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1 **Insulin: its Role in the Central Control of Reproduction**

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32 **Abstract**

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

43

44 **Highlights**

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

50

51 **Key words**

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

53

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

55

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

68

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

80

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

82

83 *2.1. Insulin: its major role in controlling glucose homeostasis*

84

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

97

98 *2.2. Evidence for a role for insulin in the control of the HPG axis*

99

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

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

114

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

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

142

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

145 In addition to GnRH/LH pulses, the GnRH/LH surge is also sensitive to metabolic cues. Models of
146 negative energy balance induced by fasting, caloric restriction and lactation are accompanied by a
147 decrease in circulating insulin and disruption of estrous cyclicity in a number of species such as rats
148 [26, 27], ewes [28], heifers [29] and monkeys [30]. Specifically, in adult female rats, short-term food
149 deprivation blocks the LH surge [31, 32]. Short-term fasting during the luteal phase of the estrous
150 cycle in sheep, increased serum concentrations of progesterone and delayed or diminished the pre-
151 ovulatory LH surge [33, 34]. Data on the role of insulin in these disruptions are lacking, however,
152 insulin replacement during lactational anestrus- a model of severe undernutrition did not restore
153 estrous cyclicity [35]. Therefore, it is likely that other metabolic signals, such as hypoglycemia, leptin,
154 or even the activation of the stress axis may be responsible for these disruptions [35, 36]. Experimental
155 diabetes induced in female rats with STZ [37], a state of extreme hypoinsulinemia, results in impaired
156 ovulation rates over an extended period of observation, disruption of the positive feedback effects of
157 estradiol, and absent or delayed LH surges [20, 38, 39]. However, in this model, reproductive
158 abnormalities are at least partially reversed after peripheral insulin administration [40]. These results
159 are similar to those described above, in that effects on pulsatile and surge secretion are not reversed by
160 insulin during negative energy balance but are at least partially reversed in diabetic models
161 (hypoinsulinemia vs. extreme hypoinsulinemia).

162

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

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

179
180 Similar conclusions can be drawn from experiments in sheep that have been prenatally exposed to
181 excess testosterone and exhibit metabolic and reproductive deficits similar to those seen in women
182 with polycystic ovarian syndrome (PCOS) [49, 50]. Prenatal testosterone treated female ewes display
183 hyperinsulinemia and insulin resistance [50], as well as defects in steroid feedback control of LH
184 secretion, including delayed and reduced amplitude LH surges [51]. Interestingly, in this model,
185 restoration of cumulative plasma insulin levels with an insulin sensitizer, rosiglitazone, was able to
186 restore the amplitude but not the timing of the LH surge [52]. Taken together, data from these two
187 sheep models (IIH and prenatal testosterone exposure) imply that hyperinsulinemia does not abolish
188 the LH surge but does reduce its amplitude. Whether this is a result of decreased GnRH release and/or
189 reduced pituitary responsiveness to GnRH remains to be determined, however, there is evidence that
190 both these sites are involved. For example, in women with PCOS pituitary response to GnRH is
191 suppressed under a euglycemic, hyperinsulinemic clamp [53] and this may account for the reduced
192 surge amplitude observed in the prenatal testosterone treated ewe model. Similarly, substantial
193 evidence suggests that insulin acts directly within the hypothalamus, and specifically via insulin
194 receptor (IR) containing cells to influence GnRH excitability (see section 4.2). The site(s) of action
195 (neural vs pituitary) of insulin in regulating both pulsatile and surge secretion of GnRH/LH may be
196 best addressed by future studies using the sheep model, where a specific advantage is the ability to
197 repeatedly measure GnRH in portal blood in awake animals [54].

198
199

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

201
202 There is a growing body of both epidemiological and experimental evidence indicating that
203 environmental factors can act early in the development to shape relationships between the regulation
204 of energy status and reproduction later in life. The concept that early environmental factors can
205 permanently organize or imprint physiological and behavioral systems is called fetal or early
206 programming [55-58]. This hypothesis originated from studies indicating that low birth weight is
207 associated with an increased biological risk for coronary heart disease in adult life [55]. Later studies
208 performed by Philips and collaborators [59-61] demonstrated a strong correlation between low birth
209 weight, high cortisol levels and development of hypertension and Type 2 diabetes. There is now a
210 good body of evidence, both from epidemiological data and experimental studies in animals, linking

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

213

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

227

228 Another explanation of the relationship between body weight at birth and adult metabolic syndrome
229 comes from the “Thrifty Phenotype Hypothesis”, according to which a fetus that endures poor
230 nutrition during gestation, would spare the growth of vital organs, e.g. the brain, at the expense of
231 tissues such as the muscle and the endocrine pancreas [66]. Thus, the fetus would adapt its metabolism
232 to conditions of limited nutrition with permanent changes in insulin and glucose metabolism,
233 increasing the risk of adult Type 2 diabetes and the metabolic syndrome [66]. In the light of evidence
234 discussed above it would be of particular interest to identify the regions of the brain affected by early
235 nutritional insults. We speculate that hypothalamus, where information about nutritional status is
236 “read”, and which plays a key role in governing reproduction, could be one such region. In support of
237 this, recent studies using an intrauterine growth restriction rat model (maternal low protein restriction)
238 found impaired insulin signaling in the hypothalamus in 20 days old pups [67]. Specifically, tyrosine
239 phosphorylation levels of IRS2 and PIK3 p85 α were impaired, changes which could potentially block
240 insulin signal transduction. However, it is not known if these hypothalamic changes in insulin
241 signaling pathway components persist into puberty and adulthood, or whether they play a causal role
242 in affecting later metabolic or reproductive function.

243

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

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

266

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

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

297

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

308

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

310

311 *4.1 Is there local production of insulin in the CNS? Is there regulated transport across the blood-brain*
312 *barrier?*

313

314 The first studies indicating a possible role of insulin within the CNS were performed in the 1960-70s
315 [101, 102]. For example, Havrankova et al. [103] found that insulin is present in whole brain extracts
316 of rats, and its concentration on average was 25 times higher than seen in plasma, with the
317 hypothalamus being identified as the brain region with the highest insulin levels. This finding was
318 consistent with previous data [104] showing that insulin receptors are widely distributed in the CNS of

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

337

338 Transport of peripheral insulin across the brain-blood barrier (BBB) may also be a factor in its action
339 in the brain. In studies of intravenous infusions of insulin performed in dog, it was found that insulin
340 levels also increased in the cerebrospinal fluid (CSF; [110]). Additionally, it was revealed that the
341 increase in concentration of insulin in the CSF was not proportional to its increase in plasma, leading
342 to the suggestion that insulin passes into the CSF by the way of saturable transport system [110-113].
343 Of relevance to the current review, it was noted that the BBB shows regional differences in insulin
344 permeability, with the hypothalamus being one of the brain regions with the highest permeability,
345 where insulin is transported over twice as fast as into the whole brain [114]. Insulin transport was also
346 shown to be regulated by physiological state (e.g. fasting) and altered in genetically obese Zucker rats
347 [92, 115], who also show lower levels of insulin in their brain compared to lean Zucker rats [116].
348 However, in contrast to Zucker rats, animals with diabetes induced by injections of alloxan or
349 streptozotocin had an increased saturable transport of insulin across the BBB [117]. This discrepancy
350 between these two models of diabetes can in part be explained by differences in the levels of insulin in
351 the blood. Whereas the Zucker rats are insulin resistant and have elevated levels of insulin in serum,
352 animals with diabetes induced with alloxan and streptozotocin are insulinopenic [112]. It was also
353 proposed that one of the mechanism by which stress, manifested as increased glucocorticoid levels,

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

356

357 *4.2. Where does insulin act in the brain to regulate reproduction?*

358

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

372

373 *4.3. GnRH neurons*

374

375 As the final common pathway in the control of mammalian reproductive neuroendocrine function,
376 GnRH neurons were an obvious candidate as a target for insulin action. Based on cell line
377 observations, insulin was originally thought to be acting directly on GnRH neurons via a functional IR
378 [124, 125]. However, a recent study suggests otherwise. Deletion of IR from GnRH neurons had no
379 effect on adult reproductive function in mice, as indicated by normal expression of estrous cyclicity
380 [126]. Interestingly, in the sheep, even though there is an abundance of IR β in the POA, GnRH
381 neurons located there were devoid of immunoreactive IR β [121]. These data taken together indicate
382 that the influence of insulin on GnRH secretion is most likely not mediated directly, but instead via
383 inputs other insulin-responsive neurons. One such afferent source that has been speculated to play this
384 role is that of kisspeptin neurons of the POA and hypothalamus.

385

386 *4.4. Kisspeptin neurons*

387

388 The first known biological function of kisspeptin was in suppression of tumor metastasis, described in
389 1996 by Lee et al.[127]. Later, in 2003, two independent groups of scientists, discover, that mutations

390 of the kisspeptin receptor KISS1R (GPR54), lead hypogonadotropic hypogonadism in humans, and a
391 failure to enter puberty [128, 129]. These findings not only revolutionized the field of reproduction but
392 also provided a missing link in understanding the neural regulation of the GnRH system. Fruitful
393 studies of many researchers revealed a crucial role for kisspeptin in regulation of GnRH secretion
394 [130], including the control of both GnRH pulses and the GnRH surge. There are two major
395 populations of kisspeptin neurons in the mammalian diencephalon: one located in the preoptic region
396 and the other in the ARC. The rostral population in rodents is located in the rostral periventricular
397 region of the third ventricle (RP3V) and has been strongly implicated in the functional control of the
398 GnRH and LH surge [131]. The caudal (ARC) population of kisspeptin cells express two other
399 neuropeptides important for reproduction, neurokinin B (NKB) and dynorphin [132] Because of its co-
400 expression of the three distinct neuropeptides, these cells have been termed KNDy
401 (Kisspeptin/NKB/Dynorphin) neurons [133], and they are thought to play a critical role in the
402 generation of GnRH pulses [134, 135]. Because of their expression of nuclear steroid hormone
403 receptors, both kisspeptin populations are believed to conveying the influence of gonadal steroids,
404 such as estradiol and progesterone, onto GnRH neurons, and they are also believed to be important
405 mediators for other types of signals that regulate GnRH neuronal activity. In this regard, much
406 attention has been focused upon their potential role in transmitting metabolic cues to GnRH neurons
407 [1]. As puberty and reproduction are closely connected with metabolism, recent studies focused on
408 role of Kisspeptin and its receptor, KISS1R, in metabolic control of both puberty and fertility.

409
410 Sufficient body energy stores are indispensable for the reproductive axis to start functioning at
411 puberty, and, not surprisingly, under-nutrition and the resulting state of negative energy balance is
412 closely associated with a lack of or delay in puberty onset in animals [136, 137] and humans [138].
413 Castellano et al., [139] using RT-PCR of whole hypothalamic fragments from prepubertal male and
414 female rats collected after 72h of fasting found a decrease in hypothalamic Kiss1 mRNA levels and an
415 increase in Kiss1R mRNA expression levels. Administration of kisspeptin i.c.v. to immature,
416 undernourished female rats was sufficient to restore vaginal opening (a marker of puberty) in about
417 60% of animals, and induce gonadotropin and estrogen secretion. Results of these studies suggest that
418 negative energy balance caused by fasting induces a decrease in the kisspeptin expression, and that
419 this decrease may in part be responsible for the pubertal deficit. Similar studies conducted by Roa et
420 al. [140] in adult rats also showed that intracerebral infusion of kisspeptin-10 in animals subjected to
421 chronic undernutrition increased ovarian weights and circulating LH levels. After 7 days of kisspeptin
422 infusion, no differences were found between vehicle-treated, and kisspeptin-treated animals subjected
423 to continued under-nutrition. These data indicate that chronic undernutrition in adult female rats
424 markedly altered the ability of the chronic kisspeptin infusion to restore normal reproductive functions
425 (e.g. normal pattern of gonadotropin response to continuous infusion of kisspeptin-10).

426 Despite the evidence supporting a role for kisspeptin in linking metabolism and reproduction, there is
427 controversy as to whether insulin is the mediating signal. For example, uncontrolled long term
428 diabetes in female rats is characterized by lowered LH secretion and decreased hypothalamic kiss1
429 mRNA[141]. Furthermore, the disturbance observed in the kisspeptin system appears to be causative
430 to altered LH secretion as i.c.v kisspeptin administration reversed gonadotropin defects, despite
431 prevailing metabolic perturbations [141]. However, insulin does not appear to be the upstream
432 mediator of decreased kisspeptin, as insulin infusion in male rats was not able to reverse the diabetes
433 induced kiss1 mRNA and LH decrease [142]. Similarly, 50% caloric restriction or lactational negative
434 energy balance decreased kiss1 mRNA in POA kisspeptin cells, and both kisspeptin and NKB mRNA
435 in ARC KNDy cells of the rat, and this decrease was not reversed by sc insulin injections [35, 143]. In
436 addition, *in vitro* studies showed that insulin failed to stimulate kisspeptin expression in hypothalamic
437 cell line N6 [144]. By contrast, hyperinsulinemia produced by a bolus injection of insulin in the late
438 follicular phase dramatically increased c-fos expression in ARC kisspeptin cells of sheep [145],
439 although this effect could reflect either direct or indirect actions. In addition, recent studies have
440 shown a high percentage of IR colocalization in KNDy cells but not preoptic kisspeptin cells, nor in
441 GnRH cells, in the sheep brain [121]. However, a recent study using transgenic techniques to
442 specifically delete IR from kisspeptin cells produced mice that display a normal onset of puberty onset
443 [146]. Thus, while studies to date suggest that kisspeptin and KNDy cells may be mediators of insulin
444 action, they are by themselves likely not a critical component in insulin's influence on reproduction, at
445 least with respect to puberty.

446

447 4.5. *AgRP/POMC neurons*

448

449 In addition to KNDy cells, two additional populations of ARC neurons have been strongly implicated
450 as mediators of insulin action: cells which express AgRP and NPY, and a separate population that
451 expresses POMC and cocaine amphetamine related transcript (CART; [49]). AgRP/NPY and
452 POMC/CART neurons are well established as key regulators of glucose homeostasis, energy
453 metabolism and body weight [147], and may also act as a link between metabolism and reproduction
454 [148]. First, these cells contain IR in sheep [149] and rodents [150]. While deletion of IR alone in
455 AgRP or POMC neurons is reported to produce no gross reproductive abnormalities [151], deletion of
456 both IR and leptin receptors in POMC neurons produced mice with ovarian abnormalities and elevated
457 serum testosterone levels that resemble the symptoms of PCOS [152]. Second, insulin directly
458 regulates the electrophysiological properties of these neurons; POMC neurons are activated [153] and
459 NPY/AgRP inhibited by insulin [154]. Third, recent evidence suggests that NPY/AgRP and POMC
460 derived peptides such as alpha-melanocyte-stimulating hormone (α -MSH), are able to directly
461 influence GnRH neuron excitability [155]. Fourth, there is anatomical evidence that projections from

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

470

471 **5. Clinical relevance**

472

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

480

481 In addition to primary metabolic deficits, diabetic patients show disruptions of reproductive function
482 manifested as hypogonadism or infertility [159-161]. Most drugs available to treat diabetes mellitus
483 act either in the pancreas by increasing insulin secretion, or in tissues such as the liver or muscle by
484 improving insulin sensitivity. However, in view of recent studies discussed above suggesting that the
485 brain also plays a critical role in the regulation of glucose homeostasis, this organ has also received
486 attention as a promising new target of drugs aiming to treat both diabetes mellitus type 1 and type 2
487 [5]. However, although the clinical association between insulin deficiency/resistance and reproductive
488 defects is well established, whether the underlying mechanisms include actions of insulin or insulin
489 resistance at a neural level remains to be determined.

490

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

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

499

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

516

517 There is much less evidence concerning impact that hyperinsulinemia has on male fertility,
518 particularly at a CNS level. It is known that insulin acts at very early stages of testicular development
519 as modulator of specific genes, e.g. Sry and Sox9, which are essential for male sex determination
520 [177]. In addition to its early actions, insulin also plays a role in the postnatal testes, regulating germ
521 cell production before and after puberty, affecting testes size and FSH production [178]. Interestingly,
522 the testes is an extra-pancreatic source of insulin [179], and STZ-induced diabetes has been shown to
523 diminish testicular insulin expression in the rat [180]. To investigate the role of the testicular insulin,
524 a diabetic model of Akita mouse was created with nonfunctional insulin gene (*ins2*) in both testes and
525 pancreas. Homozygous mice showed onset of diabetes prior to puberty and thereafter were infertile
526 with small sized testes and arrested spermatogenesis. Exogenous insulin treatment improved testicular
527 size and function, but because of the blood-testis barrier it was presumed that insulin in this study was
528 exerting its effects indirectly. The authors suggested one possible site of action responsible for the
529 restoration of testicular function was the hypothalamus; however, other sites of action were also
530 possible [179].

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

533 other hand, obese men with insulin resistance frequently exhibit reduced levels of gonadotropins and
534 testosterone, impaired semen parameters, altered androgen-to-estrogen ratios, and erectile problems
535 [182]. However, again, whether any of these changes are due to the primary effects of changes in
536 insulin signaling at a neural level are not known.

537 **6. Conclusions**

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

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

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

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

576

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