20 YEARS OF LEPTIN

Leptin and reproduction: past milestones, present undertakings, and future endeavors

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Abstract

The association between leptin and reproduction originated with the leptin-mediated correction of sterility in *ob*/*ob* mice and initiation of reproductive function in normal female mice. The uncovering of a central leptin pathway regulating food intake prompted the dissection of neuroendocrine mechanisms involving leptin in the metabolic control of reproduction. The absence of leptin receptors on GnRH neurons incited a search for intermediary neurons situated between leptin-responsive and GnRH neurons. This review addresses the most significant findings that have furthered our understanding of recent progress in this new field. The role of leptin in puberty was impacted by the discovery of neurons that co-express kisspeptin, neurokinin B, and dynorphin and these could act as leptin intermediates. Furthermore, the identification of first-order leptin-responsive neurons in the premammilary ventral nucleus and other brain regions opens new avenues to explore their relationship to GnRH neurons. Central to these advances is the unveiling that agouti-related protein/neuropeptide Y neurons project onto GnRH and kisspeptin neurons, allowing for a crosstalk between food intake and reproduction. Finally, while puberty is a state of leptin sensitivity, mid-gestation represents a state of leptin resistance aimed at building energy stores to sustain pregnancy and lactation. The mechanisms underlying leptin resistance in pregnancy have lagged; however, the establishment of this natural state is significant. Reproduction and energy balance are tightly controlled and backed up by redundant mechanisms that are critical for the survival of our species. It will be the goal of the following decade to shed new light on these complex and essential pathways.

Key Words
- leptin
- gonadotropin-releasing hormone
- reproduction
- hypothalamus
- neurotransmitters

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Introduction

The survival of any species depends on its ability to reproduce, otherwise it will become extinct. Reproduction is an energy-demanding process and its physiological costs vary widely in the animal kingdom, but there is no doubt that the burden of reproduction lies with females. The ability to reproduce starts at different ages in humans but invariably involves a period of sexual maturation that culminates with the first menstrual cycle. In most inbred strains of mice, the first ovulation occurs between 6 and 8 weeks of age; however, in humans, puberty is attained in females at ~12–13 years of age. While the onset of puberty hallmarks the reproductive lifespan of a female, pregnancy, parturition, and lactation are the crux of

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reproductive biology and remain the most physiologically complex and energy-demanding life processes.

The adipose mass of an organism represents an energy storage reservoir. In organisms that undergo periods of hibernation or torpor, buildup of the adipose mass sustains the low metabolic rates and is essential for reproductive function, as best exemplified in ground squirrels (Forger *et al*. 1986). Except in marsupials, torpor in mammals is not a requirement for survival, but the ability to fatten up at critical periods is an essential component of normal physiology. In this review, we will address the leptin-mediated effects and associated mechanisms that pertain to the accumulation of adipose mass at critical times during the reproductive events of a female, namely at puberty and pregnancy.

The seminal discovery that leptin is secreted from adipocytes (Zhang *et al*. 1994) raised an interest in the mechanisms of food intake and adipose tissue accumulation and their impact on the obesity epidemic. However, the link between adipose tissue and reproduction predates obesity, which often confounds and aggravates studies of leptin and the reproductive axis. The inspirational papers of Gordon Kennedy pioneered the link between energy intake and reproduction by placing these two pathways within the hypothalamic network (Kennedy & Mitra 1963*a*,*b*,*c*). Later, Frisch & McArthur (1974) unveiled their critical weight hypothesis and demonstrated that perturbations in the adipose mass affect female fertility negatively (Vigersky *et al*. 1977, Frisch *et al*. 1980, 1981). While much of important physiology was unveiled in these early days, it is unfortunate that the groundbreaking parabiosis experiments of the late Doug Coleman (1973) between *db/db* and *ob/ob* mice did not involve investigation of the reproductive axis during the months' long response of *ob/ob* mice to the *db/db*-originated leptin. Rose Frisch studies were critical in providing a basis for the role of leptin in reproduction and were analogous to Coleman's groundbreaking parabiosis experiments that provided a framework for the roles of leptin and leptin receptor. It was not until leptin rescued the sterility of *ob/ob* mice (Chehab *et al*. 1996, Mounzih *et al*. 1997) and advanced the onset of puberty in normal mice (Ahima *et al*. 1997, Chehab *et al*. 1997, Yura *et al*. 2000) that Kennedy and Frisch's works on the link between the adipose tissue and reproduction were revived and a tying metabolic factor identified in leptin. As a result, the reproductive field has widened and leptin, acknowledged as a permissive puberty factor (Cheung *et al*. 1997), could activate a torrent orchestrated by multiple factors to culminate in the pulsatile secretion of gonadotropin-releasing hormone (GnRH). Another key observation that built on the link between leptin and reproduction is the secretion of leptin from human placenta (Masuzaki *et al*. 1997), further establishing an association between leptin and pregnancy, another state of high energy demands. The demonstration that mid-pregnancy in mice is a state of leptin resistance (Mounzih *et al*. 1998) added further mechanistic insights into the pathways responsible for increasing energy storage during pregnancy. The interplay of leptin, mediating the regulation of orexigenic and anorexigenic pathways (Boston *et al*. 1997, Cowley *et al*. 2001) and those inducing a state of leptin resistance during pregnancy, indicates symbiotic and likely overlapping associations between these two pathways.

Overall, initial studies have emphasized two essential physiological functions of leptin pertaining to its role in reproduction, namely in puberty and pregnancy. Although leptin affects both processes, their mechanisms are distinct in that the former is a state of leptin sensitivity highlighted by a leptin surge in rodents and primates (Ahima *et al*. 1998, Suter *et al*. 2000), while the latter imparts on increasing food intake via a leptin-resistant state (Mounzih *et al*. 1998). Thus, this review will focus on the progress aimed mostly at increasing our understanding of a leptin-sensitive state in puberty and to a lesser extent a leptin-resistant state during pregnancy.

Leptin in puberty

Our knowledge of the mechanisms and cues that control the onset of puberty have considerably increased in the 20th century with much of the work originating from rodent and primate animal models. There is no doubt that the onset of puberty is gated by multiple metabolic factors, which altogether inform the reproductive system about the extent of energy stores in an organism in order to activate the GnRH pulse generator. In fact, increases in adipose tissue mass advance the onset of menarche (Wattigney *et al*. 1999, Kimm *et al*. 2001), whereas fat depletion delays it (Frisch *et al*. 1980). Paradoxically, leptin-deficient subjects are obese but, due to the lack of leptin, fail to advance to puberty unless treated with exogenous leptin (Montague *et al*. 1997, Farooqi *et al*. 1999). These studies highlight the critical role of leptin in the onset of human reproductive function. There is also little doubt that any serious puberty-triggering factor must be able to influence directly or indirectly the secretion or regulation of GnRH. A requirement for such a factor is that mutations in its gene, whether in human and/or mouse models, must result in infertility or significant

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reproductive dysfunction. Leptin fulfills both of these requirements as shown by the infertility of leptin-deficient mice and humans and their leptin-mediated rescue (Chehab *et al*. 1996, Farooqi *et al*. 1999, Gibson *et al*. 2004) as well as its indirect stimulation of GnRH (Nagatani *et al*. 1998, Reynoso *et al*. 2003) and the potentiation of luteinizing hormone (LH) release from pituitary gonadotropes in the presence of GnRH (Avelino-Cruz *et al*. 2009). Leptin also appears to exert GnRH-independent effects on the reproductive system as shown by its ability to induce ovulation in *Gnrh* (*Gnrh1*)-deficient mice (Barkan *et al*. 2005), an intriguing but interesting finding. Overall, leptin is a metabolic factor that bridges the regulation of the fat mass with reproduction.

The central question is the nature of the critical metabolic factor(s) that signals to GnRH neurons the appropriate physiological readiness to initiate GnRH release and activate the puberty cascade. Nutritional and metabolic factors have long been suspected to participate in the process and could elicit a direct or indirect release of GnRH. While mouse and human studies convincingly demonstrate an essential role of leptin in puberty (Chehab *et al*. 1996, 1997, Ahima *et al*. 1997, Cheung *et al*. 1997, Farooqi *et al*. 1999), the absence of signaling-competent leptin receptor expression on GnRH neurons strongly suggests that an intermediary factor or pathway mediates the essential effects of leptin on the activation of GnRH neurons. Potential intermediate candidates are insulin, insulin-like growth factor 1 (IGF1), growth hormone (GH), ghrelin, neuropeptide Y (NPY), orexin, melanin-concentrating hormone (MCH), adiponectin, kisspeptin, and possibly gut peptides. Which of these factors is the single, if any, stimulus and what are its backup or co-factors? From an evolutionary perspective, it makes sense that reproduction, a fundamental component of a species survival, has developed redundant systems. To put this concept into the perspective of critical metabolic and nutritional factors, we can turn to knockout mouse models to determine the impact on reproduction of a specific gene encoding a metabolic factor.

Insulin/IGF1

The insulin/IGF1/GH axis is the hallmark of anabolic hormones that affect GnRH neurons at puberty. This is best demonstrated by the infertility of neuron-specific insulin receptor knockout female mice (NIRKO), which exhibit a 90% reduction in LH but remain responsive to a GnRH agonist (Bruning *et al*. 2000). Thus, neuronal expression of the insulin receptor appears to be critical for reproduction. In a milieu of elevated leptin levels at puberty, it is conceivable that leptin and insulin together influence GnRH secretion. The critical role of IGF1 is also shown by the postnatal lethality of *Igf1* null mice (Liu *et al*. 1993, Powell-Braxton *et al*. 1993). However, a mixed genetic background of *Igf1* null mice rescues their lethal phenotype and these mice exhibit growth retardation and a failure to attain puberty (Liu & LeRoith 1999). In human, homozygous deletion of the *IGF1* gene resulted, as in genetically heterogeneous mice, in postnatal survival and retarded growth but normal, if not, early puberty (Woods *et al*. 1996, 2000). Thus, unlike in NIRKO mice, IGF1 does not appear to be critical and necessary for the onset of puberty but remains an important factor as it correlates with growth. An elegant experiment (Divall *et al*. 2010) targeted the deletion of *Ir* or *Igf1r* on GnRH neurons and determined puberty and fertility outcomes in knockout mice. Mice with a targeted deletion of *Ir* on GnRH neurons displayed normal puberty and fertility. However, male and female mice with a deletion of the *Igf1r* on GnRH neurons showed delayed puberty but normal fertility. Interestingly, IGF1 administration advanced puberty in normal but not *Igf1r* knockout mice. Although *Igf1r* knockout mice showed delayed but not a block in puberty, IGF1R signaling on GnRH neurons appears to be an important contributor to the timing of puberty.

Orexigenic and anorexigenic factors

Orexigenic factors such as ghrelin, NPY, MCH, agouti-related protein (AgRP), and orexin constitute a food intake-regulatory circuit that could influence the hypothalamic–pituitary–gonadal (HPG) axis. Ghrelin, an endogenous ligand for the GH secretagogue receptor (Howard *et al*. 1996), is similar to leptin in that they are both secreted from the periphery, the former from stomach and the latter from adipose tissue, and exert their major effects on the arcuate nucleus (ARC; Cowley *et al*. 2001, Hewson *et al*. 2002), including the HPG axis (Steiner *et al*. 2003, Kluge *et al*. 2007). The fact that leptin and ghrelin are antagonistic in their effects on food intake, their respective anorexigenic and orexigenic roles make them ideal candidates to regulate the reproductive system in times of nutritional variation. Ghrelin exerts a negative effect on GnRH (Fernandez-Fernandez *et al*. 2005), whereas leptin stimulates it or facilitates its secretion (Nagatani *et al*. 1998, Reynoso *et al*. 2003). However, unlike leptin-deficient mice, ghrelin knockout mice are neither obese nor reproductively impaired (De Smet *et al*. 2006). Similarly, orexin knockout mice suffer no energy or

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reproductive peculiarities (Chemelli *et al*. 1999). Interestingly, knockout mice for NPY are fertile and lean (Erickson *et al*. 1996*a*). However, *ob*/*ob* mice deficient in NPY or in the NPY-Y4 receptor show an attenuation of obesity and improved fertility (Erickson *et al*. 1996*b*, Sainsbury *et al*. 2002), suggesting that a lack of NPY or its Y4 receptor alleviates the restraint placed by a deficiency of leptin on the reproductive axis and, most importantly, demonstrating a central role for NPY in reproduction. Although NPY has long been known to stimulate GnRH secretion (Khorram *et al*. 1988, Sutton *et al*. 1988), it was also found to have both inhibitory and stimulatory effects on GnRH, via the NPY-Y1 and NPY-Y4 receptors respectively (Roa & Herbison 2012). Thus, it would be revealing to determine the physiological states and mechanisms under which NPY exerts this variable effect on GnRH neurons. Another observation that links NPY to GnRH is their migration origin. Both GnRH and NPY neurons originate from the olfactory placode and migrate into the CNS during embryogenesis (Hilal *et al*. 1996), implying that their mutual relocation to the hypothalamus could be somewhat evolutionary and functionally linked.

AGRP is expressed in leptin-responsive neurons of the ARC and exerts significant effects on energy intake when overexpressed in transgenic mice (Ollmann *et al*. 1997), but has no obvious effects on adiposity or fertility in *Agrp* knockout and double *Agrp*/*Npy* knockout mice (Qian *et al*. 2002, Xu *et al*. 2005). However, ablation of AGRP-expressing neurons in leptin-deficient *ob*/*ob* and leptin receptor-deficient *db*/*db* mice, remarkably, restores fertility (Israel *et al*. 2012, Wu *et al*. 2012, Sheffer-Babila *et al*. 2013). Thus, the findings that NPY and AgRP play critical roles in reducing sterility in leptin- or leptin signaling-deficient states (Erickson *et al*. 1996*b*, Israel *et al*. 2012, Wu *et al*. 2012) uncover an essential link between energy balance and reproductive pathways. While it is reasonable to assume that AgRP/NPY projections into GnRH neurons might serve as the long-sought leptin intermediate, a more plausible hypothesis is that they represent a secondary alternate reproductive pathway, namely because single or double *Agrp*/*Npy* knockout mice without leptin or leptin receptor mutations fail to exhibit any reproductive defect. However, AgRP stimulates GnRH release (Stanley *et al*. 1999) and thus this secondary pathway is fundamental and may underlie yet unexplained mechanisms of fertility in *ob*/*ob* mice such as when food restricted or bred on mixed genetic backgrounds (Lane & Dickie 1954, Ewart-Toland *et al*. 1999, Qiu *et al*. 2001). The facilitating and rescuing effects of this secondary pathway may also explain fertility in a female with a leptin receptor mutation (Nizard *et al*. 2012).

MCH is another orexigenic peptide expressed in the lateral hypothalamus, where leptin-responsive neurons are located. However, *Mch* knockout mice are lean, hypophagic, and remain fertile (Shimada *et al*. 1998), owing most probably to the presence of enough adipose tissue that secretes leptin. Furthermore, *ob*/*ob* mice lacking *Mch* remain infertile despite attenuation of their obesity caused by an increase in energy expenditure and not decreased hyperphagia (Segal-Lieberman *et al*. 2003).

Anorexigenic factors also affect the reproductive system. It is well known that perturbations in energy distribution and balance such as in lipodystrophy and anorexia nervosa cause interruptions of the menstrual cycles. Notable anorexigenic factors secreted by the gut in response to nutrient ingestion include protein tyrosine (PYY), pancreatic polypeptide, cholecystokinin (CCK), glucagon-like peptide 1 (GLP1), oxyntomodulin (OXM), and apolipoprotein A-IV (apoA-IV). *Pyy* knockout mice are obese and fertile (Batterham *et al*. 2006). *Cck* knockout mice are normal and fertile (Lacourse *et al*. 1999). *Glp1r* knockout mice show impairments in behavioral and stress responses and minor reproductive disturbances, such as reduced gonadal weights in males and slight puberty delay in females, but overall normal fertility (MacLusky *et al*. 2000). Similar to GLP1, OXM results from the proglucagon (*Gcg*) gene, acts as an agonist at GLP1R and stimulates weight loss (Kosinski *et al*. 2012), but has not yet been shown to exhibit any reproductive effect. Similarly, apoA-IV, which is secreted by the small intestine in response to fat absorption, was suggested as a satiety factor but *apoA-IV* (*Apoa4*) knockout mice showed only mild dyslipidemia and no reproductive defects (Weinstock *et al*. 1997).

POMC neurons that activate the anorexigenic arm of the central leptin pathway are direct leptin targets in the ARC. Mutations of the *POMC* gene in human and its ablation in mice result in obesity but no apparent effects on the reproductive axis (Krude *et al*. 1998, Yaswen *et al*. 1999). In addition, targeted deletion of the leptin receptor on POMC neurons resulted in no reproductive defects (Balthasar *et al*. 2004, van de Wall *et al*. 2008). However, deletions of both leptin and insulin receptors from POMC neurons caused insulin resistance and reduced fertility, a condition that resembled characteristics of the polycystic ovary syndrome (Hill *et al*. 2010). Thus, POMC neurons also mediate normal function of the reproductive axis in females. α-MSH is the POMC-derived ligand that binds to MC4R located on second-order leptin-responsive hypothalamic neurons, which stimulate the anorexigenic arm of the pathway. *Mc4r* knockout mice are obese and subfertile (Huszar *et al*. 1997) and, interestingly, their

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reproductive dysfunction can be rescued with increasing exercise (Irani *et al*. 2005). This observation is consistent with the ability of a melanocortin receptor antagonist to reverse reduced food intake in leptin-treated *ob*/*ob* mice, without, however, affecting the activation of their reproductive axis, suggesting a dissociation of the melanocortin pathway from the reproductive system (Hohmann *et al*. 2000). These observations are also consistent with normal fertility of adult obese subjects carrying heterozygous and homozygous deleterious mutations in the *MC4R* gene (Farooqi *et al*. 2003). Thus, while melanocortin neurons could play a secondary role in reproduction, they are unlikely to serve as the critical leptin intermediates.

Overall, it appears that while the orexigenic and anorexigenic factors listed above could play single handedly a critical role in fertility, it is more likely that altogether they would be the gatekeepers of reproduction. To gain further insight into their individual role, a more informative approach would be required to generate double and triple knockout alleles in a single mouse and then determine their combined effects on obesity and reproduction. While this strategy is quite laborious for traditional knockout strategies, the recent advent of genomic editing technologies such as the CRISPR/Cas9 system (Wang *et al*. 2013) makes it likely to generate such informative experimental model systems.

Kisspeptins

The exciting discovery of the kisspeptin system offered a great candidate for a leptin intermediate acting between leptin-responsive neurons and GnRH neurons. Kisspeptin, a product of the *KISS1* gene, is expressed in various mammalian species, predominantly in a large population of neurons in the ARC and to a lesser extent in the preoptic area (POA), the rostral periventricular region of the third ventricle, as well as in scattered regions of the brain outside of the hypothalamus (Lehman *et al*. 2010). Kisspeptin neurons connect to NPY and POMC neurons (Backholer *et al*. 2010), which are first-order leptin-responsive neurons in the hypothalamus, enhancing the coordination of the nutritional leptin axis to its reproductive counterpart.

Kisspeptin emerged as a primary puberty candidate factor following the findings that subjects with hypogonadotropic hypogonadism carry mutations in the kisspeptin receptor *GPR54* (*KISS1R*; de Roux *et al*. 2003, Seminara *et al*. 2003, Semple *et al*. 2005). The first indication that kisspeptin could indeed be the long-sought leptin intermediate was demonstrated in leptin-deficient *ob*/*ob* mice that express low levels of *Kiss1* mRNA, that are then stimulated to be increased with leptin treatment (Smith *et al*. 2006). Targeted deletions in mice of *Kiss1* or the Kiss1 receptor *Gpr54* gene revealed infertility and abnormal sexual maturations (d'Anglemont de Tassigny *et al*. 2007, Lapatto *et al*. 2007), affirming a critical role for the kisspeptin system in the activation of the reproductive axis. Consistently, the finding that i.c.v. administration of kisspeptin stimulated maturation of the reproductive axis in hypoleptinemic states of prepubertal rats and hyperleptinemic *fa*/*fa* Zucker rats with a mutation in the leptin receptor (Navarro *et al*. 2004), suggesting that Kiss1 advances puberty when administered centrally and that its GnRH-triggering function is upstream of GnRH neurons, which is what would be expected from a leptin intermediate. Most importantly, kisspeptins are potent stimulators of GnRH release (Irwig *et al*. 2004, Thompson *et al*. 2004, Messager *et al*. 2005, Pielecka-Fortuna *et al*. 2008). While these above findings were close to sealing the nature of the leptin intermediate, it was surprisingly found that deletion of the leptin receptor from Kiss1 neurons (Donato *et al*. 2011) or ablation of >95% of kisspeptin neurons before puberty resulted in normal puberty and fertility (Mayer & Boehm 2011). Furthermore, transgenic expression of the kisspeptin receptor in neurons of leptin receptor-deficient mice failed to rescue their sterility (Cravo *et al*. 2013). Thus, these studies dampened the excitement of a kisspeptin–leptin intermediate hypothesis, despite the fact that a small amount of kisspeptin might actually be sufficient to trigger puberty (Popa *et al*. 2013). While kisspeptin expressing neurons are unlikely to be the leptin intermediate neurons, they nevertheless play a critical role in reproduction, perhaps in the maintenance of fertility after puberty. Thus, it would be interesting to tease out the effects of kisspeptin at puberty and subsequently for the maintenance of fertility. In addition to leptin, insulin receptors are located on kisspeptin neurons and could contribute to their effect on reproduction. Interestingly, deletion of the insulin receptor from kisspeptin neurons resulted in delayed puberty, but thereafter, normal fertility (Qiu *et al*. 2013), adding insulin as a potential mediator of reproduction from kisspeptin neurons.

The involvement of kisspeptin in sexual maturation and reproduction extends to an exciting set of neurons in the ARC, which co-express kisspeptin, neurokinin B, and dynorphin. These neurons, termed KNDy, project on GnRH neurons, stimulate GnRH release (Hrabovszky *et al*. 2010, Ramaswamy *et al*. 2010), and get activated during the LH surge (Merkley *et al*. 2012). Furthermore, central administration of a NK3 receptor (NK3R) agonist in

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prepubertal females elicits LH secretion, whereas infusion of an NK3R antagonist delays vaginal opening and LH pulse amplitude (Navarro *et al*. 2012, Li *et al*. 2014). The proportion of kisspeptin neurons that express the long form of the leptin receptor (LepRb) is controversial and ranges from 6% (Louis *et al*. 2011) to 40% (Smith *et al*. 2006), implying that leptin action on KNDy neurons could be minimal or substantial. Analogous to the fact that a small amount of kisspeptin is required to trigger puberty (Popa *et al*. 2013), it is equally conceivable, but remains to be determined, that a small number of KDNy neurons expressing LepRb could elicit GnRH release. The small proportion of neurons required to trigger reproductive function is reminiscent of the fact that implantation of preoptic slices containing only 3–140 GnRH neurons is enough to correct hypogonadism in *Gnrh*-deficient mice (Silverman *et al*. 1985). While STAT3 signaling is not required for reproduction (Bates *et al*. 2003), only 15% of KNDy neurons in the ARC phosphorylate STAT3 upon leptin exposure (Cravo *et al*. 2011). Thus, KDNy neurons that bind leptin, express LepRb, but suppress STAT3 phosphorylation will be critical for unraveling a leptin–kisspeptin STAT3-independent pathway for initiating reproductive function.

Premammilary ventral nucleus

An illuminating finding that throws new light on the search for a leptin intermediate was the identification that the premammilary ventral nucleus (PMV), a site in the hypothalamus that exhibits broad expression of receptors involved in energy metabolism, encompasses leptin-responsive neurons (Donato *et al*. 2010). Lesions in the PMV resulted in decreased activation of GnRH and Kiss1 neurons, causing low estrogen secretion and deficient feedback of GnRH on proestrus (Donato *et al*. 2013), demonstrating that the PMV is a critical area that mediates the effects of leptin on reproduction. Another tantalizing finding is the uncovering that neurons directly responsive to leptin, located in various brain sites including the PMV, POA, ARC, and dorsomedial hypothalamus, release the neurotransmitter nitric oxide (Donato *et al*. 2010). While it has been previously known that targeted inactivation of the mouse nitrous oxide synthase (*Nos1*) gene caused infertility in the presence of normal leptin levels (Gyurko *et al*. 2002), recent studies have demonstrated that deletion of the neuronal *Nos* gene, or its pharmacological inhibition, blunted the ability of exogenous leptin to restore fertility to *ob*/*ob* mice (Bellefontaine *et al*. 2014). In the same study, leptin has been found to coordinate fertility by acting on neurons in the POA to induce NO synthesis via activation of neuronal NOS. Thus, leptin-responsive nitric oxide-releasing neurons define a new class of PMV neurons that provide input to the neuroendocrine regulation of reproduction.

Overall, it appears that the identification of a single critical leptin intermediate, which indirectly affects GnRH secretion, remains to be fully elucidated, although the primary candidates are KNDy neurons and NO leptin-responsive neurons. Consistent with the notion of redundancies in reproductive pathways, it is quite likely that a single leptin intermediate may not exist, but rather that a web of neuronal connections coordinate the firing of a complex system that is fundamental to species survival. The experimental dissection of these pathways will prove to be difficult but not impossible considering the increasing use of genome-editing tools to knock in and knock out multiple genes in the same cell and generate corresponding mouse models (Wang *et al*. 2013). Another possibility, which remains to be fully explored and distinct from the stimulatory hypothesis of a leptin intermediate, is the removal of a restraint imposed on GnRH neurons, such as from epigenetic mechanisms. All these approaches should help us in the following decade to expand on novel findings and derive conclusive mechanisms for the reproductive side of leptin.

Leptin in maternal nutrition during pregnancy

The adipose tissue mass plays another role in reproduction, essentially for building up adequate energy reserves to sustain a pregnancy and for the subsequent energy demands of lactation. The first evidence of leptin playing a role in pregnancy originated from the report that pregnant women secrete elevated levels of leptin from the placenta into the maternal circulation (Masuzaki *et al*. 1997). The effects of leptin during pregnancy were subsequently assessed in leptin-treated and mated male and female *ob*/*ob* mice, thus resulting in *ob*/*ob* pregnancies that were controlled with exogenous leptin (Mounzih *et al*. 1998). In this study, withdrawal of leptin treatment at 0.5, 6.5, 10.5, and 19.5 days post coitum (p.c.) of pregnant *ob*/*ob* mice did not affect implantation, gestation, or parturition. However, the food intake of *ob*/*ob* females continuously treated with leptin during pregnancy resulted, as of day 10.5 p.c., in increased food intake compared with previous days, demonstrating an attenuation effect of the leptin treatment and the establishment of a leptin-resistant state. Consistently, during the late pregnancy of rats, surges in food intake are associated

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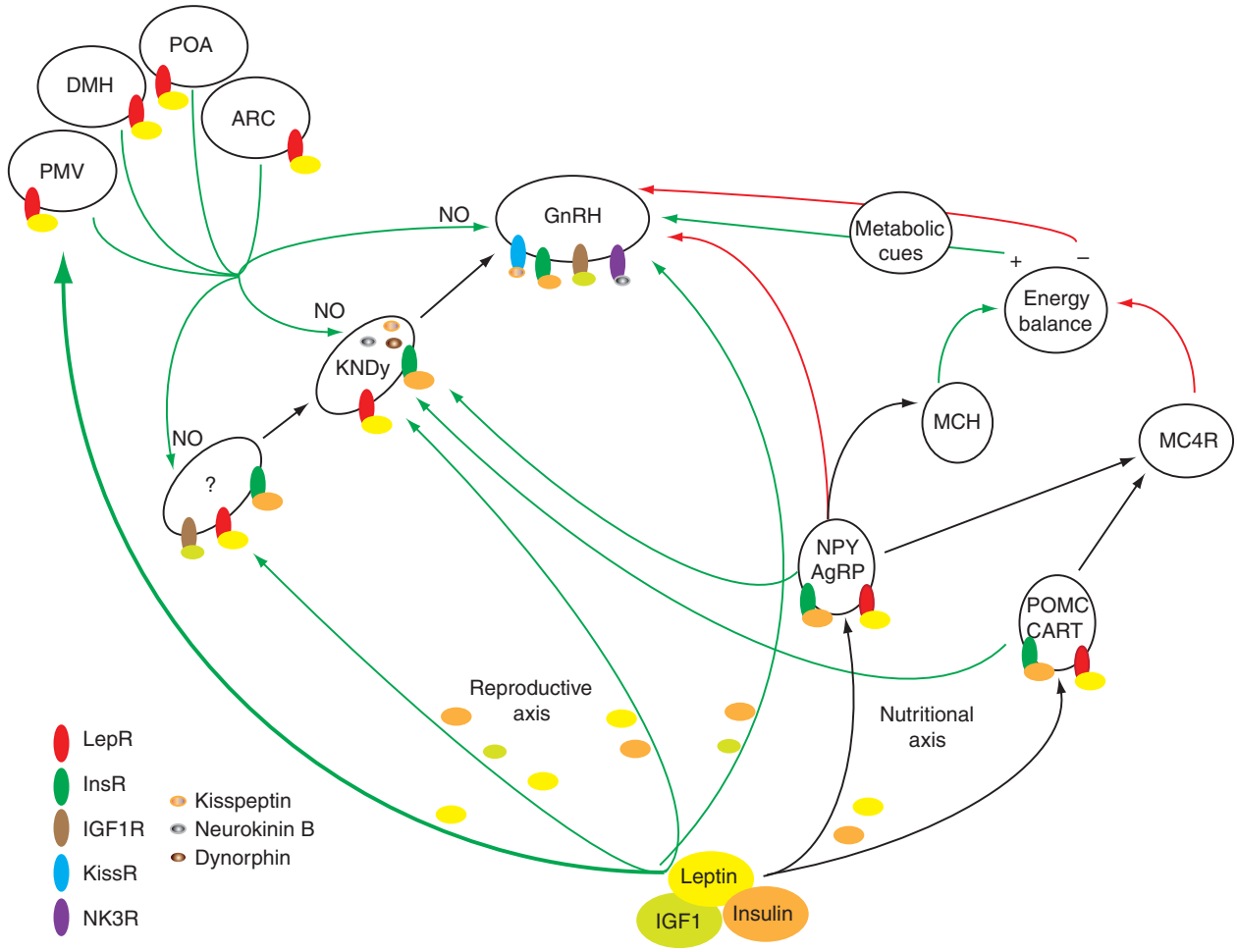

Figure 1

Schematic and proposed model for the metabolic regulation and coordination of the leptin-mediated reproductive and nutritional axes. Leptin, insulin, and IGF1 are shown as the most critical circulatory factors that act on hypothalamic and other brain networks to regulate food intake and GnRH secretion. The pathway, rich in redundancies, depicts the various direct and indirect inputs that converge on GnRH neurons. The crosstalks of the reproductive and nutritional axes are represented by the projections of the AgRP/NPY and POMC neurons to the KNDy neurons and by the AgRP/NPY neurons onto GnRH neurons. The first-order leptin responsive neurons in the PMV and other hypothalamic areas are denoted with a thick green arrow to highlight their essential reproductive function and the release of the NO neurotransmitter. Ligands and receptors are shown on each set of neurons and green/red arrows represent stimulatory and inhibitory effects respectively. The postulated elusive leptin intermediate neurons are denoted with a question mark. Acronyms in the figure are the same as in the text.

with elevated plasma leptin levels and central administration of leptin during this period is less effective at reducing food intake compared with cyclic rats, again demonstrating a period of reduced leptin sensitivity (Johnstone & Higuchi 2001). The onset of leptin resistance in pregnancy was appropriately associated with decreased STAT3 phosphorylation in the VMH (Ladyman & Grattan 2004, 2005) and by the dysregulation of AgRP/NPY and melanocortin neurons (Ladyman *et al.* 2009). Thus, mid-gestation in the mouse represents the beginning of a leptin-resistant state that could conceivably be derived from synaptic plasticity and reprograming of neuronal projections into the hypothalamus. These changes would be aimed at establishing a body weight set point that results in increased food intake and adipose mass accumulation. Maternal food intake, whether increased or decreased, was found to program postnatal leptin expression, as demonstrated by elevated leptin expression in adipose tissue and plasma secretion in female pig offspring, whose mothers were allowed for higher food consumption during the second quarter of pregnancy (Eckert *et al.* 2000). While decreased maternal nutrition during pregnancy can have devastating effects on fetal reprograming, one effect is that the postnatal leptin surge (Ahima *et al.* 1998)

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is severely attenuated by maternal under nutrition (Delahaye *et al*. 2008), presumably resulting in delayed growth and puberty.

The mechanisms underlying leptin resistance in pregnancy may be underlined by the same mechanisms as in obesity; however, the triggering factors in either case are likely to be distinct owing to the differences of both physiological states. The onset of leptin resistance in pregnancy is a natural process, which could be carried *post partum* in subsets of women with obesity (Gunderson & Abrams 2000). The timing for the onset of leptin resistance in pregnancy is predictable and therefore could ease the uncovering of the triggering mechanisms and the associated factors.

Conclusions and future perspectives

Puberty and mid-gestation are physiological states of leptin sensitivity and leptin resistance respectively. Studies centering on understanding the mechanisms that underlie the onset of puberty, and to a lesser extent those involved in leptin resistance during pregnancy, have been exhaustive in the past 20 years. Our knowledge of the triggering neurons has been substantial and potential leptin intermediates have emerged and enlightened the role of leptin in reproduction. First, the location of a previously unappreciated site for leptin action in the PMV opens new avenues to investigate neuronal projections, synaptic plasticity, and neurotransmitters that signal the timing and firing of GnRH neurons to trigger the reproductive cascade. Secondly, the characterization of the kisspeptin system, specifically the KNDy neurons, which are upstream of GnRH, and the potential role leptin plays in these neurons continue to be an exciting pathway to decipher and dissect. Thirdly, the revealing role of AgRP/NPY neurons that influence GnRH neurons is a critical step that bridges the central nutritional pathway elicited by the binding of leptin to first-order neurons to the leptin-responsive neurons in the reproductive axis. The essential criteria for neurons to qualify, as leptin intermediary neurons, is that they would have to respond to leptin, initiate leptin signaling, not necessarily via STAT3 phosphorylation, and stimulate GnRH release most probably through KNDy neurons. In addition, site-specific deletion of the leptin receptor from these neurons would have to result in sterility, irrespective of the presence or absence of obesity. Furthermore, *ob*/*ob* and *db*/*db* mice should display dysregulation of these intermediary neurons, stemming from their hypoactivation in leptin or leptin signaling deficiency.

While reproductive disturbances are largely dissociated from common obesity, nutritional factors and reproduction are closely connected, as exemplified in states of negative energy balance, when food intake corrects amenorrhea. For example, exogenous leptin induces menstruation in hypothalamic amenorrhea and is thus an appropriate fertility treatment for this disorder (Welt *et al*. 2004, Chou *et al*. 2011). Furthermore, in lipodystrophy, leptin treatment, withdrawal, and reinstatement have effects on the progression, interruption, and regain of puberty (Kamran *et al*. 2012).

A proposed pathway that recapitulates the salient findings summarized in this review is shown in Fig. 1 and outlines the essential neuronal connections that involve leptin, insulin, and IGF1 or their surrogate neurons to converge onto GnRH neurons. As central pathways are critical for any leptin-mediated effect, the focus of future years will be on new first-order sets of leptin-responsive neurons in the PMV and other brain regions that project on GnRH neurons. These neurons will undoubtedly uncover mechanisms that will begin to unravel the complexities of a redundant system essential for species survival.

Finally, a provocative question is whether the primary function of leptin pertains to its metabolic regulation of the reproductive axis. Then conceivably, dysregulation in segments of this pathway, perhaps those that involve the leptin intermediate(s), could cause overweight disorders. While innumerable and challenging arguments would certainly be raised on either side of this hypothesis, it remains worthy of consideration. We still have intriguing questions to ask for many more years and additional lessons to be learnt from the role of leptin in the metabolic control of neuroendocrine reproductive biology.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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