2001, Kluge *et al*. 2007, 2009, 2012, 2013, Kluge 2012). Although ghrelin is predominantly produced in the stomach, ghrelin-expressing neurons have also been identified in the hypothalamus (Cowley *et al*. 2003), yet it remains unclear whether the actions of brain-derived ghrelin are the same as those for circulating ghrelin. Under normal conditions, circulating ghrelin exhibits a circadian pattern whereby its concentration becomes elevated in anticipation of meals. However, in women exhibiting chronic under-nutrition, chronically elevated basal ghrelin levels are observed and are correlated with suppressed reproductive function (Scheid & De Souza 2010). Ghrelin deficiency, on the other hand, does not significantly affect fertility or feeding behavior in mice (Sun *et al*. 2003). The latter observation is likely due to the fact that the ghrelin receptor (the growth hormone secretagogue receptor

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type 1a (GHSR1a)) has constitutive activity (Holst *et al.* 2003, Holst & Schwartz 2004), so even in the absence of ghrelin peptide, ghrelin signaling is not abolished. Considering ghrelin's inhibitory actions on GNRH release, the lack of fertility phenotype observed in the ghrelin-knockout mice is perhaps not surprising. It would arguably have been more informative to determine whether the ghrelin-knockout mice were protected against a fasting-induced reduction in LH secretion.

Chronic overexpression of ghrelin, on the other hand, caused an acute increase in food intake and body weight in mice, but failed to cause long-term impacts (Qi *et al.* 2015), likely due to compensation by other factors. Unfortunately, reproductive parameters were not assessed in these mice. Nevertheless, under normal physiological conditions, the increase in circulating ghrelin concentrations that accompanies nutritional stress is thought to antagonize leptin's and insulin's effects, thereby acting as a 'brake' on fertility. In support of this, inhibition of ghrelin signaling improves the reproductive phenotype of leptin-deficient mice (Zhu *et al.* 2013). Furthermore, exercising women with amenorrhea exhibit plasma ghrelin levels that are approximately 85% greater than those observed in their normally cycling peers (De Souza *et al.* 2004), and women with disordered eating and amenorrhea also exhibit chronically elevated ghrelin levels (Schneider & Warren 2006, Schneider *et al.* 2008), which may be due to reduced leptin-induced inhibition of ghrelin (Kalra *et al.* 2005). These studies strongly implicate ghrelin as a key player in the mechanism whereby low energy availability suppresses the neuroendocrine reproductive axis.

Other nutritional signals

Although leptin, insulin and ghrelin appear to be the major signals whereby peripheral metabolic status is relayed to the neuroendocrine reproductive axis, there are many other nutritional factors that also influence HPG axis function, as thoroughly reviewed by Comninos and coworkers (Comninos *et al.* 2014). For example, the anorectic gut peptides glucagon-like peptide (GLP)-1 and peptide YY3-36 (PYY) are both stimulated by food intake and appear to facilitate GNRH release and HPG function (Beak *et al.* 1998, MacLusky *et al.* 2000, Pinilla *et al.* 2006). Adiponectin, a protein hormone secreted by white adipocytes, is also implicated in the metabolic control of fertility and has been shown to have a predominantly inhibitory effect on the HPG axis (Klenke *et al.* 2014). However, the roles of these metabolic hormones in conveying nutritional status centrally do not appear to be as influential as those of leptin, insulin and ghrelin.

The GNRH neuronal network

Although GNRH neurons serve as the final gatekeepers of the neuroendocrine reproductive axis, they are in turn regulated by a network of hormone-sensitive afferent neurons (termed the GNRH neuronal network) and it is primarily through this specialized network that the influence of leptin, insulin and ghrelin, as well as other peripheral signals of nutritional status (Comninos *et al.* 2014), appears to converge on the neuroendocrine reproductive axis.

Indeed, GNRH neurons themselves *can* directly detect some nutritional signals, including insulin and ghrelin (Farkas *et al.* 2013, Evans *et al.* 2014*a*, DiVall *et al.* 2015), as well as glucose (Zhang *et al.* 2007), PYY (Pinilla *et al.* 2007) and adiponectin (Klenke *et al.* 2014). Although it might be speculated that disruption of metabolic regulation at the level of the final common output of the GNRH neuronal network would have more profound effects than at the level of individual afferent neurons, in most cases, direct hormone–GNRH communication does not play a critical role when it comes to the regulation of fertility by nutritional factors. For example, although mice exhibiting brain-specific deletion of InsR (Bruning *et al.* 2000) or forebrain neuron-specific deletion of LepR (Quennell *et al.* 2009) were subfertile and infertile, respectively, GNRH-specific deletion of InsR (GNRH-IRKO mice) (Divall *et al.* 2010) or LepR (Quennell *et al.* 2009) did not result in any reproductive perturbations. Interestingly, however, GNRH-IRKO mice were protected from high-fat diet-induced infertility (DiVall *et al.* 2015), suggesting direct insulin–GNRH signaling may play a role in obesity-related infertility. With regard to ghrelin, although it can modulate GNRH neurons directly, at least in brain slice preparations (Farkas *et al.* 2013), many of its neuroendocrine effects appear to be mediated through hypothalamic circuitry afferent to GNRH neurons (Forbes *et al.* 2009, Schaeffer *et al.* 2013). It thus appears the neuroendocrine integration of nutritional signals on reproduction largely occurs upstream of GNRH neurons.

Although this indirect mechanism of action may seem redundant and inefficient, it probably allows the metabolic control of fertility to be more fine-tuned and fail-safe. Instead of GNRH neurons acting as the receivers, processers and integrators of individual nutritional signals, it appears information regarding peripheral energy status

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is first channeled through upstream circuitry. Information encoded by individual nutritional signals thus becomes contextualized among the information encoded by the others, yielding a comprehensive snapshot of peripheral energy status. The GNRH neurons then receive this integrated information and can respond accordingly, thereby maintaining their role as the final gatekeepers of reproductive function.

Characterizing the neuropeptide and/or neurotransmitter identities of this 'fuel-gauging' network subserving GNRH neurons, as well as their role in mediating the metabolic control of fertility, remains an active area of research. Although many questions remain unanswered, a lot of headway has been made (Table 1). Perhaps not surprisingly, many of the same neuronal populations previously identified to play a role in energy balance homeostasis have now been identified to play a role in modulating reproductive function as well. Conversely, many neuronal populations previously known to modulate GNRH release are now recognized as integrators of nutritional information.

GABA-expressing neurons

While emphasis is often placed on the role of neuropeptide-expressing populations with regard to the integration of metabolic and reproductive status, the contribution of neurotransmitters also deserves attention. It has been known since the 1980s that gamma-amino butyric acid (GABA) (the main inhibitory neurotransmitter in the mammalian central nervous system) is involved in the regulation of GNRH release both before and after puberty (Adler & Crowley 1986, Nikolarakis *et al*. 1988, Donoso *et al*. 1994, Mitsushima *et al*. 1994), and that GABAergic afferents compose a major group of synaptic inputs to GNRH neurons. Similarly, it has been known for some time that GABAergic neurons are modulated by nutritional factors, including leptin (Ovesjo *et al*. 2001). However, it was not until Sullivan and coworkers demonstrated that GABAergic neurons play a role in conveying metabolic information to GNRH neurons (Sullivan *et al*. 2003, Sullivan & Moenter 2004) that the role of GABAergic neurons in the integration of nutritional and reproductive status became appreciated. Indeed, the GNRH neurons express both $GABA_A$ and $GABA_B$ receptor isoforms. Binding of GABA to the $GABA_B$ receptor causes an inhibition of pulsatile GNRH release, but the effect of $GABA_A$ receptor activation can be either excitatory or inhibitory depending on the chloride concentration within the GNRH neuron (Herbison & Moenter 2011).

To investigate the role(s) of leptin and insulin signaling via GABAergic neurons in the control of HPG axis function, our lab generated mice exhibiting GABA-specific deletion of LepR (Zuure *et al*. 2013) and InsR (Evans *et al*. 2014*b*) and demonstrated that leptin signaling, but not insulin signaling, via GABAergic neurons is a major component of the circuitry whereby peripheral metabolic status is relayed to the neuroendocrine reproductive and metabolic axes. Mice lacking LepR from GABA neurons were profoundly obese and exhibited delayed puberty and subfertility (Zuure *et al*. 2013), whereas mice lacking InsR from GABA neurons exhibited a very mild metabolic phenotype and normal pubertal development and fertility (Evans *et al*. 2014*b*). Although it remains a possibility that the reproductive impairments observed in the GABA-specific LepR-knockout mice were directly related to their obesity phenotype, our lab and others have demonstrated that reproductive competency can be maintained despite profound obesity (Bates *et al*. 2003, Singireddy *et al*. 2013), suggesting leptin–GABA signaling is directly involved in the control of HPG axis function. In contrast, insulin–GABA signaling does not appear to be critically involved in regulating fertility.

Although leptin signaling via GABAergic neurons appears to play a critical role in the regulation of fertility, many GABAergic neurons co-express other transmitters (Table 2), including NPY and AgRP (Meister 2007), kisspeptin (Cheong *et al*. 2015) and dopamine (Olson & Nestler 2007, Marshall *et al*. 2016), for example, and it is therefore possible that both the neuropeptide(s) and/or neurotransmitter(s) released from these neurons act as the effectors on GNRH neurons. At this stage, it is not clear whether leptin signaling via GABAergic neurons critically regulates fertility by modulating GABA release or the release of other transmitters, or both. However, evidence for the latter is strong. Furthermore, although insulin signaling via GABAergic neurons themselves does not appear to play a critical role (Evans *et al*. 2014*b*), insulin was shown to increase the number of synaptic $GABA_A$ receptors to postsynaptic domains, which is the limiting factor in eliciting a greater synaptic response (Wan *et al*. 1997, Bell-Horner *et al*. 2006). Therefore, insulin actions on GABAergic efferent neurons, via modulation of the GABA postsynaptic response, may explain some of insulin's central effects on GNRH release.

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Table 1 Gene deletion studies: summary of the metabolic control of fertility.

Genetic background	Genetic phenotype	Manipulation	Metabolic phenotype	Reproductive phenotype	References
Leptin					
Sf1-cre + *LepR*-flox	SF-1-specific LepR KO	–	Overweight	Normal	Bingham *et al.* (2008)
Agrp-cre + *LepR*-flox	AgRP-specific LepR KO	–	Overweight	Normal	van de Wall *et al.* (2008)
Pomc-cre + *LepR*-flox	POMC-specific LepR KO	–	Overweight	Normal	van de Wall *et al.* (2008), Shi *et al.* (2010)
Gnrh-cre + *LepR*-flox	GNRH-specific LepR KO	–	Normal body weight	Normal	Quennell *et al.* (2009)
Camk2a-cre + *LepR*-flox	Forebrain neuron-specific LepR KO	–	Obese	Infertile	Quennell *et al.* (2009)
Kiss1-cre + *LepR*-flox	Kisspeptin-specific LepR KO	–	Normal body weight	Normal	Donato *et al.* (2011)
nNOS-cre + *LepR*-flox	Nitric oxide neurons	–	Obese	Impaired estrus cyclicity, normal fertility	Leshan *et al.* (2012)
Vgat-cre + *LepR*-flox	GABA-specific LepR KO	–	Obese	Delayed puberty, subfertile	Zuure *et al.* (2013)
Vglut2-cre + *LepR*-flox	Glutamate-specific LepR KO	–	Overweight	Normal	Zuure *et al.* (2013)
ob/ob	Leptin-deficient	–	Obese	Infertile	Ingalls *et al.* (1950), Zhang *et al.* (1994)
ob/ob	Leptin-deficient	Leptin-treated	Restored body weight control	Rescued fertility	Mounzih *et al.* (1997)
ob/ob	Leptin-deficient	Agrp-ablated	Restored body weight control	Rescued fertility	Wu *et al.* (2012)
ob/ob	Leptin-deficient	Agrp-deficient	Obese	Rescued fertility	Sheffer-Babila *et al.* (2013)
ob/ob + *Nos1*–/–	Leptin-deficient	–	Improved body weight control	Infertile	Bellefontaine *et al.* (2014)
ob/ob + *Nos1*–/–	Leptin-deficient	Leptin-treated	Restored body weight control	Infertile	Bellefontaine *et al.* (2014)
db/db	LepR-deficient	–	Obese	Infertile	Hummel *et al.* (1966)
Agrp-Cre + *LepR*-stop-flox	Agrp-restricted LepR expression	–	Overweight	Rescued fertility	O Egan & GM Anderson (unpublished observations)
POMC-cre + *LepR*-stop-flox	POMC-restricted LepR expression	–	Overweight	–	Huo *et al.* (2009)
LepR neo/neo + PMV-AAV-Flp	PMV-restricted LepR expression	–	Obese	Rescued fertility	Donato *et al.* (2011)
Insulin					
Nestin-cre + *InsR*-flox	Brain-specific InsR KO	–	Mildly overweight	Normal	Bruning *et al.* (2000)
Gnrh-cre + *InsR*-flox	GNRH-specific InsR KO	–	Normal body weight	Normal	Divall *et al.* (2010)
Gnrh-cre + *InsR*-flox	GNRH-specific InsR KO	HFD feeding	Obese	Protected from HFD-induced infertility	DiVall *et al.* (2015)
Agrp-cre + *InsR*-flox	AgRP-specific LepR KO	–	Normal body weight	Normal	Konner *et al.* (2007)
Pomc-cre + *InsR*-flox	POMC-specific InsR KO	–	Normal	Normal	Konner *et al.* (2007)
Kiss1-cre + *InsR*-flox	Kisspeptin-specific InsR KO	–	Normal body weight	Normal	Evans *et al.* (2014*a*)
Vgat-cre + *InsR*-flox	GABA-specific InsR KO	–	Mildly overweight	Normal	Evans *et al.* (2014*b*)
Vglut2-cre + *InsR*-flox	Glutamate-specific InsR KO	–	Normal body weight	Normal	Evans *et al.* (2014*b*)

(Continued)

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Table 1 Continued.

Genetic background	Genetic phenotype	Manipulation	Metabolic phenotype	Reproductive phenotype	References
Camk2a-cre + *InsR*-flox	Forebrain neuron-specific InsR KO	–	Mildly overweight	Normal	Evans *et al.* (2015)
Leptin + insulin					
Pomc-cre + *InsR*-flox + *LepR*-flox	POMC-specific LepR and InsR KO	–	Overweight	Subfertile	Hill *et al.* (2010)
Kiss1-cre + *InsR*-flox + *LepR*-flox	Kisspeptin-specific LepR and InsR KO	–	Normal body weight	Altered pubertal timing	Qiu *et al.* (2015)
Other					
Ghrelin–/–	Ghrelin-deficient	–	Normal body weight	Normal	Sun *et al.* (2003)
Ghsr–/–	Ghrelin receptor-deficient	–	Underweight	–	Sun *et al.* (2008)
Nestin-Cre + *Sf1* flox/–	Brain-specific SF1 KO	–	Normal body weight	Impaired estrus cyclicity, subfertile	Kim *et al.* (2010)

Glutamate-expressing neurons

Glutamate is the primary excitatory neurotransmitter used by the majority of neurons in the CNS, and a body of evidence demonstrates glutamate regulates GNRH neurons' excitability and thus secretion. As reviewed by Iremonger and coworkers (Iremonger *et al.* 2010), many studies have shown that glutamate agonists and antagonists injected into the brain can stimulate or inhibit LH secretion, respectively (Lopez *et al.* 1990, Brann & Mahesh 1995, Ping *et al.* 1997). Glutamate likely exerts its influence via direct effects on GNRH neurons, as Tena-Sempere's lab demonstrated that glutamate-stimulated LH secretion occurs in a kisspeptin-independent manner (Garcia-Galiano *et al.* 2012). Furthermore, neurons in the ventral premamillary nucleus (PMV) of the hypothalamus, which mediate some of leptin's metabolic influences on GNRH function (Donato *et al.* 2011), are almost exclusively glutamatergic (Kocsis *et al.* 2003), supporting a role for glutamate-expressing neurons in the metabolic control of fertility.

To investigate the direct effects of leptin and insulin signaling via glutamatergic neurons in the metabolic regulation of fertility, our lab generated glutamate-specific LepR KO and InsR KO mice. In contrast to the

Table 2 Neuropeptide and transmitter co-expression.

Primary neuropeptide/ transmitter population	Sub-population	Co-expression	Estimated co-expression (%)	References
AgRP/NPY	ARC	GABA	≤100	Marshall *et al.* (2016)
POMC/CART	ARC	GABA	<40	Wittmann *et al.* (2013)
	ARC	Glutamate	<60	Wittmann *et al.* (2013)
Kisspeptin	AVPV	Glutamate	<25	Cheong *et al.* (2015)
	AVPV	Dopamine	≤50	Skrapits *et al.* (2015)
	ARC	GABA	<25	Cheong *et al.* (2015)
	ARC	Glutamate	<75	Cheong *et al.* (2015)
	ARC	GABA	<15	Marshall *et al.* (2016)
nNOS	ARC	GABA	<50	Marshall *et al.* (2016)
SF1	VMH	Glutamate	≤75	Tong *et al.* (2007*a*,*b*)
	VMH	GABA	≤20	Tong *et al.* (2007*a*,*b*)
Dopamine	ARC	GABA	≤80	Marshall *et al.* (2016)
	VTA	GABA	≤40	Merrill *et al.* (2015)
	VTA	Glutamate	≤20	Kawano *et al.* (2006)
	AVPV	Kisspeptin	≤50	Skrapits *et al.* (2015)

AgRP, agouti-related peptide; ARC, arcuate nucleus; AVPV, antero-ventral peri-ventricular nucleus; CART, cocaine-amphetamine-related-transcript; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; SF1, steroidogenic factor 1; VMH, ventro-medial hypothalamus; VTA, ventral tegmental area.

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GABA-specific LepR KO mice, which exhibited delayed puberty and reduced fecundity, the glutamate-specific LepR KO mice exhibited normal puberty onset and fertility (Zuure *et al*. 2013). Glutamate-specific InsR KO mice also exhibited normal HPG axis function (Evans *et al*. 2014*b*). Although these studies suggest glutamate is not a critical mediator of the metabolic control of fertility, some evidence suggests otherwise. For example, LepR-null mice exhibiting PMV-restricted re-expression of endogenous LepR showed rescued puberty onset and improved fertility (Donato *et al*. 2011). As mentioned previously, the PMV is almost exclusively glutamatergic, and these data suggest the PMV is a key site for leptin's permissive action at the onset of puberty. Although speculative, it appears leptin signaling in glutamate neurons may be sufficient to permit puberty onset and reproductive function, but unlike leptin signaling in GABA neurons, it is not critically involved.

NPY/AgRP-expressing neurons

A subset of GABAergic neurons in the arcuate nucleus (ARC) of the hypothalamus co-express neuropeptide Y (NPY) and agouti-related peptide (AgRP), which are both orexigenic neuropeptides shown to have predominantly inhibitory effects on GNRH neurons (McShane *et al*. 1992, Cone 2005, Vulliemoz *et al*. 2005, Roa & Herbison 2012). NPY/AgRP neurons, in turn, are directly modulated by insulin (Konner *et al*. 2007) and leptin (Elias *et al*. 1999) and are also innervated by ghrelin-expressing neurons (Cowley *et al*. 2003). Insulin and leptin inhibit NPY/AgRP-expressing neurons (Sato *et al*. 2005, Konner *et al*. 2007), thereby reducing the inhibitory tone of NPY/AgRP on GNRH neurons. It should be noted that the inhibitory effects of AgRP on GNRH neurons are likely mediated, at least in part, via AgRP's antagonism of stimulatory melanocortin receptor (MC4) signaling (Ollmann *et al*. 1997, Cowley *et al*. 1999). In contrast to leptin and insulin, ghrelin stimulates NPY/AgRP neurons, thereby promoting NPY/AgRP-induced suppression of GNRH release (Lebrethon *et al*. 2007). NPY/AgRP neurons are thus aptly equipped to integrate metabolic and reproductive function.

Physiologically, fasting causes an increase in *Agrp* (Korner *et al*. 2001) and *Npy* (Brady *et al*. 1990) mRNA expression and concomitant suppression of GNRH drive (Kalamatianos *et al*. 2008). Interestingly, although NPY-knockout mice did not exhibit a fertility phenotype under normal conditions, they were protected against the fasting-induced suppression of LH release that was observed in wild-type control mice (Hill & Levine 2003), suggesting NPY plays a critical role in the mechanism whereby HPG function becomes suppressed in response to low energy availability. The decreased leptin and insulin concentrations observed in response to fasting are thought to mediate this effect, as treatment with insulin or leptin decreased *Npy* mRNA and peptide expression in fasted animals (Schwartz *et al*. 1991, Wang & Leibowitz 1997, Korner *et al*. 2001). Fasting is also accompanied by an increase in circulating ghrelin concentration, which may further promote the increased NPY expression observed in response to a fast (Asakawa *et al*. 2001). However, as mentioned previously, ghrelin receptors have constitutive activity (Holst *et al*. 2003, Holst & Schwartz 2004), so even in the absence of fasting, the ghrelin receptor promotes tonic NPY/AgRP neuronal activation. Accordingly, insulin-deficient diabetic rats show increased hypothalamic expression of *Npy* mRNA and peptide, despite being energy replete, and insulin administration restored NPY levels (Abe *et al*. 1991). This suggests insulin is critically involved in restraining tonic NPY/AgRP activity.

Further supporting the idea that insulin and/or leptin must restrain NPY/AgRP neurons to permit fertility, it was shown that ablating NPY/AgRP-expressing neurons in leptin-deficient (*ob/ob*) diabetic mice was able to restore their fertility, food intake regulation and glucose tolerance (Wu *et al*. 2012). Additionally, AgRP deficiency alone (vs neuronal ablation) in LepR-null (*db/db*) mice was also able to rescue their HPG axis function (Sheffer-Babila *et al*. 2013). Lastly, our lab recently showed that fertility can be completely reinstated in otherwise LepR-deficient mice by restoring leptin sensitivity exclusively in NPY/AgRP neurons (O Egan & GM Anderson, unpublished observations), demonstrating that leptin-NPY/AgRP signaling alone can sufficiently block NPY/AgRP-induced inhibition of reproductive function. As both NPY-knockout mice and AgRP-deficient mice were shown to exhibit improved reproductive phenotypes in response to nutritional stress (fasting and LepR deficiency, respectively), it appears that they are both independently involved in the suppression of GNRH. These data highlight both the powerful inhibitory tone NPY/AgRP-expressing neurons exert on reproductive function and also reveal that leptin signaling exclusively in NPY/AgRP neurons is sufficient to permit fertility (Vulliemoz *et al*. 2005).

As mentioned previously, reactivation of LepR exclusively in the PMV was also sufficient to induce puberty onset (Donato *et al*. 2011). However, unlike the PMV-LepR 'rescue' mice, the NPY/AgRP-LepR 'rescue' mice exhibited

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a slightly attenuated body weight phenotype. Although it thus remains a possibility that the improved reproductive function observed in the NPY/AgRP-LepR 'rescue' mice might be due in part to their improved body weight, this seems unlikely as, in addition to the PMV-LepR 'rescue' experiment, there are several accounts demonstrating that fertility can be maintained in morbidly obese mice (Bates *et al*. 2003, Singireddy *et al*. 2013).

POMC/CART-expressing neurons

Acting in opposition to the NPY/AgRP neurons are ARC neurons encoding pro-opiomelanocortin (POMC, a precursor of the anorexigenic melanocortin peptides) and cocaine–amphetamine-related transcript (CART) (POMC/CART neurons). These neurons play a key role in energy homeostasis by promoting satiety and suppressing feeding (Baskin *et al*. 1999, Meister 2007); yet, they have also been shown to promote GNRH neuron activity, presumably via direct actions (Leranth *et al*. 1988, Roa & Herbison 2012, True *et al*. 2013). Sub-populations of POMC neurons have been shown to co-express GABA and glutamate (Table 2). Perhaps not surprisingly, POMC/CART neurons are regulated by insulin, leptin and ghrelin (Havel *et al*. 2000, Cowley *et al*. 2001, 2003, Balthasar *et al*. 2004, Williams *et al*. 2010). Peripheral ghrelin peptide indirectly inhibits the activity of POMC/CART neurons in mice (Cowley *et al*. 2003), but POMC/CART neurons may also be directly modulated by ghrelin-expressing neurons via synaptic communication (Guan *et al*. 2008). Leptin activates POMC neurons to promote satiety, and in the absence of leptin-POMC signaling, an obesity phenotype results (Balthasar *et al*. 2004). Insulin's appetite-suppressing effects are also largely mediated via POMC neurons (Benoit *et al*. 2002); yet, in the absence of direct insulin-POMC signaling, mice exhibit normal body weight regulation (Konner *et al*. 2007), presumably due to the developmental compensation provided by leptin. These data suggest leptin actions on POMC neurons are required for normal energy homeostasis, whereas insulin actions are not.

With regard to fertility, insulin or leptin actions *alone* on POMC neurons do not appear to play a critical role in conveying energy status to the HPG axis, as mice exhibiting POMC-specific deletion of either LepR (Balthasar *et al*. 2004) or InsR (Konner *et al*. 2007) exhibited completely normal fertility. Interestingly, mice exhibiting POMC-specific deletion of both LepR and InsR did present with reduced fertility, but not infertility (Hill *et al*. 2010). This suggests leptin and insulin exert overlapping control of POMC neurons, such that in the absence of leptin-POMC signaling, sufficient compensation occurs via insulin, and vice versa. However, despite exhibiting subfertility, the mice lacking both leptin- and insulin-POMC signaling were still able to reproduce, suggesting insulin and leptin can act via other circuitry to permit sufficient activation of the HPG axis, further highlighting how redundancy in the mechanisms whereby nutritional signals reach GNRH neurons confers reproductive resilience.

Kisspeptin-expressing neurons

The endogenous G-protein-coupled receptor 54 (GPR54) ligand, kisspeptin, has been identified as the most potent secretagogue of GNRH (Irwig *et al*. 2004) and is likewise implicated in the timing of puberty onset (Han *et al*. 2005) and the mechanism whereby energy status is relayed to the reproductive axis (Tena-Sempere 2006, Castellano *et al*. 2010, De Bond *et al*. 2016). Although kisspeptin gene (*Kiss1*) expression is primarily regulated by gonadal steroid hormones (Smith 2009), it is also modulated by metabolic status such that its expression becomes reduced in response to metabolic stress, such as fasting (Castellano *et al*. 2005) or prolonged high-fat diet feeding (Quennell *et al*. 2011). Approximately 40% of ARC kisspeptin neurons were shown to express the gene for LepR (Smith *et al*. 2006) in one report, but subsequent work suggests a more modest level of co-expression (Louis *et al*. 2011, Cravo *et al*. 2013) and failed to show direct leptin–kisspeptin signaling (Quennell *et al*. 2011). InsR protein expression was also observed in a subset of kisspeptin immunoreactive neurons (Evans *et al*. 2014*a*). Physiologically, kisspeptin treatment to women with acquired GNRH deficiency due to low energy availability was able to stimulate GNRH/LH release (Jayasena *et al*. 2009, 2010), suggesting that neuroendocrine integration of nutritional signals converge upstream of, or directly on, kisspeptin neurons. Accordingly, leptin treatment to *ob/ob* mice increased *Kiss1* mRNA expression (Smith *et al*. 2006), whereas ghrelin administration was shown to reduce kisspeptin expression and LH pulse frequency in *ad libitum* fed rats (Forbes *et al*. 2009). Also, insulin-dependent diabetic rats exhibit reduced kisspeptin mRNA levels (Castellano *et al*. 2006). Interestingly, insulin administration did not rescue the reduced kisspeptin gene expression observed in these diabetic rats; yet, leptin administration was sufficient to restore *Kiss1* mRNA levels and LH concentration (Castellano *et al*. 2006).

Although ample evidence suggests leptin and insulin modulate kisspeptin neurons, their direct influences are

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not required for normal reproductive control, as mice exhibiting kisspeptin-specific deletion of either InsR (Evans *et al*. 2014*a*) or LepR (Donato *et al*. 2011) retain normal HPG axis function. Mice exhibiting kisspeptin-specific deletion of both InsR and LepR were also completely fertile, yet, had delayed pubertal timing (Qiu *et al*. 2015). However, Manfredi-Lozano and coworkers recently documented a novel leptin–melanocortin–kisspeptin–GNRH signaling pathway, and it is thus likely that leptin's effects on kisspeptin are mediated indirectly via POMC neurons (Manfredi-Lozano *et al*. 2016). Conversely, kisspeptin was shown to directly excite POMC neurons and indirectly inhibit NPY neuronal activity (Fu & van den Pol 2010), so it appears kisspeptin's role in the mechanism whereby nutritional status is conveyed to GNRH neurons could be both upstream and downstream of POMC neurons. Interestingly, over half of ARC kisspeptin neurons were recently shown to arise from *Pomc*-expressing progenitors (Sanz *et al*. 2015). This may explain, at least in part, why prenatal nutritional insults, which are known to impair hypothalamic development of the melanocortin system and thus cause disturbances to adulthood energy homeostasis (Wattez *et al*. 2013), can cause reproductive impairments (Dupont *et al*. 2012).

GALP-expressing neurons

Galanin-like peptide (GALP)-expressing neurons represent another population of neurons that contributes to the metabolic control of fertility (Cunningham 2004, Crown *et al*. 2007, Shioda *et al*. 2011). Anatomically, GALP cell bodies are primarily located in the ARC, but their fibers make apparent contacts with GNRH neurons in the rat hypothalamus (Takatsu *et al*. 2001). GALP-positive nerve terminals were also found to make axo-somatic and axo-dendritic synaptic contacts with GNRH neurons in transgenic rats in which GNRH neurons were tagged with enhanced green fluorescent protein (Takenoya *et al*. 2006), suggesting GALP can directly regulate GNRH neurons. In turn, GALP neurons express LepR and are regulated by leptin (Jureus *et al*. 2000, Takatsu *et al*. 2001, Cunningham *et al*. 2002) and are also directly modulated by insulin (Fraley *et al*. 2004). GALP neurons are thus equipped to transduce information regarding nutritional status to the HPG axis.

Physiologically, GALP administration was shown to stimulate LH release in mice (Kauffman *et al*. 2005), which occurs in a kisspeptin-independent manner (Garcia-Galiano *et al*. 2012), and GALP infusion was sufficient to rescue the onset of puberty in food-restricted weanling male and female rats (Mohr *et al*. 2012), demonstrating GALP is modulated by nutritional signals and also facilitates fertility *in vivo*. Accordingly, rats exhibiting insulin-dependent diabetes have reduced *Galp* mRNA compared to control animals, and brain insulin administration reversed this effect (Fraley *et al*. 2004). *Galp* mRNA levels were also reduced in rats exposed to a 48-h fast, and insulin or leptin administration increased *Galp* mRNA levels (Jureus *et al*. 2000, Fraley *et al*. 2004), suggesting insulin and leptin exhibit overlapping control of GALP expression. Furthermore, subcutaneous insulin and leptin co-administration rescued reproductive function in insulin-dependent diabetic rats; yet, this effect was prevented when a GALP antibody was administered, demonstrating that GALP mediates the ability of leptin and insulin to rescue reproductive function in these rats (Stoyanovitch *et al*. 2005).

GALP's effects on HPG function may be direct, as GALP was able to directly stimulate GNRH neurons isolated from the rat (Kuramochi *et al*. 2005) and, as mentioned previously, GALP neurons directly innervate GNRH neurons. However, GALP's effects may also be mediated via kisspeptin neurons, as GALP administration increased *Kiss1* mRNA in food-restricted rats (Mohr *et al*. 2012). However, it does not appear ARC POMC/CART or NPY/AgRP neurons are involved in mediating GALP's effects on fertility, as GALP was not able to directly modulate their activity (Kuramochi *et al*. 2005). In contrast, NPY neurons in the dorsomedial nucleus of the hypothalamus *were* shown to mediate GALP's acute orexigenic feeding actions (Kuramochi *et al*. 2006), suggesting GALP and NPY neurons may exhibit bi-directional communication to coordinate their effects on feeding behavior and fertility.

SF1-expressing neurons

Like GABAergic neurons, many glutamatergic neurons co-express neuropeptides (Table 2), such as steroidogenic factor 1 (SF1) (Tong *et al*. 2007*a*). SF1 expression is restricted to, and largely comprises, the ventro-medial hypothalamus (VMH), which is known to play a key role in glucose homeostasis and appetite control (Tong *et al*. 2007*b*), as well as reproduction (Kim *et al*. 2010). Interestingly, brain-specific deletion of SF1 peptide expression leads to impaired fertility and late-onset obesity (Kim *et al*. 2010). However, these SF1-deficient mice also exhibited impaired hypothalamic development and neuronal organization, so it remains unknown whether the observed phenotype was a direct result of SF1 peptide deletion. In a more refined experiment, it was

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demonstrated that SF1 neuron-specific deletion of LepR leads to obesity without causing reproductive impairments (Bingham *et al*. 2008). The former finding, albeit with its considerable caveats, may suggest SF1 peptide is critically involved in the regulation of fertility; yet, the latter result suggests that leptin signaling via SF1-expressing neurons is not, despite its critical role in regulating energy homeostasis. Leptin's roles in the control of metabolism and reproduction therefore appear dissociated, further highlighting the complexity of the neuroendocrine integration of metabolic and reproductive function.

Tyrosine hydroxylase-expressing neurons

Tyrosine hydroxylase (TH)-expressing neurons, which synthesize and release catecholamines (either noradrenaline or dopamine), are also modulated by leptin, insulin and ghrelin (Hommel *et al*. 2006, Konner *et al*. 2011, Zhang & van den Pol 2016) and are involved in the regulation of energy homeostasis (Khanh *et al*. 2014) and fertility (Clarkson & Herbison 2011). Several distinct TH-expressing neuronal populations have been identified and anatomically characterized; yet, the major populations thought to be involved in the regulation of energy homeostasis are the ARC and ventral tegmental area (VTA) dopaminergic populations. The TH neurons in the ARC, referred to as tuberoinfundibular dopamine (TIDA) neurons, were recently shown to play an orexigenic role in homeostatic feeding behavior (Zhang & van den Pol 2016). Accordingly, ghrelin was shown to evoke direct excitatory effects in these neurons. A subset of these neurons were also shown to co-express GABA, which was involved in mediating some of the orexigenic effects (Zhang & van den Pol 2016). The TH-expressing neurons in the VTA of the midbrain are thought to be involved in the regulation of hedonic (vs homeostatic) feeding behavior (Volkow *et al*. 2011). Accordingly, intra-VTA leptin infusion was shown to decrease dopamine neuron firing and reduce feeding (Trinko *et al*. 2011), whereas ghrelin infusion into the VTA stimulated dopamine release and dramatically increased food intake (Abizaid 2009). Although insulin does not appear to directly modulate dopamine release, it was shown to increase dopamine reuptake and thus, like leptin, caused an overall reduction in dopamine signaling, leading to suppressed food intake (Mebel *et al*. 2012). Although dopamine appears to be the major effector of these observations, sub-populations of VTA dopamine neurons have been shown to co-express GABA (Olson & Nestler 2007) and glutamate (Alsio *et al*. 2011), and it thus remains a possibility that these neurotransmitters contribute to the regulation of hedonic eating. Nevertheless, these studies identify a role for both the ARC and VTA TH-expressing neurons in the regulation of feeding behavior.

With regard to fertility, dopamine has been shown to exert both acute and tonic inhibition of GNRH neuron activity (Liu & Herbison 2013). Using electrophysiology in mouse brain slices, Liu and Herbison demonstrated that bath application of dopamine potently inhibited the firing rate of ~50% of GNRH neurons, whereas application of dopamine receptor antagonists increased the basal firing of ~1/3 of GNRH neurons. Approximately 20% of GNRH neurons were shown to receive dopaminergic innervation from the anteroventral periventricular nucleus (Liu & Herbison 2013); however, identifying whether the ARC or VTA dopamine population(s) are involved in the regulation of GNRH remains unclear.

In order to characterize the functional role of insulin and leptin signaling via dopamine neurons, mice exhibiting TH-specific deletion of InsR or dopamine-specific deletion of LepR were generated. Interestingly, although the TH-specific InsR KO mice exhibited an increased body weight phenotype (Konner *et al*. 2011), the dopamine-specific LepR KO mice showed completely normal feeding behavior and body weight, even when challenged with a high-fat diet (Liu *et al*. 2011). Unfortunately, their reproductive phenotypes were not assessed.

NOS-expressing neurons

Nitric oxide synthase (NOS) neurons, which produce nitric oxide, comprise a subset of hypothalamic neurons recently shown to mediate leptin's effects on the HPG axis. As mentioned previously, leptin treatment restores HPG axis function in leptin-deficient mice; yet, in the absence of nitric oxide signaling, leptin was unable to induce puberty in *ob/ob* mice (Bellefontaine *et al*. 2014). This suggests nitric oxide signaling facilitates leptin's permissive effects on puberty onset and fertility. Furthermore, neuronal NOS-knockout mice are hypogonadic and infertile (Gyurko *et al*. 2002), and mice exhibiting NOS neuron-specific deletion of LepR exhibit delayed puberty and hyperphagic obesity (Leshan *et al*. 2012). However, even though NOS expression identified the population of neurons involved in mediating leptin's effects on metabolic and reproductive function, the authors suggest it is unlikely that nitric oxide signaling itself was responsible for leptin's observed metabolic effects because NOS-deficient mice show no obvious metabolic phenotype (Huang 2000). Sub-populations of

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NOS neurons co-express GABA (Marshall *et al*. 2016) and glutamate (Lin *et al*. 2004), and it is therefore possible that one of these neurotransmitters is responsible for leptin's downstream metabolic effects.

Other neuronal populations

Although the aforementioned neuronal populations are arguably the key populations involved in the metabolic control of fertility, there is also evidence supporting the involvement of others. Among these, for example, are RFamide-related peptide (RFRP), melanin-concentrating hormone (MCH) and orexin-expressing neurons. A small subpopulation of RFRP neurons, which are the mammalian orthologs to avian gonadotropin-inhibiting hormone neurons, were recently shown to co-express LepR (Poling *et al*. 2014), for example. However, leptin's regulation of RFRP neurons appears to occur via indirect signaling (Rizwan *et al*. 2014). MCH and orexin neurons are also implicated in the mechanism whereby low energy availability suppresses GNRH drive (Chen *et al*. 2007, Backholer *et al*. 2009, Wu *et al*. 2009, Skrapits *et al*. 2015), yet, the influence of NPY/AgRP in the suppression of reproductive function appears to be more critically involved (Wu *et al*. 2012). Nevertheless, the influence of these 'lesser involved' populations should not be ignored as under normal physiological conditions, they still contribute to the net impact of all the inhibitory and stimulatory inputs from the GNRH neuronal network that ultimately regulate GNRH drive.

Other considerations

Intracellular signaling molecules Although emphasis is often placed on identifying the specific hormone–neuron interactions involved in the integration of metabolic and reproductive function, the role of intracellular signaling molecules should not be ignored. Leptin and insulin might act via unique receptors to exert their influence on specific neuronal populations, for example, yet a high degree of overlap and interaction between the intracellular pathways activated in response to their respective receptors being activated has been demonstrated (Niswender *et al*. 2001, 2003, Niswender & Schwartz 2003, Mirshamsi *et al*. 2004, Benomar *et al*. 2005, Carvalheira *et al*. 2005, Morris & Rui 2009, Berthou *et al*. 2011, Burgos-Ramos *et al*. 2011). Furthermore, critical roles for discrete signaling pathways have been established with regard to the metabolic control of fertility. For example, disruption of neural signal transducer and activator of transcription 3 (STAT3) causes obesity, diabetes and infertility in mice (Gao *et al*. 2004). As leptin induces STAT3 activation, one might assume the phenotype observed in these STAT3-knockout mice might be due to the inactivation of leptin-induced signaling. However, when STAT3 inactivation was restricted to LepR-expressing cells, reproductive function remained intact (Singireddy *et al*. 2013), highlighting that STAT3 signaling other than leptin-induced STAT3 activation may be required for reproductive function. Similarly, the mammalian target of rapamycin (mTOR), which can be activated in response to a multitude of factors, including insulin and leptin, has been identified as a central regulator of metabolic and reproductive function. Roa and coworkers demonstrated that mTOR inactivation inhibited the gonadotropic axis at puberty and also blunted the ability of leptin to promote puberty in food-restricted female rats (Roa *et al*. 2009, Roa & Tena-Sempere 2014). In addition to STAT3 and mTOR signaling, phosphatidylinositol-3-kinase (PI3K) signaling is also considered a key integrator of metabolic and reproductive function. Impairments in leptin- and/or insulin-induced PI3K signaling are observed in response to chronic under- and over-nutrition, and these impairments are thought to play a role in the mechanism whereby reproductive function becomes suppressed, as reviewed by Acosta-Martinez (2011). These data, along with others, demonstrate the importance of a more complete understanding of the roles that intracellular signaling molecules play in the neuroendocrine integration of nutritional signals on fertility.

Cellular energy sensing Nutritional signals and the intracellular signaling cascades they initiate undoubtedly play an important role in the metabolic control of fertility; yet, there is also ample evidence suggesting glucose availability plays a role. As reviewed by Roland and Moenter (2011), a decrease in glucose availability mediates the effects of fasting to suppress GNRH-stimulated LH release (Bucholtz *et al*. 1996, Howland 1980). *In vivo* experiments show GNRH neurons express ATP-sensitive K^+ (K_{ATP}) channels and are sensitive to metabolic perturbations via these K_{ATP} channels (Zhang *et al*. 2007). For example, blockade of K_{ATP} channels with tolbutamide led to an increase in LH concentration regardless of energy balance (i.e. fasted or fed), yet, was unable to override the fasting-induced suppression of GNRH-induced LH secretion (Huang *et al*. 2008). Furthermore, there is evidence suggesting nutritional signals modulate hypothalamic glucose sensing, which in turn could

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influence GNRH neuronal function (Bruning *et al*. 2000, Diggs-Andrews *et al*. 2010). Insulin and leptin, for example, have been shown to activate K_{ATP} channels (Spanswick *et al*. 2000, Mirshamsi *et al*. 2004).

Adenosine monophosphate (AMP)-activated protein kinase (AMPK), long considered a cell-autonomous energy sensor, can also act as an integrative metabolic sensor in the brain (Ramamurthy & Ronnett 2006) and may therefore modulate GNRH neuronal activity, either directly or indirectly by stimulating or inhibiting neurons in the GNRH neuronal network. The activity of AMPK is normally regulated by immediate glucose availability, but is also increased in the presence of orexigenic stimuli (e.g. AgRP and ghrelin) and decreased in the presence of anorexigenic stimuli (e.g. insulin and leptin) (Minokoshi *et al*. 2004). This permits the possibility that whole body energy status (i.e. fuel reserves) acts in concert with immediate glucose availability to modulate the activity of neurons. Minokoshi and coworkers propose a model for how orexigenic and anorexigenic signals, through AMPK activation and suppression, respectively, result in an integrated metabolic response required for the regulation of food intake and energy balance (Minokoshi *et al*. 2004), and this could presumably be the case for reproductive function as well.

Astroglial cells It is also possible that astroglial cells are involved in the mechanism whereby nutritional signals modulate GNRH neuronal activity (Baroncini *et al*. 2007, Ojeda *et al*. 2008). Firstly, astrocytes play an important role in glucose metabolism (Yi *et al*. 2011), which, as discussed previously, is an important regulator of GNRH neuronal function. Secondly, leptin and insulin signaling via astrocytes has been demonstrated (Yi *et al*. 2011). Astrocytic insulin signaling modulates glucose metabolism in primary human astrocytes (Heni *et al*. 2011) as well as *in vivo* (Garcia-Caceres *et al*. 2016), which suggests insulin actions on astrocytes could indirectly mediate the effects of glucose availability on GNRH neuronal function. Leptin signaling in astrocytes was also recently shown to play an active role in the hypothalamic control of feeding (Kim *et al*. 2014). Recently, astrocytes were shown to control food intake by inhibiting AgRP neuron activity (Yang *et al*. 2015), which suggests astrocytes are indirectly able to promote fertility as restraining AgRP neurons would presumably facilitate GNRH drive, as mentioned previously. Furthermore, Sandau and coworkers demonstrated that the synaptic cell adhesion molecule, SynCAM1, mediates astrocyte-to-GNRH neuron adhesiveness in the mouse hypothalamus (Sandau *et al*. 2011*b*), and it was shown that astrocyte-to-GNRH neuron adhesiveness via SynCAM1 is important in the control of female sexual development. For example, female mice expressing a SynCAM1 dominant negative form specifically in astrocytes exhibited delayed onset of puberty, disrupted estrus cyclicity and reduced fecundity (Sandau *et al*. 2011*a*). Astroglial cells, therefore, play a role in GNRH neuronal function and may facilitate the nutritional regulation of fertility.

Tanycytes, which are specialized bipolar glial cells located in the ARC and median eminence, also play a role in GNRH release via extension and retraction of their end-foot processes between GNRH synaptic terminals (Rodriguez *et al*. 2005, Baroncini *et al*. 2007). As reviewed by Levin and coworkers, tanycytes express tight junctions that regulate the permeability of the brain parenchyma to CSF and may therefore have the capacity to integrate a number of signals associated with energy status (Levin *et al*. 2004). Tanycytes express glucose transporters, as well as K_{ATP} channels (Garcia *et al*. 2003), which supports the possibility that they act as glucose sensors and as a possible conduit whereby peripheral nutritional signals could modulate GNRH neuronal activity. Accordingly, tanycytes were shown to respond rapidly to changes in glucose and ATP concentration (Frayling *et al*. 2011).

Stress responses as metabolic modulators of HPG axis function Although the evidence supporting the linkage between metabolic impairments and reproductive disturbances is strong, others have proposed the idea of a stress-related mechanism in which reproductive function is compromised secondary to the activation of the stress-response systems: the hypothalamic–pituitary–adrenal (HPA) and sympathoadrenal axes (Tilbrook *et al*. 2000, 2002). Although this review has focused on metabolic stress, it has also been shown that immune stress (i.e. inflammation), psychosocial stress, environmental stress and physical stress are associated with alterations in GNRH secretion and subsequent reproductive disturbances (O'Byrne *et al*. 1988, Chen *et al*. 1992, Li *et al*. 2004*a*,*b*, Berga 2006, 2008, Kinsey-Jones *et al*. 2009, Knox *et al*. 2009). It therefore stands to reason that stress itself exerts an inhibitory effect on reproductive function. However, some evidence suggests the influence of stress cannot be easily separated from its impact on nutritional signals, suggesting the final common pathway leading to compromised reproductive function may be perceived low energy availability (Loucks & Redman 2004, Berga 2008).

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The theory behind this hypothesis is that activation of the body's stress-response systems stimulates the synthesis and secretion of glucocorticoids and catecholamines into the bloodstream (Tilbrook *et al*. 2000), which are counter-regulatory hormones that alter fuel regulation and signal adaptive metabolic changes that are characteristic of a hypo-metabolic state (Laughlin & Yen 1996). In other words, stress has a metabolic influence, and the stress-induced metabolic profile is similar to that of chronic low energy availability.

Conversely, however, nutritional infertility could be caused by the activation of the stress axes. For example, fasting-induced suppression of LH pulse frequency and amplitude in rats was prevented by the blockade of adrenergic signaling in the paraventricular nucleus of the hypothalamus and by blockade of HPA axis activation (Maeda *et al*. 1994). It therefore remains a possibility that the final pathway whereby reproductive function is suppressed might be a convergence of both metabolic and stress factors. In support of this, the combined impact of minor metabolic and psychosocial stressors (that on their own had little impact on HPG function) was shown to exert a synergistic effect on the suppression of reproductive function (Williams *et al*. 2007). Therefore, what remains to be determined is whether suppression of reproductive function due to metabolic stress occurs primarily via the direct signaling of hormones like leptin, insulin and ghrelin on the GNRH neuronal network or whether activation of the sympathoadrenal and HPA secretory responses is also required.

Summary

Due to the complexity and inherent redundancy of the metabolic control of fertility, impaired signaling of individual metabolic hormones through any one neuronal mediator does not appear to jeopardize neuroendocrine reproductive function (Fig. 1). This is likely due to compensation via other pathways (e.g. other nutritional signals and/or neuronal mediators) and often undermines the true physiological importance of the targeted pathway. However, widespread deletion of central leptin signaling consistently causes reproductive impairments (Mounzih *et al*. 1997, Quennell *et al*. 2009, Zuure *et al*. 2016), highlighting leptin is absolutely required for normal reproductive control. Furthermore, although the deletion of LepR signaling from discrete neuronal populations consistently failed to markedly impair HPG axis function, restoring LepR signaling exclusively in the PMV (Donato *et al*. 2011) or in AgRP neurons (O Egan and GM Anderson, unpublished observations) in LepR-null mice was sufficient to reinstate puberty and reproductive competency, respectively. This latter finding, along with the demonstration that AgRP ablation (Wu *et al*. 2012) and AgRP deficiency (Sheffer-Babila *et al*. 2013) in *ob/ob* mice also restore fertility, highlights that unrestrained

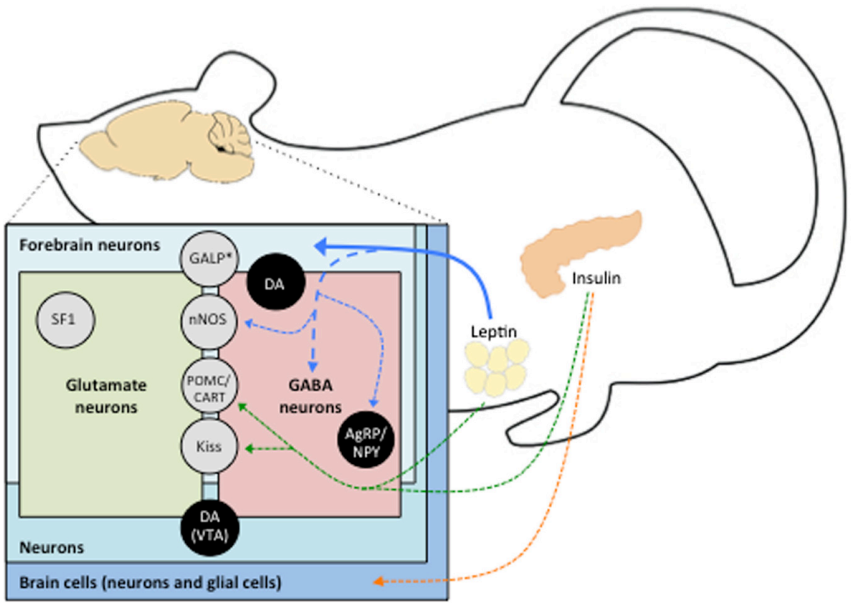

Figure 1
Schematic diagram illustrating the major neuronal populations targeted by nutritional signals to promote (grey circles) or suppress (black circles) the neuroendocrine reproductive axis. Solid arrows represent pathways identified to be absolutely required for fertility, whereas dotted arrows represent pathways shown to be critically involved but not required (as identified through Cre-LoxP gene deletion experiments). The thickness of arrow represents an estimate of the potency of the effect based on the reviewed literature. Blue arrows indicate leptin effects, orange arrows indicate insulin effects and green arrows indicate combined leptin and insulin effects. Pathways not represented by arrows are not critically involved (e.g. effects of ghrelin are not shown as the deletion of ghrelin or its receptor do not disrupt reproductive drive). AgRP/NPY, agouti-related peptide/neuropeptide Y; DA, dopamine; GALP, galanin-like peptide; Kiss, kisspeptin; nNOS, neuronal nitric oxide synthase; POMC/CART, pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript; SF1, steroidogenic factor-1; VTA, ventral tegmental area. *, co-expression data is not available.

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AgRP expression is critically involved in the suppression of HPG function due to nutritional stress.

In contrast, widespread deletion of central insulin signaling (Bruning *et al*. 2000, Evans *et al*. 2014*b*, 2015) or ghrelin signaling (Sun *et al*. 2008) does not cause infertility, demonstrating they are not required to permit sufficient HPG activation. This is not to say they are not important modulators of GNRH drive, but rather highlights sufficient compensation can occur in their absence to preserve reproductive function. However, pathological insulin signaling via GNRH neurons due to diet-induced obesity promotes infertility (DiVall *et al*. 2015), demonstrating insulin can directly influence HPG axis function. Furthermore, it remains a possibility that insulin critically regulates reproductive function through its combined actions on the hypothalamus and pituitary (Brothers *et al*. 2010).

In conclusion, the neuroendocrine integration of metabolic and reproductive function is complex, and many nutritional signals play a role in conveying information regarding peripheral energy status centrally to the GNRH neuronal network. Due to the inherent redundancies in the metabolic control of fertility, the use of transgenic models to test the functional role of individual metabolically relevant signaling pathways has largely been unsuccessful. Nevertheless, these investigations have importantly demonstrated how adaptable and robust the neuroendocrine reproductive axis is in regard to its ability to integrate and respond to multiple metabolic cues.

Declaration of interest

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

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Author contribution statement

M C E drafted the manuscript and G M A provided support and edited the final manuscript.

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