Insulin: its Role in the Central Control of Reproduction

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Abstract

Insulin has long been recognized as a key regulator of energy homeostasis via its actions at the level of the brain, but in addition, plays a role in regulating neural control of reproduction. In this review, we consider and compare evidence from animal models demonstrating a role for insulin for physiological control of reproduction by effects on GnRH/LH secretion. We also review the role that insulin plays in prenatal programming of adult reproduction, and consider specific candidate neurons in the adult hypothalamus by which insulin may act to regulate reproductive function. Finally, we review clinical evidence of the role that insulin may play in adult human fertility and reproductive disorders. Overall, while insulin appears to have a significant impact on reproductive neuroendocrine function, there are many unanswered questions regarding its precise sites and mechanisms of action, and their impact on developing and adult reproductive neuroendocrine function.

Highlights

- Insulin plays a key role in the regulation of reproduction in addition to metabolism
- Insulin regulates both pulsatile and surge secretion of GnRH/LH
- Insulin may be a signal in prenatal programming of adult reproductive function
- Insulin targets in the brain include kisspeptin, AgRP and POMC neurons
- Insulin resistance in human disease is associated with reproductive dysfunction

Key words

Insulin receptors, hypothalamus, GnRH, kisspeptin, AgRP, POMC

1. Reproduction and energy balance: the functional connection in health and disease

Reproduction is a crucial function of the organism and is controlled by complex interactions between the hypothalamus, pituitary and gonads, the so-called hypothalamic-pituitary-gonadal (HPG) axis [1]. However, reproduction and the survival of offspring is also an energetically demanding process, and the relationship between reproductive success and energy balance is well established. Energy is stored primarily as fat and glycogen, and together with glucose, allows organisms to grow and reproduce [2]. However, an animal's energy stores depend not only on the availability of energy sources (food), but also on energy expenditure. Pregnancy, parturition, lactation and maternal behavior are all energetically demanding states and in order to be successful in reproducing, the organism must be able to monitor energy status. Thus, negative energy balance either due to hypophagia (e.g. fasting, anorexia nervosa, and cachexia) or excessive energy expenditure (e.g. excessive exercize-induced amenorrhea and lactation) is linked to a suppression of reproductive function and ovarian cyclicity in a variety of species including humans [2, 3].

Since its discovery by Banting and Best in 1921 [4], insulin has been recognized as a key circulating signal mediating energy homeostasis. While major role of insulin is to maintain peripheral glucose homeostasis, via stimulation of glucose uptake, oxidation and storage [5], there is also strong evidence that insulin plays a role in regulating reproduction and may serve as a major signal linking metabolism and reproductive status. In this review we will focus on the role of insulin as an important factor in the control of reproduction, through actions occurring not only in the periphery but also in the central nervous system (CNS). We will review evidence primarily from animal studies demonstrating a role of insulin in the regulation of reproduction during adulthood, as well as during fetal development. In addition, we will examine evidence for potential CNS targets of insulin action specifically related to reproduction. Finally we will discuss the clinical relevance of the relationship between insulin and reproduction, with a specific focus on potential neural sites of action.

2. Insulin as a signal in the metabolic control of adult reproduction

2.1. Insulin: its major role in controlling glucose homeostasis

In a simple sense, physiological maintenance of the regulation of blood glucose levels is the result of the coordinated function of three organs: the pancreas, which secretes insulin in response to increases in blood glucose; the liver, which decreases glucose production in response to raising levels of insulin; and skeletal muscle (and other tissues) that respond to insulin by increasing glucose uptake. In addition to this role, insulin also plays an important function in fat and protein metabolism, as it promotes the transport of amino acids from the bloodstream into muscle and other tissues/cells. Acting within cells, insulin increases the rate of incorporation of amino acids into protein and reduces protein breakdown. Moreover, insulin stimulates lipid (fat) synthesis from carbohydrate (in the process called lipogenesis) and decreases fatty acid release from tissue (in the process called lipolysis), leading to an increase in total body lipid stores [6]. Finally, insulin also possesses important vascular actions, such as vasodilatation, which leads to increase in the blood flow, and subsequent augmentation of glucose disposal in classic insulin target tissues [7, 8].

2.2. Evidence for a role for insulin in the control of the HPG axis

Metabolism and reproduction are closely interlinked and a large body of research has focused on elucidating the mechanism by which signals from the periphery are conveyed to the HPG axis under various metabolic states. The master molecule for the control of the HPG system in mammals is the decapeptide, gonadotropin-releasing hormone (GnRH). GnRH is synthesized by a relatively small

number of neurons, whose cell bodies are dispersed over an area that extends from the rostral ventral forebrain to the caudal hypothalamus and varies between species [9-11]. GnRH neurons send a major axonal projection to the median eminence [12-14], where GnRH is secreted into the pituitary portal system, and subsequently causes the release of luteinizing hormone (LH) from gonadotrophs of the anterior pituitary [16]. There are two major modes of GnRH secretion: the tonic or episodic secretion of GnRH that is seen in both males and females, and the preovulatory surge secretion of GnRH which is responsible for triggering ovulation and occurs only in females Both pulsatile and surge modes of GnRH secretion are sensitive to metabolic signals [18-22] and pathological situations which lead to acute and/or chronic hypo- or hyperinsulinemia are frequently coupled with disturbed GnRH/LH pulse and surge release patterns.

The importance of insulin as a regulator of GnRH/LH pulses remains to be fully elucidated as results vary considerably between studies and the effects of insulin per se are difficult to tease apart from the role of accompanying peripheral signals and metabolites. For example, in diabetic male rats, there was a 50% reduction in LH pulse frequency and amplitude compared to non-diabetic controls [18]. Those deficits were completely reversed by twice daily insulin treatment [18]. Similarly, in Streptozotocin-induced (STZ-induced) diabetic male lambs, 24h withdraw from insulin supplementation decreased LH pulse frequency and acute re-supplementation reversed the inhibition [19]. However, in this study, a longer-term insulin withdrawal (96h) exaggerated the effects on LH (with a further reduction in LH pulses) during which insulin and glucose plasma concentrations remained constant. Therefore, other suppressors such as non-esterified fatty acids and ketone bodies cannot be ruled out [19]. By contrast, studies of other hypoinsulinemic models such as fasting yield different results. In adult (non-diabetic) male rhesus monkeys that underwent 24 h of fasting a profound suppression of LH was recorded, and rapid re-feeding reversed those deficits. To test the possible role of insulin, on the day of re-feeding, post-meal insulin secretion was partially suppressed by diazoxide (40-99%). However, this treatment did not block the LH increase observed after feeding [23] indicating that insulin alone was could not account for the observed inhibition. Similarly a central role of insulin in regulating GnRH/LH pulses remains controversial. Hileman et al. [24] reported that central injection of insulin (lateral ventricle) did not increase LH secretion in the growth-restricted, hypogonadotropic lamb. By contrast, Miller et al. [25] found that the infusion of insulin into the third ventricle stimulated pulsatile LH secretion in adult male sheep. In a male diabetic sheep model, insulin infusion in the lateral ventricle reversed the decrease in LH pulse frequency but not to the same extent as peripheral insulin, providing further evidence that insulin alone cannot account for the diabetes-induced deficit in LH pulses. The reason for the discrepancy between these studies is not known, however, differences may be due to the type of model used (diabetic vs. fasting models), the doses, infusion site, and rate of insulin administered (pharmacological vs. supraphysiological; lateral vs. third ventricle; acute vs. chronic), the species

(polygastric vs. monogastric animals) and the level of hypoinsulinemia [complete (diabetes) vs. partial (fasting)].

Despite variable results, the above studies taken together suggest that the peripheral and central actions of insulin are permissive rather than necessary for normal GnRH/LH pulsatile secretion.

In addition to GnRH/LH pulses, the GnRH/LH surge is also sensitive to metabolic cues. Models of negative energy balance induced by fasting, caloric restriction and lactation are accompanied by a decrease in circulating insulin and disruption of estrous cyclicity in a number of species such as rats [26, 27], ewes [28], heifers [29] and monkeys [30]. Specifically, in adult female rats, short-term food deprivation blocks the LH surge [31, 32]. Short-term fasting during the luteal phase of the estrous cycle in sheep, increased serum concentrations of progesterone and delayed or diminished the pre-ovulatory LH surge [33, 34]. Data on the role of insulin in these disruptions are lacking, however, insulin replacement during lactational anestrous- a model of severe undernutrition did not restore estrous cyclicity [35]. Therefore, it is likely that other metabolic signals, such as hypoglycemia, leptin, or even the activation of the stress axis may be responsible for these disruptions [35, 36]. Experimental diabetes induced in female rats with STZ [37], a state of extreme hypoinsulinemia, results in impaired ovulation rates over an extended period of observation, disruption of the positive feedback effects of estradiol, and absent or delayed LH surges [20, 38, 39]. However, in this model, reproductive abnormalities are at least partially reversed after peripheral insulin administration [40]. These results are similar to those described above, in that effects on pulsatile and surge secretion are not reversed by insulin during negative energy balance but are at least partially reversed in diabetic models (hypoinsulinemia vs. extreme hypoinsulinemia).

Another commonly used experimental model for metabolic stress is insulin-induced hypoglycemia (IIH). This model mimics the detrimental effects of an acute decrease in energy availability, but also the effects of iatrogenic insulin overdose in diabetic patients. Even though the individual roles of supraphysiological amounts of insulin and the acute hypoglycemia are difficult to tease apart, there are several pieces of evidence that suggest that insulin does indeed contribute to the GnRH surge disruption during IIH. Studies carried out in ewes have determined that IIH during the activation, transmission or secretory phases of the GnRH surge mechanism [41, 42] initiates a sudden activation of the hypothalamic-pituitary-adrenal (HPA) axis [43, 44] resulting in a delayed and reduced amplitude LH surge in the majority of treated ewes [42, 43, 45]. By contrast, there are reports of no effect of IIH on the LH surge of proestrous rats [46] and monkeys [47]; however, the doses of insulin used in these studies were significantly less than those used in the sheep experiments, suggesting that a specific threshold of hyperinsulinemia/hypoglycemia may need to be reached for deleterious effects to occur. Glucose replacement in the IIH sheep model reverses the effects of IIH on the timing of the

surge [48] but not on its amplitude [42]. Therefore, even though the timing of the LH surge appears to be sensitive to glucose availability, surge amplitude does not, and it may be that hyperinsulinemia in this experimental model is responsible for this effect.

Similar conclusions can be drawn from experiments in sheep that have been prenatally exposed to excess testosterone and exhibit metabolic and reproductive deficits similar to those seen in women with polycystic ovarian syndrome (PCOS) [49, 50]. Prenatal testosterone treated female ewes display hyperinsulinemia and insulin resistance [50], as well as defects in steroid feedback control of LH secretion, including delayed and reduced amplitude LH surges [51]. Interestingly, in this model, restoration of cumulative plasma insulin levels with an insulin sensitizer, rosiglitazone, was able to restore the amplitude but not the timing of the LH surge [52]. Taken together, data from these two sheep models (IIH and prenatal testosterone exposure) imply that hyperinsulinemia does not abolish the LH surge but does reduce its amplitude. Whether this is a result of decreased GnRH release and/or reduced pituitary responsiveness to GnRH remains to be determined, however, there is evidence that both these sites are involved. For example, in women with PCOS pituitary response to GnRH is suppressed under a euglycemic, hyperinsulinemic clamp [53] and this may account for the reduced surge amplitude observed in the prenatal testosterone treated ewe model. Similarly, substantial evidence suggests that insulin acts directly within the hypothalamus, and specifically via insulin receptor (IR) containing cells to influence GnRH excitability (see section 4.2). The site(s) of action (neural vs pituitary) of insulin in regulating both pulsatile and surge secretion of GnRH/LH may be best addressed by future studies using the sheep model, where a specific advantage is the ability to repeatedly measure GnRH in portal blood in awake animals [54].

3. Insulin: its potential role in prenatal programming of reproduction

There is a growing body of both epidemiological and experimental evidence indicating that environmental factors can act early in the development to shape relationships between the regulation of energy status and reproduction later in life. The concept that early environmental factors can permanently organize or imprint physiological and behavioral systems is called fetal or early programming [55-58]. This hypothesis originated from studies indicating that low birth weight is associated with an increased biological risk for coronary heart disease in adult life [55]. Later studies performed by Philips and collaborators [59-61] demonstrated a strong correlation between low birth weight, high cortisol levels and development of hypertension and Type 2 diabetes. There is now a good body of evidence, both from epidemiological data and experimental studies in animals, linking

the intrauterine environment with the development of hypertension, diabetes, elevated blood cholesterol and PCOS in adulthood (for review see [55, 62, 63]).

Several not mutually exclusive hypotheses have been developed to explain a link between a low body weight at birth and later adult risk of metabolic syndrome. “The Fetal Insulin Hypothesis” states that pancreatic beta cell dysfunction can lead to defects in glucose stimulated insulin secretion, which in turn lead to reduced insulin mediated fetal growth and a low birth weight [64]. Those alterations at early stages would later in adulthood result in defects in beta cells and decreased insulin sensitivity, and thereby affect whole body glucose metabolism. Interestingly, however, Ng et al. [65], using a rat model of chronic high fat diet, reported that not only maternal but also paternal metabolic status could affect offspring health. Specifically, they found that the female offspring of males fed a high fat diet in adulthood showed impaired glucose tolerance and insulin secretion. Moreover, the gene-expression profile of the insulin-secreting pancreatic islet cells obtained from the daughters was abnormal, with changes in multiple gene networks and cellular pathways. The authors speculated that exposure to a high fat diet may have affected spermatogenesis in those fathers, re-programming the gametes possibly via epigenetic mechanisms.

Another explanation of the relationship between body weight at birth and adult metabolic syndrome comes from the “Thrifty Phenotype Hypothesis”, according to which a fetus that endures poor nutrition during gestation, would spare the growth of vital organs, e.g. the brain, at the expense of tissues such as the muscle and the endocrine pancreas [66]. Thus, the fetus would adapt its metabolism to conditions of limited nutrition with permanent changes in insulin and glucose metabolism, increasing the risk of adult Type 2 diabetes and the metabolic syndrome [66]. In the light of evidence discussed above it would be of particular interest to identify the regions of the brain affected by early nutritional insults. We speculate that hypothalamus, where information about nutritional status is “read”, and which plays a key role in governing reproduction, could be one such region. In support of this, recent studies using an intrauterine growth restriction rat model (maternal low protein restriction) found impaired insulin signaling in the hypothalamus in 20 days old pups [67]. Specifically, tyrosine phosphorylation levels of IRS2 and PIK3 p85α were impaired, changes which could potentially block insulin signal transduction. However, it is not known if these hypothalamic changes in insulin signaling pathway components persist into puberty and adulthood, or whether they play a causal role in affecting later metabolic or reproductive function.

Of particular interest for the current review are also findings suggesting that the adverse environmental factors are related to intrauterine growth retardation (IUGR) and low birth weight may predispose individuals to the later onset of development of metabolic syndrome, and that those individuals may

also have reproductive system abnormalities. Based on those findings, a hypothesis termed the developmental origins of health and disease (DOHaD) has been developed [68], which states that an adverse perinatal environment programs or imprints the development of several tissues. In agreement with this concept, perinatal perturbations of the fetus/neonate nutrient supply might be a crucial determinant of individual programming of body weight set-point. The best-known example of the influence of negative metabolic status is the Dutch Famine Study, in which fetuses exposed to famine during early pregnancy had a higher energy intake and adiposity in adulthood [69, 70]. Importantly, prenatal growth restraint, followed by postnatal catch-up growth has been associated with relative hyperinsulinism, increased visceral fat, FSH hypersecretion, development of exaggerated adrenarche with reduced uterine and ovarian size, reduced ovulation rate in adolescent girls and early post-menarche (for review see [71, 72]) as well as an advanced tempo of pubertal development and menarche [72]. Moreover, during the post-menarcheal period, girls born with low body weight have increased risk of developing PCOS, a disorder of androgen excess (in particular elevated free testosterone levels) as well as ovarian and metabolic dysfunctions [73-75]. Furthermore, women with PCOS demonstrated higher risk of developing of gestational diabetes mellitus (GDM) [76] and approximately 40% of PCOS women are insulin resistant [77]. Although PCOS manifests clinically during adolescence, the disease may originate in intrauterine life [78]. Importantly, experiments in sheep that have been prenatally exposed to excess testosterone lead to adult metabolic and reproductive deficits similar to those seen in women with PCOS [49, 50].

Thus, both epidemiological studies and animal models indicate that nutritional status during gestation has long-term effects on central and peripheral systems that regulate energy balance and reproduction in the developing offspring. Moreover, perinatal nutrition impacts susceptibility to developing metabolic disorders and plays a role in programming body weight set points (for an review see [79]). Those observations led to the hypothesis of metabolic imprinting, according to which a stimulus or insult occurring during a critical period of development has a long-term effect on the physiologic and metabolic responses of the offspring (for review see [80]). However, the role of altered neural organization in effects of prenatal programming by nutrients has not been studied in the same degree of detail as the role of peripheral organ function. Insulin, which is increased in offspring of fat-fed dams [81], and insulin-like growth factors, are thought to be pivotal to neuronal differentiation, as well as synapse formation and consolidation, in the hypothalamus [82] which plays a crucial role in regulation of appetite and food intake [83, 84]. As insulin and leptin are two important hormonal signals, which are secreted into the bloodstream in proportion to the amount of adipose tissue, they are often studied in the animal models discussed above [85]. Those hormones are blood-borne and cross the brain-blood barrier to act upon the brain, including the arcuate nucleus of the hypothalamus (ARC). Within the ARC, neuropeptide Y (NPY), agouti-related peptide (AgRP) and

proopiomelanocortin (POMC) are synthesized and released [86-89]. NPY acts to stimulate food intake and reduce energy expenditure via interactions with receptors located in the paraventricular nucleus of the hypothalamus (PVN) and the lateral hypothalamus area (LHA). POMC neurons release alpha-melanocyte-stimulating hormone (α-MSH), which acts in the PVN and LHA on melanocortin receptors to decrease intake and increase energy expenditure. AgRP released from ARC NPY/AgRP neurons acts as a functional antagonist of α-MSH at melanocortin receptors [87, 90]. In healthy organisms, adipocyte stores are correlated with a rise in the levels of insulin and leptin. Insulin and leptin, in turn, inhibit NPY/AgRP and stimulate POMC neurons, providing a feedback influence which acts to inhibit food intake [89]. However offspring of diabetic pregnant rats displayed increased hypothalamic insulin levels, and both at weaning [91] and as adults [82] had increased number of NPY-positive neurons in ARC. Thus changes in hypothalamic appetite regulatory peptides may contribute to the development of obesity and metabolic disturbances in the offspring of diabetic female rats [91] although experimental manipulations are needed in this model to convincingly demonstrate this role.

In summary, current evidence suggests that insulin may play a role in the programming of both metabolism and reproductive systems during development, and these early alterations could lead to peripheral and central abnormalities in both systems during puberty and adulthood. A possible target for early insulin action is the hypothalamus, where information about metabolic status is conveyed to the reproductive functions. Thus, in case of prenatal programming by nutrients, where insulin functions are impaired, the disruptions of reproductive system are also observed. Importantly both under- and over-nutrition could lead to obesity and diabetes, diseases associated with insulin abnormalities, in which secondary abnormalities including disruptions of reproductive functions are present. Moreover, in support of long-term programming effects, studies have shown a perpetuation of type 2 diabetes into second-generation offspring in response to maternal under-nutrition [92-100].

4. Insulin: reproductive effects at the level of the brain

4.1 Is there local production of insulin in the CNS? Is there regulated transport across the blood-brain barrier?

The first studies indicating a possible role of insulin within the CNS were performed in the 1960-70s [101, 102]. For example, Havrankova et al. [103] found that insulin is present in whole brain extracts of rats, and its concentration on average was 25 times higher than seen in plasma, with the hypothalamus being identified as the brain region with the highest insulin levels. This finding was consistent with previous data [104] showing that insulin receptors are widely distributed in the CNS of

rats. These observations raised the question of the source of insulin found in the brain. It was proposed that pancreatic insulin present in the plasma and cerebrospinal fluid was taken up and stored by cells in the brain [103]. However, the possibility of extrapancreatic insulin production in the brain was suggested by immunocytochemical studies revealing the presence in the brain of C-peptide (connecting peptide), a metabolic product of insulin biosynthesis. Insulin-like immunoreactivity was shown to be present in the brain of human, rats, mice, frogs and tortoise [105]. Additionally, *post mortem* studies on human brain revealed that concentration of insulin and C-peptide is much higher compared to its blood levels. Moreover, the highest concentration of insulin and C-peptide was found to be present in the hypothalamus [106, 107]. Importantly, it was also shown that metabolic status influenced the presence of C-peptide-like immunoreactivity in the brain. Rats fasted for 72 h showed a decrease in the hypothalamic C-peptide-like immunoreactivity, which was reversed by glucose administration. In addition to the presence of C-peptide immunoreactivity, preproinsulin mRNA was also detected in the brain. Both *in situ* hybridization and immunocytochemisty showed the presence of preproinsulin mRNA and peptide in isolated enriched cultures of rabbit brain, restricted to neurons and absent in the glia [108]. Using *in situ* hybridization, the mRNA encoding preproinsulin was detected in the PVN but not in other regions of the rat brain [109]. However, these early findings have not been replicated, and whether local neuronal synthesis of insulin contributes to physiological actions of insulin remains to be demonstrated.

Transport of peripheral insulin across the brain-blood barrier (BBB) may also be a factor in its action in the brain. In studies of intravenous infusions of insulin performed in dog, it was found that insulin levels also increased in the cerebrospinal fluid (CSF; [110]). Additionally, it was revealed that the increase in concentration of insulin in the CSF was not proportional to its increase in plasma, leading to the suggestion that insulin passes into the CSF by the way of saturable transport system [110-113]. Of relevance to the current review, it was noted that the BBB shows regional differences in insulin permeability, with the hypothalamus being one of the brain regions with the highest permeability, where insulin is transported over twice as fast as into the whole brain [114]. Insulin transport was also shown to be regulated by physiological state (e.g. fasting) and altered in genetically obese Zucker rats [92, 115], who also show lower levels of insulin in their brain compared to lean Zucker rats [116]. However, in contrast to Zucker rats, animals with diabetes induced by injections of alloxan or streptozotocin had an increased saturable transport of insulin across the BBB [117]. This discrepancy between these two models of diabetes can in part be explained by differences in the levels of insulin in the blood. Whereas the Zucker rats are insulin resistant and have elevated levels of insulin in serum, animals with diabetes induced with alloxan and streptozotocin are insulinopenic [112]. It was also proposed that one of the mechanism by which stress, manifested as increased glucocorticoid levels,

enhances appetite and increases body weight could be related to the inhibition of insulin transport into the brain [118].

4.2. Where does insulin act in the brain to regulate reproduction?

Regardless of whether insulin is produced locally or not, there is strong evidence that many of insulin's action on the brain's reproductive system are mediated through direct actions on neuronal insulin receptors (IR). The most compelling evidence comes from the CNS-specific IR knockout mouse that exhibits hypogonadotropic hypogonadism [15]. Moreover, intracerebroventricular (i.c.v.) insulin administration has been shown to restore normal LH surges in STZ treated rats, despite the maintenance of peripheral diabetes-induced metabolic signals and metabolites (including hypoinsulinemia; [119]). IRs are widely distributed in the brain with highest concentrations in the olfactory bulb, hypothalamus, cerebral cortex, cerebellum and hippocampus [120]. Interestingly, an abundance of IRs are localized in areas that are well known to play a key role in reproduction such as the ARC, ventromedial hypothalamic nucleus (VMH), and preoptic area (POA; [15, 121-123]). These hypothalamic areas consist of potential sites for the action of insulin to control reproduction, and most recent attention has focused on specific identified subsets of neurons in these regions known to be involved in reproductive neuroendocrine control.

4.3. GnRH neurons

As the final common pathway in the control of mammalian reproductive neuroendocrine function, GnRH neurons were an obvious candidate as a target for insulin action. Based on cell line observations, insulin was originally thought to be acting directly on GnRH neurons via a functional IR [124, 125]. However,a recent study suggests otherwise. Deletion of IR from GnRH neurons had no effect on adult reproductive function in mice, as indicated by normal expression of estrous cyclicity [126]. Interestingly, in the sheep, even though there is an abundance of IRβ in the POA, GnRH neurons located there were devoid of immunoreactive IRβ [121]. These data taken together indicate that the influence of insulin on GnRH secretion is most likely not mediated directly, but instead via inputs other insulin-responsive neurons. One such afferent source that has been speculated to play this role is that of kisspeptin neurons of the POA and hypothalamus.

4.4. Kisspeptin neurons

The first known biological function of kisspeptin was in suppression of tumor metastasis, described in 1996 by Lee et al.[127]. Later, in 2003, two independent groups of scientists, discover, that mutations

of the kisspeptin receptor KISS1R (GPR54), lead hypogonadotropic hypogonadism in humans, and a failure to enter puberty [128, 129]. These findings not only revolutionized the field of reproduction but also provided a missing link in understanding the neural regulation of the GnRH system. Fruitful studies of many researchers revealed a crucial role for kisspeptin in regulation of GnRH secretion [130], including the control of both GnRH pulses and the GnRH surge. There are two major populations of kisspeptin neurons in the mammalian diencephalon: one located in the preoptic region and the other in the ARC. The rostral population in rodents is located in the rostral periventricular region of the third ventricle (RP3V) and has been strongly implicated in the functional control of the GnRH and LH surge [131]. The caudal (ARC) population of kisspeptin cells express two other neuropeptides important for reproduction, neurokinin B (NKB) and dynorphin [132] Because of its co-expression of the three distinct neuropeptides, these cells have been termed KNDy (Kisspeptin/NKB/Dynorphin) neurons [133], and they are thought to play a critical role in the generation of GnRH pulses [134, 135]. Because of their expression of nuclear steroid hormone receptors, both kisspeptin populations are believed to conveying the influence of gonadal steroids, such as estradiol and progesterone, onto GnRH neurons, and they are also believed to be important mediators for other types of signals that regulate GnRH neuronal activity. In this regard, much attention has been focused upon their potential role in transmitting metabolic cues to GnRH neurons [1]. As puberty and reproduction are closely connected with metabolism, recent studies focused on role of Kisspeptin and its receptor, KISS1R, in metabolic control of both puberty and fertility.

Sufficient body energy stores are indispensable for the reproductive axis to start functioning at puberty, and, not surprisingly, under-nutrition and the resulting state of negative energy balance is closely associated with a lack of or delay in puberty onset in animals [136, 137] and humans [138]. Castellano et al., [139] using RT-PCR of whole hypothalamic fragments from prepubertal male and female rats collected after 72h of fasting found a decrease in hypothalamic Kiss1 mRNA levels and an increase in Kiss1R mRNA expression levels. Administration of kisspeptin i.c.v. to immature, undernourished female rats was sufficient to restore vaginal opening (a marker of puberty) in about 60% of animals, and induce gonadotropin and estrogen secretion. Results of these studies suggest that negative energy balance caused by fasting induces a decrease in the kisspeptin expression, and that this decrease may in part be responsible for the pubertal deficit. Similar studies conducted by Roa et al. [140] in adult rats also showed that intracerebral infusion of kisspeptin-10 in animals subjected to chronic undernutrition increased ovarian weights and circulating LH levels. After 7 days of kisspeptin infusion, no differences were found between vehicle-treated, and kisspeptin-treated animals subjected to continued under-nutrition. These data indicate that chronic undernutrition in adult female rats markedly altered the ability of the chronic kisspeptin infusion to restore normal reproductive functions (e.g. normal pattern of gonadotropin response to continuous infusion of kisspeptin-10).

Despite the evidence supporting a role for kisspeptin in linking metabolism and reproduction, there is controversy as to whether insulin is the mediating signal. For example, uncontrolled long term diabetes in female rats is characterized by lowered LH secretion and decreased hypothalamic kiss1 mRNA[141]. Furthermore, the disturbance observed in the kisspeptin system appears to be causative to altered LH secretion as i.c.v kisspeptin administration reversed gonadotropin defects, despite prevailing metabolic perturbations [141]. However, insulin does not appear to be the upstream mediator of decreased kisspeptin, as insulin infusion in male rats was not able to reverse the diabetes induced kiss1 mRNA and LH decrease [142]. Similarly, 50% caloric restriction or lactational negative energy balance decreased kiss1 mRNA in POA kisspeptin cells, and both kisspeptin and NKB mRNA in ARC KNDy cells of the rat, and this decrease was not reversed by sc insulin injections [35, 143]. In addition, *in vitro* studies showed that insulin failed to stimulate kisspeptin expression in hypothalamic cell line N6 [144]. By contrast, hyperinsulinemia produced by a bolus injection of insulin in the late follicular phase dramatically increased c-fos expression in ARC kisspeptin cells of sheep [145], although this effect could reflect either direct or indirect actions. In addition, recent studies have shown a high percentage of IR colocalization in KNDy cells but not preoptic kisspeptin cells, nor in GnRH cells, in the sheep brain [121]. However, a recent study using transgenic techniques to specifically delete IR from kisspeptin cells produced mice that display a normal onset of puberty onset [146]. Thus, while studies to date suggest that kisspeptin and KNDy cells may be mediators of insulin action, they are by themselves likely not a critical component in insulin's influence on reproduction, at least with respect to puberty.

4.5. AgRP/POMC neurons

In addition to KNDy cells, two additional populations of ARC neurons have been strongly implicated as mediators of insulin action: cells which express AgRP and NPY, and a separate population that expresses POMC and cocaine amphetamine related transcript (CART; [49]). AgRP/NPY and POMC/CART neurons are well established as key regulators of glucose homeostasis, energy metabolism and body weight [147], and may also act as a link between metabolism and reproduction [148]. First, these cells contain IR in sheep [149] and rodents [150]. While deletion of IR alone in AgRP or POMC neurons is reported to produce no gross reproductive abnormalities [151], deletion of both IR and leptin receptors in POMC neurons produced mice with ovarian abnormalities and elevated serum testosterone levels that resemble the symptoms of PCOS [152]. Second, insulin directly regulates the electrophysiological properties of these neurons; POMC neurons are activated [153] and NPY/AgRP inhibited by insulin [154]. Third, recent evidence suggests that NPY/AgRP and POMC derived peptides such as alpha-melanocyte-stimulating hormone (α-MSH), are able to directly influence GnRH neuron excitability [155]. Fourth, there is anatomical evidence that projections from

NPY/AgRP and POMC/CART neurons directly contact GnRH cells in a number of species [156, 157]. Finally, there is preliminary evidence of local connections from AgRP and POMC neurons onto kisspeptin (KNDy) neurons in the ARC [158]. Thus, both AgRP/NPY and POMC/CART neurons appear to be well positioned to influence GnRH secretion directly as well as by indirect routes. The manner in which each of these ARC populations, together with KNDy/kisspeptin neurons and perhaps other neuronal populations, contribute to insulin's effects on GnRH neuroendocrine function will need to be fully elucidated by future work.

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5. Clinical relevance

Diabetes is usually lifelong (chronic) disease with two major types. Type 1 diabetes mellitus may result primarily from the pancreas' failure to produce enough insulin, while type 2 diabetes mellitus result from a condition of insulin resistance. Both conditions are of great concern, but 90% of all diabetes cases are type 2 diabetes mellitus, which affects more than 285 million people worldwide. Thus, understanding the role of insulin both acting peripherally as well as within the CNS and its dysfunction in conditions such as diabetes could lead to development of better clinical treatments and improvement of heath of millions of people worldwide.

In addition to primary metabolic deficits, diabetic patients show disruptions of reproductive function manifested as hypogonadism or infertility [159-161]. Most drugs available to treat diabetes mellitus act either in the pancreas by increasing insulin secretion, or in tissues such as the liver or muscle by improving insulin sensitivity. However, in view of recent studies discussed above suggesting that the brain also plays a critical role in the regulation of glucose homeostasis, this organ has also received attention as a promising new target of drugs aiming to treat both diabetes mellitus type 1 and type 2 [5]. However, although the clinical association between insulin deficiency/resistance and reproductive defects is well established, whether the underlying mechanisms include actions of insulin or insulin resistance at a neural level remains to be determined.

There is substantial evidence that hyperinsulinemia and insulin resistance when associated with obesity has a negative impact on human female fertility. For example, weight reduction in obese, infertile women is associated with an increase in the frequency of ovulation and the likelihood of pregnancy. Even among ovulatory women, increasing body mass index (BMI) is associated with decreasing spontaneous pregnancy rates, with the mechanism thought to be related to adverse effects of elevated insulin levels on ovarian function [162, 163]. In addition, there is a causal association

between maternal obesity and pregnancy complications, with the risk of pregnancy complications increasing with obesity.

Obesity has also a negative influence on the outcome of treatments for infertility (e.g. insufficient follicular development, lower oocyte counts, poorer outcomes from in vitro fertilization) [166-168]. Weight loss in obese subfertile women leads to favorable hormonal changes and an improvement in fertility. Metformin treatment of obese patients with infertility due to PCOS facilitates ovulation, supporting the idea that insulin resistance impairs normal oocyte development [169]. In this view, hyperinsulinemia stimulates ovarian androgen secretion directly and indirectly (by stimulating LH release or increasing ovarian LH receptors) [170, 171]. Extreme hyperinsulinemia (in hereditary cases caused by insulin receptor mutations or lipodystrophy) excessively stimulates the IGF-1 signal transduction pathway in ovarian theca cells, and results in increased androgen production by blocking the normal cellular down-regulation of response to LH [172, 173]. In general, all treatments that lower insulin levels, including weight loss or treatment with insulin sensitizers, improve female reproductive function and clinical pregnancy but there is still no evidence that metformin improves live birth rates. Therefore, the role of metformin in improving reproductive outcomes in women with PCOS appears to be limited [174, 175]. While there is clear evidence that ovary is a major target of insulin action in these interventions, the possibility also exists that some of the clinical improvements seen in these patients are due to normalizing insulin actions in the CNS [53, 59, 176].

There is much less evidence concerning impact that hyperinsulinemia has on male fertility, particularly at a CNS level. It is known that insulin acts at very early stages of testicular development as modulator of specific genes, e.g. Sry and Sox9, which are essential for male sex determination [177]. In addition to its early actions, insulin also plays a role in the postnatal testes, regulating germ cell production before and after puberty, affecting testes size and FSH production [178]. Interestingly, the testes is an extra-pancreatic source of insulin [179], and STZ-induced diabetes has been shown to diminish testicular insulin expression in the rat [180]. To investigate the role of the testicular insulin, a diabetic model of Akita mouse was created with nonfunctional insulin gene (*ins2)* in both testes and pancreas. Homozygous mice showed onset of diabetes prior to puberty and thereafter were infertile with small sized testes and arrested spermatogenesis. Exogenous insulin treatment improved testicular size and function, but because of the blood-testis barrier it was presumed that insulin in this study was exerting its effects indirectly. The authors suggested one possible site of action responsible for the restoration of testicular function was the hypothalamus; however, other sites of action were also possible [179].

While reports of genetic syndromes of severe insulin resistance have included prominent descriptions of ovarian dysfunction [181], changes in male reproductive function have rarely been reported. On the

other hand, obese men with insulin resistance frequently exhibit reduced levels of gonadotropins and testosterone, impaired semen parameters, altered androgen-to-estrogen ratios, and erectile problems [182]. However, again, whether any of these changes are due to the primary effects of changes in insulin signaling at a neural level are not known.

6. Conclusions

While there is ample evidence to support insulin as a key regulator of reproductive function, current knowledge of its neural actions with respect to reproduction is in many instances incomplete and rudimentary. Insulin is clearly an important regulator of pulsatile and surge GnRH/LH secretion, but whether these effects of due to insulin, per se, or whether changes in accompanying peripheral signals and metabolites may be involved, remains to be determined. Insulin appears to play a primarily permissive role in the control of pulsatile GnRH secretion, and those effects are due to different aspects (amplitude vs. timing) of the generation of the GnRH/LH surge responsible for ovulation.

There is much epidemiological and experimental evidence to suggest a role for insulin in fetal programming of the metabolic and reproductive axes, but it is not known whether these long-term effects are due to primary actions on the developing brain. Recent preliminary evidence in the sheep suggests that there may be a convergence of insulin and gonadal hormones early in development responsible for programming of reproductive neuroendocrine circuitry. Specifically, co-treatment of insulin sensitizer blocked the effect of prenatal testosterone on arcuate AgRP cell number in female sheep hypothalamus suggesting that a common mediator involving both insulin and androgen signaling is responsible for the prenatal programming of this hypothalamic circuitry. However, again, whether these effects are due to primary actions of insulin on the developing brain, or due to effects on maternal or placental function, remains to be explored.

At a neural level, the specific brain targets of insulin have been examined which may be involved in relaying its influence in reproduction: these include GnRH neurons, the final common pathway mediating control of the hypothalamic-pituitary gonadal axis, as well as upstream neurons, such as those containing the neuropeptides, kisspeptin, AgRP, and POMC. While data is mixed as to whether insulin receptors are present in GnRH neurons, there is clear evidence of their presence in the other cell types. While kisspeptin neurons may not by themselves be critical components in insulin's effects on reproduction [146], insulin receptors in POMC neurons may be more important since deletion of IR leads to adult female reproductive deficits [152], and the contribution of AgRP neurons to this influence have yet to be specifically investigated. Multiple anatomical interconnections among these neuronal subpopulations, however, suggest that they may comprise a redundant network that mediates insulin's reproductive actions upon GnRH neuronal activity and neuroendocrine output.

Finally, clinical evidence clearly implicates insulin deficiency/resistance in adult human female fertility, but whether these effects are due to primary actions upon reproductive neuroendocrine circuitry, or are exerted at the level of the pituitary or gonads, is not known. Further, while there is growing consensus of the importance of insulin signaling in the central control of reproduction, well-defined experimental models are needed both in the adult and development nervous system to determine insulin's mechanisms of action independent of associated changes in metabolic signals. The ability to selectively manipulate components of insulin signaling in a cell-specific manner within defined neuronal subpopulations by transgenic approaches [138] presents such an opportunity, but will also need to coupled with careful and detailed physiological models of adult reproductive function in order to ensure the effective clinical translation of this knowledge in the future.

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