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REVIEW

GnRH NEURON BIOLOGY AND CONGENITAL HYPOGONDOTROPIC HYPOGONADISM

Hypogonadotropic hypogonadism and metabolic syndrome: insights from the high-fat diet experimental rabbit animal model

Annamaria MORELLI [1] *, Linda VIGNOZZI [2], Mario MAGGI [2]

[1]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy; [2]Department of Experimental, Clinical and Biomedical Sciences "Mario Serio", University of Florence, Florence, Italy

*Corresponding author: Annamaria Morelli, Anatomy and Histology Section, Department of Experimental and Clinical Medicine, University of Florence, Largo Brambilla 3, 50139 Florence, Italy.

ABSTRACT

The etiology of metabolic syndrome (MetS) is complex and involves the interplay between environmental, lifestyle and genetic determinants. MetS in men can be associated with a biochemical pattern of partial hypogonadotropic hypogonadism (HH). A similar pattern has been noted in both men and women with a variety of acute illnesses and chronic diseases, and there is ongoing debate regarding whether this phenomenon might adaptive (e.g. diverting resources from reproduction into survival), or maladaptive (e.g. anemia, sarcopenia, osteopenia and fatigue of androgen-deficiency amplify and widen the adverse consequences of the original disease-trigger). In women with hypothalamic amenorrhea (HA-HH secondary to chronic bioenergetic deficit from dietary restriction and/or intensive exercise), a genetic link to congenital HH (CHH) was recently established; women carrying monoallelic CHH gene mutations will typically not develop CHH, but are significantly more susceptible to HA. However, the male reproductive axis seems to be more resistant to similar environmental insults. In contrast, MetS-associated HH (mHH) is specifically a male phenomenon; the reproductive phenotype of females with MetS tending instead towards hyperandrogenism, rather than hypogonadism. The underlying pathogenic mechanisms responsible for mHH have not been clearly identified and, as yet, there has been no investigation of a potential role for CHH mutation carriage in its etiology. Over the decades, the use of either genetic- or diet-induced obesity and/or MetS animal models has greatly helped to illuminate the complex etiology of metabolic dysregulation, but the strong relationship between obesity/MetS and mHH in males has been largely neglected, with little or no information about the regulation of reproductive function by metabolic factors under conditions of bioenergetic excess.
However, the pathogenic link between MetS and HH in males has been recently investigated in an animal model of high fat diet (HFD)-induced MetS, which perfectly recapitulates the human phenotype. Interesting insights derived by these studies have added novel information about the causative role played by hypothalamic alterations driven by metabolic disturbances in mHH. In particular, it appears that HFD-induced inflammatory injury at the hypothalamic level negatively affects GnRH neuron content, with the reduction of circulating gonadotropins and sex hormones being related to MetS severity.

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The pathogenic mechanisms responsible for the close association between metabolic syndrome (MetS) and a form of partial hypogonadotropic hypogonadism (HH) in the male population are complex and not fully elucidated. MetS is a combination of different metabolic and cardiovascular alterations, whose aetiology is multifactorial and, as in most diseases, comprises both genotype and lifestyle factors. In this context, hormonal disturbances

(low testosterone, and/or increased estrogen/testosterone ratio) may be a consequence of metabolic dysregulation, since lifestyle modifications that lead to weight loss improve both cardiovascular profile and sexual function, with increasing serum testosterone (T) levels.[1] However, the converse may also apply, with MetS being a consequence of T deficiency and hypogonadism *per se* potentially representing a novel risk factor for MetS and cardiovascular disease.[2, 3] Potential candidate molecules linking HH and MetS include estrogens (E), insulin, leptin and inflammatory mediators such as tumor necrosis factor alpha (TNFα).[4-8]

Animal models can be of great help in unravelling the complex mechanisms characterizing the pathogenesis of human multifactorial disorders, but a key requirement is that they be matched as closely as possible with the corresponding human phenotype. Concerning MetS and comorbidities, the extent to which experimental targeted activations or deficiencies may strictly recapitulate the heterogeneous conditions of humans is not completely known and therefore difficult to assess in animal models. The majority of laboratory-based studies investigating pathogenic mechanisms of MetS have been performed using eugonadal animal models with either genetic- or diet-induced obesity [9] and/or MetS.[10] However, the strong relationship between obesity/MetS and male HH has been largely neglected, with little or no information about the regulation of reproductive function by the metabolic disturbance of bioenergetic excess. Moreover, although a recent study demonstrated that diet-induced obesity in male rats suppressed the reproductive hormone axis after 9 months of feeding, it did not clearly characterize the cardiometabolic profile of the model.[11] With the main aim of studying the joint contribution exerted by testosterone deficiency and MetS on cardiovascular system, a novel rodent model has been recently developed using adult orchiectomized rats fed a high fat diet (HFD).[12] However, although this manipulation generated MetS animals with primary hypogonadism, the model cannot be regarded as a proper surrogate of human phenotype, where central (or secondary) defects of testicular function are predominantly involved.

Multiple factors related to metabolic dysregulation may perturb central neuroendocrine mechanisms of the reproductive axis.[13] This system is finely regulated by a population of hypothalamic GnRH neurons, located in the preoptic region of the hypothalamus. Through the release of coordinated GnRH pulses, these neurons stimulate pituitary gonadotropin (FSH, LH) secretion, thereby promoting gonadal maturation and function.[14] Hence, conditions that perturb GnRH neurosecretion can cause alterations in the reproductive/sex hormone profile. The kisspeptin/KISS1 receptor (KISS1R/GPR54) system plays a master role in the central control of GnRH neuron activity.[15, 16] Indeed, GnRH neurons express KISS1R, through which kisspeptin, encoded by KISS1 gene and released by discrete neurons in the hypothalamus, regulates the reproductive axis at puberty, as well as along the adult life span.[17] Besides other functions, kisspeptin (Kp) neurons convey, directly or indirectly, some of the regulatory actions of key metabolic signals, such as leptin, E and insulin, to GnRH neurons.[14, 18] The stress of overnutrition has a deleterious impact on metabolic and gonadotropic function, as well as on the kisspeptin/KISS1R system in HFD-fed male rats.[11]

Regulation of GnRH neuron network by peripheral signals

The hypothalamus consists of several subnuclei, of which arcuate nucleus (ARC) is a major site of leptin action. The ARC lies within the mediobasal hypothalamus (MBH), close to the third ventricle and above the median eminence (ME), an organ with an incomplete blood–brain barrier (BBB). The ARC lies partially outside the BBB and is, therefore, sensitive to hormones and nutrients from the circulation and the cerebrospinal fluid (Figure 1).

Profound fasting has been found to cause HH in previously healthy, normal-weight male individuals, which is completely reversible by administration of GnRH,[19] although (for males

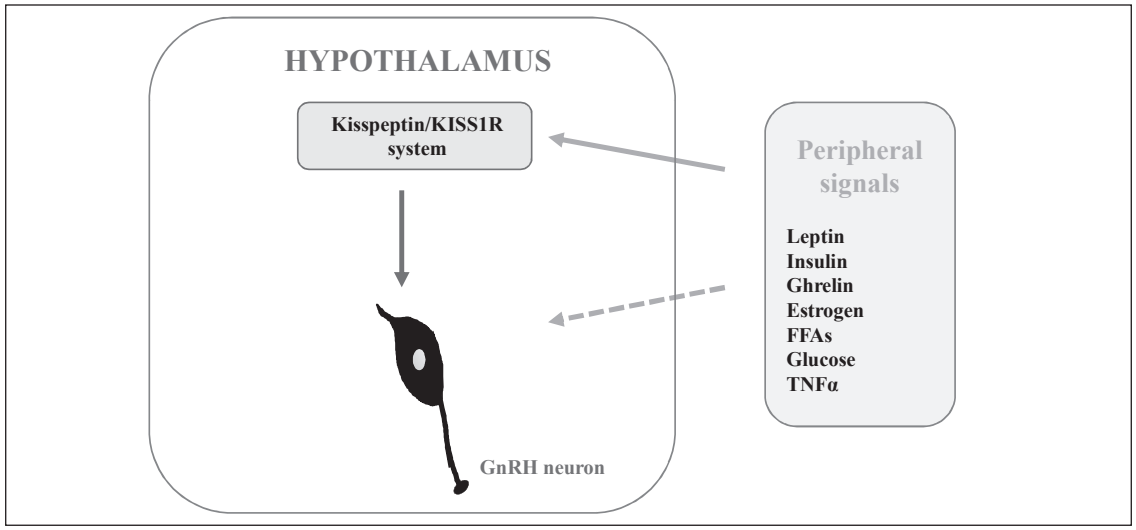

Figure 1.—Peripheral signals conveying metabolic information to the brain primarily target Kp/KISS1R system, which in turn regulate GnRH neuron activity at the onset of puberty as well as during the adult life span. Metabolic and hormonal milieu may also directly interfere with GnRH neuron function.

at least) it has not yet been established that reversal can be equally achieved with leptin, or Kp. During food restriction, increased ghrelin and reduced leptin secretion are suggested to be the underlying mechanisms of impaired GnRH secretion.[20] Ghrelin, a 28-amino acid peptide produced in gastric endocrine cells, stimulates food intake by transmitting hunger signals to the nucleus tractus solitaries (NTS) via the vagal afferent nerve. Although reports on male anorexia nervosa are scanty, three male patients with acute anorexia nervosa were longitudinally studied during weight gain, and changes of leptin over time were positively correlated with changes in LH, FSH and T level.[21] These data suggested that the male reproductive axis is sensitive to metabolic cues of bioenergetic deficit.

In the recent years, interdisciplinary research in neuroscience and immunology has linked overnutrition to the onset of inflammation within the brain, particularly within the hypothalamus. Several studies in animal models have consistently shown that hypothalamic inflammation can be induced by nutritional excess.[22] For instance, an acute central oversupply of glucose [23] or lipids [24, 25] was shown to induce hypothalamic inflammation. In rats and mice that are susceptible to diet induced obesity (DIO), consumption of a HFD rapidly induces neuron injury in a brain area critical for energy homeostasis and body weight control.[26] Indeed, inflammation within the hypothalamic ARC, a centre of feeding-regulation, develops 1-3 days after initiation of HFD feeding. HFD-induced activation of microglia results in production of a variety of proinflammatory cytokines, thereby provoking inflammatory responses that cause neuronal death within ARC.[26] Moreover, even very short-term HFD (one day of HFD feeding) resulted in inflammation within the colon, nodose ganglion and hypothalamus in mice. Interestingly, HFD-induced inflammatory responses were abolished by a single peripheral administration of ghrelin before the initiation of HFD feeding. Celiac vagotomy also suppressed inflammatory responses in the nodose ganglion and hypothalamus of HFD-fed mice. These findings suggest that the vagal afferent nerve may transfer gut-derived inflammatory signals to the hypothalamus via the nodose ganglion, and that ghrelin may protect against HFD-induced inflammation.[27] In particular, HFD induced central ghrelin resistance by reducing both ghrelin receptor expression in the hypothalamus and orexigenic neuropeptides, neuropeptide Y (NPY) and agouti-related peptide

(AgRP) neuronal responsiveness to ghrelin.[28] Activation of the innate immune system has also been proposed to mediate leptin resistance under conditions of overnutrition in DIO rodents. IκB kinase-β/nuclear factor-κB (IKKβ/NF-κB)- a key intracellular pro-inflammatory pathway of the innate immune system - is predominantly expressed in neurons of the MBH, but its signalling remains down-regulated under conditions of normal food intake.[23] In contrast, an increased IKKβ/NF-κB signalling has been described in the hypothalamus of HFD-fed rodents.[23] Interestingly, pharmacological inhibition of IKKβ/NF-κB improved leptin sensitivity in HFD-fed mice.[29]

During HFD feeding a complex inflammatory signalling process has been described within the ARC. Activation of toll-like receptor 4 (TLR4) on microglia and astrocytes initiated hypothalamic inflammation via the release of proinflammatory cytokines, which through IKKβ/NF-κB, up-regulated SOC3 expression, induce leptin resistance in AgRP neurons by providing negative feedback on leptin receptor. In addition, the secretion of pro-inflammatory cytokines may also act on microglia and astrocyte cytokine receptors to stimulate further release of inflammatory factors, in an autocrine/paracrine positive loop.[30]

However, all these studies about the effects of hypothalamic inflammation were mostly restrained to a few types of well-characterized neurons, such as anorexigenic pro-opiomelanocortin (POMC) neurons and orexigenic AgRP neurons in the arcuate nucleus and steroidogenic factor 1 (SDF-1) neurons in the ventromedial nucleus, which all act as metabolic sensing centers involved in the hypothalamic control of feeding, energy expenditure, and body weight.[31] On the contrary, the relationship between MetS-related hypothalamic inflammation and GnRH neuron injury has been poorly investigated.

One of the main problems with studying GnRH neurons is that only a modest number (700-1200 in mammals) of these neurons lie scattered within the adult hypothalamus as a diffuse network, rather than being concentrated in a discrete nucleus. With the advent of transgenesis and promoter–reporter protein expression, the study of their properties, development and function has been greatly enhanced through the visualization in animals of fluorescent GnRH neurons. Besides several lines of GnRH-green fluorescent protein (GFP) transgenic mice,[32-34] the transgenic GnRH3:EGFP zebrafish line, in which GnRH3 neurons express EGFP, has been demonstrated to be an optimal tool for investigating the GnRH system, not just in tissue slices but also *in vivo* whole-animal systems.[35] Similar to humans, overfed zebrafish, develop an obese phenotype, and, when exposed to inflammatory stimulus, exhibit a typical host defense reaction comparable to that occurring in mammals.[36] Moreover, further studies provided evidence of the ability to alter zebrafish growth and body composition through the quality of dietary lipid, supporting the application of this model to investigations of human health and disease related to fat metabolism.[37] However, the effect of over-nutrition on GnRH neuron function has not been investigated in zebrafish.

A possible link between obesity and reproductive dysfunction could be the enhanced mobilization of free fatty acids (FFAs) because of increased lipolysis. FFAs are known to bind and activate toll like receptor 2 and 4 (TLR2 and TLR4) signaling in many cell types, including LβT2 gonadotrope cells, reducing FSH secretion.[38] HFD-induced obesity also leads to central leptin resistance by decreasing the expression of both isoforms of the leptin receptor. In addition, mice with leptin deficiency exhibit markedly reduced Kp gene expression, particularly in the ARC, and reduced Kp-immunoreactive cell numbers in the rostral periventricular region of the third ventricle.

Another class of proteins that have been shown to mediate metabolic and reproductive signals to the CNS includes insulin and IGF-1. Insulin receptors are expressed in the hypothalamus, at high levels in the ARC and the para- and periventricular nuclei, but also at lower levels in the ME. A seminal paper by Bruning *et al.* in 2000, demonstrated that a neuron specific insulin receptor knock out (NIRKO) mouse developed obesity with el-

evated insulin levels and sub-fertility resulting from impaired spermatogenesis and follicular maturation.[39] Sub-fertility in these mice was characterized by impaired GnRH release and a consequent reduction in LH and FSH secretion. This study implicates signaling through brain insulin receptors as a mechanism for the regulation of reproductive function.[39]

Murine GnRH neuronal cell lines (GN11 and GT1-7 lines) and the rat GNV3 lines all expressed the insulin receptor.[40-42] In these cell lines, insulin has been demonstrated to stimulate the ERK and PI3K intracellular pathways [40-42] and to induce GnRH secretion.[40] Taken together these data suggested that insulin regulates the expression and secretion of GnRH, acting at the level of the GnRH neuron. Interestingly, insulin has been shown to stimulate GnRH promoter activity, with an insulin responsive region of the mGnRH promoter laying between -2046 and -356bp upstream of the transcriptional start site.[41] Moreover, Jennifer Hill's group first demonstrated that Kp neurons also expressed the insulin receptor. A Kp-specific IR knock out mouse [18] was characterized by a significant delay in puberty in both males and females, but no overt metabolic or reproductive phenotype in adults. Although pituitary gonadotroph cells also express the insulin receptor, gonadotroph-specific insulin receptor KO mice (PitIRKO) exhibited normal pubertal progression, normal LH and FSH levels and, most importantly, normal fertility both in male and female.[43]

IGF-1 regulation of hypothalamic GnRH and Kp neurons has also been investigated. The IGF-1 KO mouse was reported to be infertile [44, 45] with the developmental defect thought to be at the gonadal level, but neither hypothalamic nor pituitary function were examined. IGF-1 has been demonstrated to stimulate GnRH release from GT1-7 cells [46] and it also stimulates GnRH promoter activity *in vitro*.[47] However, mice lacking the IGF-1R on GnRH neurons (GnRH-IGF-1RKO) showed delayed onset of puberty, but did not show impaired reproductive function as adults. These results underscore the diversity of signals that likely contribute to the pubertal process as compared to the reproductive function in adulthood.

In rats and mice, the ARC is also the site of major estrogen-induced KISS1 down-regulation. Accordingly, we previously demonstrated in human fetal GnRH secreting neurons (FNC-B4) cells that E treatment induces a robust reduction in the Kp expression, with down-regulation being induced by very low (subnanomolar) concentrations of 17-β estradiol.[48] These data suggested that hyperestrogenism, which often occurs in the course of obesity because of the high levels of aromatase in the accumulating visceral fat, could be an additional underlying mechanism of mHH, which indeed may result from an acquired defect of enhanced hypothalamic sensitivity to E-mediated negative feedback. Accordingly, treating obese men with the aromatase inhibitor letrozole increased gonadotropin and T circulating levels.[49, 50] Similarly, treatment with selective estrogen receptor modulators (SERMs) that antagonize E-mediated effects on the hypothalamic-pituitary-testis axis, resulted in complete normalization of pulsatile gonadotropin secretion, serum T and sexual function in men with adult-onset isolated HH,[51, 52] thus representing a promising therapeutic option for mHH.[53]

In addition to overnutrition, aging can also induce molecular inflammation within the hypothalamus. With detailed investigation, it was demonstrated that hypothalamic microglia induced an innate immune reaction during aging, via an IKKβ/NFκB-pathway.[54] Furthermore, aging-dependent IKKβ/NFκB activation and hypothalamic inflammation can strongly inhibit GnRH gene transcription, thus providing an explanation for the phenomenon of age-related GnRH neurosecretory decline.[54]

Interestingly, both overnutrition-induced and aging-mediated upregulation of the IKKβ/NF-κB signaling pathway have been found not only to promote hypothalamic neurodegeneration, but also a reduced neurogenesis.[55] Indeed, neurogenesis was significantly attenuated in the hypothalamus of adult mice maintained on prolonged high-fat diet (HFD) feeding, as a consequence of HFD-induced neuroinflammatory responses. It was reported that survival, differentiation and neurogenesis

of hypothalamic neuronal stem cells (NSCs) were mechanistically mediated by IKKβ/NF-κB-controlled apoptosis and Notch signaling, thus supporting the role of inflammation in the structural remodeling of the brain. HFD-induced obesity in mice reduced adult NSC population and new neuron turnover in the small population of POMC neurons that have important functions of controlling energy balance.[56]

There is consensus that multiple metabolic factors contributes to the timing of puberty in males, as well as in females, via effects at the level of the GnRH neuron and possibly the Kp neuron. However, many of the studies highlighted here make clear that the nutritional regulation of reproductive axis is multifactorial and require non-genetic animal models that can closely resemble the human phenotype of MetS-induced hypogonadism in the adulthood.

HFD-induced rabbit model of MetS

With the main purpose to clarify the intricate relationship that associates MetS with HH, we established an animal model of high fat diet-induced MetS, which has been largely characterized.[57-68] Adult male rabbits fed a high-fat diet for twelve weeks developed the human phenotype, including visceral obesity, hypertension, dyslipidemia and glucose intolerance. Moreover, as in humans, HFD rabbits exhibit overt HH, with low plasma levels of testosterone, LH and FSH and reduced androgen-dependent organ weight, seminal vesicles and prostate. To our knowledge this is a unique animal model wherein all features of human mHH are recapitulated and, moreover, obtained without genetic manipulation. In this experimental model, sex hormone imbalance was related to MetS severity, since T decreased and E increased as a function of the number of MetS components.[61, 67] Plasma gonadotropin levels were also negatively associated with MetS, thus suggesting the occurrence of a central defects at hypothalamic and/or pituitary level.

To evaluate the contribution of the different metabolic derangements on hormonal alterations, we designed an algorithm taking into account the presence, as a dummy variable, of one or more of the different MetS components: hyperglycemia, high triglycerides, high cholesterol, high blood pressure and visceral fat accumulation. Cut-offs for each factor were calculated based on values measured in rabbits fed a regular diet (RD). Among MetS factors, both hyperglycaemia and hypercholesterolemia resulted as being the major determinants for the negative association with LH levels, while the dyslipidaemic component (high cholesterol and triglycerides), appeared to be associated with FSH reduction.[67] In addition, severity of glucose intolerance also correlated with hormonal alterations.

Pituitary gonadotrophs are controlled by hypothalamic GnRH neurons and, indeed, a reduced content of GnRH was immunohistochemically demonstrated in the hypothalami of HFD rabbits,[57] thus supporting the hypothesis of pathogenic mechanisms at this level in the course of MetS. Hence, we investigated the role played by HFD-related hypothalamic alterations in determining the documented dysfunctions of the gonadotropic axis. A strict association of MetS — and in particular the related altered glucose and lipid metabolism — with peculiar hypothalamic alterations has been identified. Indeed, increased expression of the glucose transporter GLUT4 and inflammatory features characterised the hypothalamic tissue from HFD rabbits. In particular, HFD determined low-grade inflammation within the hypothalamus, significantly inducing microglial activation and IL-6 expression. Interestingly, all these hypothalamic derangements were, in turn, associated with LH and FSH reductions, and occurred in the preoptic area of the hypothalamus lining the third ventricle, where GnRH neurons reside. Accordingly, the same hypothalamic area showed a reduced immunopositivity not only for GnRH,[57] but also for KISS1R,[67] which along with its natural ligand Kp represent the main system mediating, at central level, the effects of a range of metabolic inputs known to regulate GnRH secretion (for reviews [14, 69]).

However, an incompletely clarified issue is whether metabolic alterations act directly

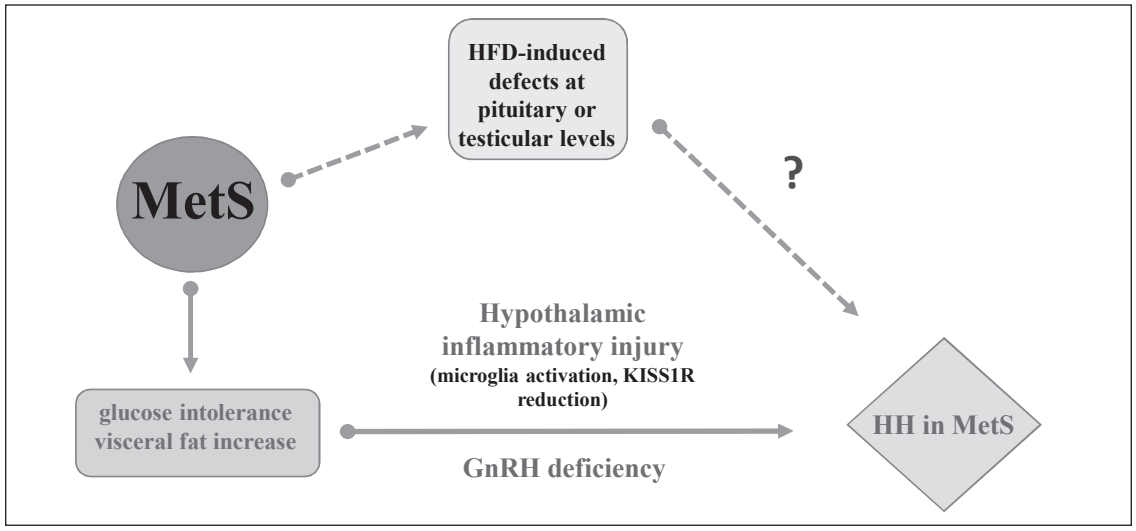

Figure 2.—Proposed pathogenic pathway linking MetS to HH, as derived by the HFD-induced animal model. Metabolic derangements, mainly guided by glucose intolerance, may promote pro-inflammatory mechanisms at hypothalamic level in the key area involved in the control of reproductive axis, thus compromising GnRH neuron activity and thereby leading to central HH. Additional defects at pituitary or testicular levels, not investigated, may contribute to the development of HH in MetS.

on GnRH neurons or are mediated by other integrating factors. Indeed, it has been demonstrated that a subpopulation of GnRH neurons projects dendrites in regions outside the blood brain barrier, where they may directly sense molecules circulating in the bloodstream.[70] Hence, the range of factors that are integrated by GnRH neurons for the control of the GnRH/gonadotropin secretion could be extended. Using a well characterized cellular model, we identified a direct inhibitory action of increasing glucose concentrations on human fetal GnRH-secreting neurons, the FNC-B4 cells,[48, 71-75] thus opening new mechanistic insights into the direct metabolic control of GnRH release.[76] FNC-B4 cells express glucose transporters (GLUT1, GLUT3 and GLUT4) and may respond to changes in glucose concentrations. Exposing FNC-B4 cells to high glucose significantly reduced the expression not only of GnRH but also of genes relevant for GnRH neuron function, such as KISS1R and leptin receptor. Although obtained *in vitro*, these findings support the idea of a deleterious direct contribution of hyperglycemia on human GnRH neurons, thus improving our understanding about the pathogenic mechanisms linking HH to metabolic disorders.

Conclusions

The mechanisms through which metabolic abnormalities may affect T secretion is still a matter of debate. However, what we learnt from animal models has greatly contributed to elucidate several aspects of the pathogenic link between MetS and HH in males. Overall, *in vivo* experimental studies indicate that metabolic derangements may have deleterious effects at the central level. In particular, proinflammatory pathways, activated under conditions of nutritional excess and occurring within the hypothalamus, may compromise a key brain area involved not only in the control of energy homeostasis but also in reproduction (Figure 2). In agreement with this possibility, *in vivo* treatment with obeticholic acid (OCA), a drug that ameliorates glucose metabolism in the rabbit MetS model, [58, 60, 62, 65] not only reverted all the HFD-induced hypothalamic alterations — including the inflammatory response, — but also increased GnRH mRNA expression.[67] However, since OCA treatment failed to restore gonadotropin and testosterone plasma levels, additional alterations at pituitary and/or testis level cannot be ruled out, as well as the direct effects of enhanced E for-

mation on hypothalamic district remain to be elucidated. The high complexity of pathogenic mechanisms linking HH to MetS requires further studies and more gains are expected from investigations in animal models.

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