

Neuroendocrine factors in the initiation of puberty: The emergent role of kisspeptin

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Published online: 6 March 2007
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Abstract Puberty is the end-point of a complex series of developmental events, defined by the dynamic interaction between genetic factors and environmental cues, ultimately leading to the attainment of reproductive capacity. The neuroendocrine basis of puberty has been the subject of extensive investigation in the last decades, and identification of the trigger(s) of puberty onset has drawn considerable attention. In this context, recognition of the fundamental role of kisspeptin (encoded by the *KiSS-1* gene) and its receptor *GPR54* as major gatekeepers of gonadotropic function in general, and puberty onset in particular, has been a major breakthrough in contemporary Neuroendocrinology. Indeed, during the last 3 years, the so-called *KiSS-1/GPR54* system has been substantiated as pivotal regulator of puberty in mammals; the lack of *GPR54* signaling being coupled to sexual immaturity (impuberism) in mice and humans. In this review, we will summarize the most salient experimental data (mostly obtained in laboratory animals) demonstrating the key roles of hypothalamic *KiSS-1* neurons in the activation of the reproductive axis at puberty, and its regulation by metabolic and, eventually, environmental factors. Whether the *KiSS-1* system is the trigger for puberty onset and/or it operates as integrator and effector of up-stream regulatory factors warrants further investigation.

Keywords Puberty · Kisspeptin · *KiSS-1* · *GPR54* · Gonadotropins · Gonadotropin-releasing hormone (GnRH) · Hypogonadism

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1 Introduction

Puberty is the culmination of a cascade of developmental events that lead to the attainment of reproductive capacity and the completion of phenotypic sexual differentiation. The complexity of this maturational process is illustrated by its multi-faceted nature, which in humans ranges from hormonal changes to significant psycho-social modifications [1]. Accordingly, multiple determinants of pubertal development have been identified, which include not only genetic factors but also endogenous signals (e.g. energy status) and environmental cues [2, 3], whose dynamic interplay is responsible for the timing of puberty onset and its eventual deviations (precocious, delayed or absent puberty). On the basis of its paramount importance, the physiology and pathophysiology of puberty have been the subject of thorough analyses during last decades, using multiple approaches and different species, including the human.

For the purpose of the present review, we will focus our attention on the neuro-hormonal mechanisms responsible for puberty onset and, specifically, on the contribution of the recently identified hypothalamic *KiSS-1* system to this phenomenon. From a general neuroendocrine perspective, puberty is defined by the full activation of the so-called hypothalamic-pituitary-gonadal (HPG) axis (or gonadotropic axis) [1, 3]. The function of this hormonal system is driven by the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH), which is episodically released into the hypophyseal portal blood system in order to activate the pulsatile secretion of both gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These, in turn, operate as major endocrine regulators of the gonads [4], which achieve complete maturation and function at the time of puberty. A vast majority of our knowledge on the signals and circuits controlling mammalian puberty has been generated in experimental species; the rat and mouse being

by far the most commonly used in neuroendocrine studies [3]. While this approach has been proven extraordinarily instrumental, it is to be noted that differences exist between humans and rodents in some aspects of prepubertal maturation, such as the infantile quiescent period of the HPG axis is fully evident in higher primates but not in rodents [5].

2 Neuroendocrinology of puberty: Brief overview

According to the hierarchical organization indicated above, it was predicted that pubertal activation of the gonadotropic axis is primarily induced by the heightening of the neurosecretory activity of the hypothalamic GnRH pulse generator; the GnRH neuron serving a central function as integrator and ultimate effector for the different modulators of puberty onset. Yet, although this contention has remained essentially invariant for decades, our understanding of the neurohormonal basis for pubertal activation of the HPG axis has undergone substantial conceptual modifications [3, 6], with emphasis being placed either in changes in the sensitivity to the feedback effects of sex steroids or modifications in the ratio between central inhibitory and stimulatory inputs to the GnRH pulse generator. On the latter, it is now globally accepted that the progressive activation of GnRH neurons along pubertal transition is brought about by the concomitant decrease in the restrain of inhibitory afferents and the increase in stimulatory inputs [3, 6]. However, the nature of these signals and their relative importance are yet to be fully elucidated.

Classical neuroendocrinological studies conducted in the 1980s and 1990s helped to identify some of the central players in the transsynaptic (inhibitory and stimulatory) control of the GnRH pulse generator at puberty, as extensively revised elsewhere [3]. These include prominent roles of γ -aminobutyric acid (GABA) and endogenous opioid peptides, as major inhibitory signals, as well as noradrenergic and glutamatergic neurotransmission, as relevant excitatory inputs [3, 6]. In addition, the involvement of other neurotransmitters and neuropeptides (e.g. neuropeptide Y) in the neuroendocrine control of puberty has been also proposed [3]. More recently, compelling data have settled the contention that the regulatory network governing GnRH secretion is not solely composed by transsynaptic inputs but also by glia-to-neuron signals, which include growth factors (such as transforming growth factor- α and neuregulins) and likely glutamatergic inputs [6–9]. Both transsynaptic and glia-to-neuron communication appears under the exquisite control of a plethora of transcription factors, whose expression is finely tuned along pubertal transition, defining an upper level of regulation, responsible for driving the secretion of subordinated cell-to-cell signals. Given the increasing complexity of the known mechanisms responsible for the precise activation of the GnRH pulse generator at

puberty, emphasis has been given recently to systems biology approaches, directed towards the identification of networks rather than single factors, in order to achieve proper understanding of the neuroendocrine basis of mammalian puberty [6]. Despite the usefulness of this holistic approach, characterization of the specific roles and physiologic relevance of the individual components of these networks remains absolutely essential.

The above formulation, with hierarchical networks of genes controlling the central activation of GnRH system [6], is fully compatible with the concept that puberty onset is mainly under genetic control. However, it is also well known that activation of the HPG axis at puberty, and its subsequent functioning in adulthood, is extremely sensitive to different endogenous and environmental cues, which interact with central regulators to precisely define the timing of puberty [2]. These include the state of energy reserves and the metabolic status of the organism [10]. This critical information is conveyed to the brain mainly through peripheral hormones originating from the adipose tissue, gastrointestinal tract and pancreas, as well as by nutritional and metabolic factors [10]. Concerning the hormonal regulators, a preponderant role has been assigned to the adipocyte-derived hormone, leptin, which signals the state of energy reserves to the centers governing not only food intake but also reproductive function; leptin being an essential permissive factor for pubertal development [11–13]. In addition, other hormones primarily involved in energy homeostasis have been recently proposed as potential modulators of puberty onset, such as the gut-derived hormone, ghrelin [14, 15].

In addition, different environmental factors are known to influence puberty onset. Among those, epidemiological studies (in humans) and experimental data (in rodents) have suggested the potential impact on the timing of puberty of the exposure, during certain stages of development, to naturally-occurring and synthetic compounds with estrogen-like activities (globally termed endocrine disruptors) [2, 16]. In addition, other environmental signals, such as different stressors, light-dark cycles and even climate conditions, have been suggested as modifiers of puberty onset [2]. Overall, puberty appears as the final end-point of the complex interplay of the individual genetic background and a large array of endogenous and exogenous regulators; puberty being now considered as a sensor of genetic and environmental interactions during development.

3 Kisspeptin and GPR54: From cancer biology to reproductive function

Despite the assumption that the essential elements responsible for the neuro-hormonal control of reproduction had been already identified, a major breakthrough in our

knowledge of this neuroendocrine system took place in late 2003, when the reproductive dimension of GPR54 (and its ligands, encoded by the KiSS-1 gene) was first unveiled [17, 18]. This review article will summarize the most relevant features of the KiSS-1/GPR54 system, with special emphasis on its essential role in puberty onset. Nonetheless, it is worthy to note that, although considered as the most important finding in reproductive physiology since the isolation of GnRH in early 1970s [19], identification of the elements of this ligand-receptor system preceded in several years the demonstration of its function as major regulator of puberty in rodents and humans.

Characterization of the elements of the KiSS-1/GPR54 system took place sequentially between 1996 and 2001. First, in 1996, KiSS-1 was identified as a metastasis-suppressor gene, using subtractive hybridization in melanoma cells with different metastatic capacity [20]. Independently, in 1999, GPR54 was cloned in the rat as an orphan receptor with a partial sequence similarity (>40%) with the transmembrane regions of galanin receptors [21]; the human ortholog being subsequently cloned, and termed AXOR12 or hOT7T175 [22, 23]. Finally, in 2001, the characterization of the peptide products of the KiSS-1 gene was completed. These were globally termed kisspeptins, as they derive from the differential proteolytic processing of a common precursor [24]. The major product of KiSS-1 gene appeared to be a 54 amino acid peptide, predominantly expressed in the placenta and termed metastin based on its ability to inhibit tumor metastasis [22]. In addition, other peptide fragments of the KiSS-1 precursor, such as kisspeptin-14, kisspeptin-13, and kisspeptin-10, were also identified [24]. From a structural stand-point, all kisspeptins share the C-terminal region of the metastin molecule, where they show a canonical Arg-Phe-NH₂ motif distinctive of the RF-amide peptide family. Upon their characterization, it was demonstrated that all kisspeptins are able to bind and activate GPR54, which was then catalogued as the cognate receptor of KiSS-1 peptides [22–24]. The major intracellular signaling systems recruited by GPR54 include activation of phospholipase C and PIP₂ hydrolysis, followed by Ca²⁺ mobilization and phosphorylation of ERK1/2 and p38 MAP kinases, as originally reported using heterologous cell systems [24], and recently confirmed in hypothalamic tissue [25].

The biological roles initially assigned to the KiSS-1/GPR54 system were confined to cancer biology. Thus, kisspeptins were shown to suppress the metastatic activity of several tumors, and loss of KiSS-1 gene expression was suggested as prognosis factor for tumor metastasis in certain types of cancer (see [26], and references therein). Yet, the actual importance of KiSS-1 in the restraint of tumor progression is still matter of debate. In addition, initial screening assays suggested that the KiSS-1 system could also conduct biological functions other than metastasis suppression, as evidenced by the wide-spread pattern

of expression of GPR54 and, to a lesser extent, KiSS-1 genes in diverse normal human and rodent tissues [22–24, 27]. Among those, prominent expression of GPR54 and KiSS-1 was demonstrated in human placenta; an observation which was followed by the analysis of the potential role of kisspeptin in the control of trophoblast invasion during placental formation [28]. In addition, the expression of GPR54 and/or KiSS-1 in different areas of the central nervous system, including hypothalamus, as well as the pituitary suggested their potential involvement in the control of diverse brain functions, including the regulation of neuroendocrine axes, such as that controlling oxytocin release [24]. Yet, the neuroendocrine dimension of kisspeptin remained virtually neglected until its role in the control of the HPG axis was first recognized.

The disclosure of the reproductive dimension of the KiSS-1/GPR54 system took place in late 2003, when two groups independently reported the presence of deletions and inactivating mutations of the GPR54 gene in patients with idiopathic hypogonadotropic hypogonadism [17, 18]. Interestingly, mouse models of genetic inactivation of GPR54 were simultaneously described; GPR54 knock-outs being a complete phenocopy of humans carrying inactivating mutations of GPR54 [18, 29]. These observations set the contention that GPR54 and its ligands, kisspeptins, must play a well-conserved, fundamental role in the control of reproductive function in mammals. Moreover, initial hormonal analyses of GPR54 null mice and humans suggested that the primary mechanism whereby inactivation of GPR54 signaling induces a state of central hypogonadism is related to defective hypothalamic GnRH secretion, likely due to the lack of an essential regulator up-stream the GnRH neuron [18]. This was in contrast to the previously known monogenic forms of hypogonadotropic hypogonadism, such as mutations of GnRH receptor or classical forms of Kallmann syndrome (inducing defective GnRH neuronal migration), and reinforced the physiologic relevance of the KiSS-1 system in the neuroendocrine control of the HPG axis.

4 Emergent roles of kisspeptin in the neuroendocrine control of reproduction

Identification of the potential role of KiSS-1 and GPR54 in the control of reproduction boosted an extraordinary interest among reproductive neuroendocrinologists, who aimed to define the effects, mechanisms of action, major regulatory elements and physiologic relevance of KiSS-1 system within the gonadotropic axis. These efforts have already resulted in a significant advancement of our knowledge of the reproductive facet of this novel system. Although this review will be specifically devoted to the analysis of the prominent role and mechanisms of action of

KiSS-1/GPR54 in the control of puberty, we consider it relevant to provide a brief overview of the whole spectrum of biological effects (on the HPG axis) that have been assigned to kisspeptin during the last 2 years, which include the control of some of the most fundamental aspects of gonadotropic function, such as negative feedback regulation, the generation of the pre-ovulatory surge of gonadotropins and the control of reproduction by metabolic and environmental cues. Detailed reviews on some of these aspects of kisspeptin physiology have been published recently elsewhere [as examples see 26, 30–32].

The initial experimental studies on the characterization of the reproductive roles of metastin and other kisspeptins already evidenced that KiSS-1 peptides are powerful elicitors of gonadotropin secretion in rodents [33–36]. These observations were later confirmed in other species, including the sheep, monkey and human [37–39]. Moreover, detailed analyses on the gonadotropin releasing ability of kisspeptin-10 in the rat evidenced that the sensitivity of the gonadotropic system to the stimulatory effect of kisspeptin is extraordinary high, as intracerebral administration of doses as low as 100 fmol–1 pmol evoked nearly maximal LH responses [40]. Likewise, FSH secretion was also significantly stimulated by kisspeptin; yet, some differences in terms of relative responses (lower for FSH), their time-course (faster for LH) and mean effective doses (EC50 values of ~4 and 400 pmol for LH and FSH, respectively) were noticed between gonadotropins [40, 41]. Overall, those data demonstrated that KiSS-1 peptides are likely the most potent elicitors of the GnRH-gonadotropin axis known to date. In line with this contention, it has been shown that systemic administration of kisspeptin, at doses as low as 0.1 µg to adult rats or 2 µg to juvenile monkeys, is sufficient to evoke clear-cut LH secretory peaks in freely-moving conditions [42, 43].

Further evidence for the physiologic relevance of KiSS-1 system in the control of gonadotropin secretion came from expression studies in models of gonadectomy. Elimination of gonadal factors in male and female rats induced not only the expected rise in circulating gonadotropin levels but also a significant increase in KiSS-1 mRNA levels at the hypothalamus [35, 44]. Both hormonal and gene expression responses were prevented by sex steroid replacement of gonadectomized animals [35]. Considering the potent stimulatory actions of kisspeptin demonstrated *in vivo*, those observations suggested that the hypothalamic KiSS-1 system is an important component of the central mechanisms whereby gonadal sex steroids carry out the negative feedback control of gonadotropins. However, detailed *in situ* hybridization analyses of changes in hypothalamic KiSS-1 expression following gonadectomy and sex steroid replacement evidenced that while negative regulation of KiSS-1 mRNA levels by sex steroids is detected at the

arcuate nucleus, at the anteroventral periventricular nucleus (AVPV) of the hypothalamus estrogen actually increases KiSS-1 gene expression [45, 46]. This observation, in face of the previously proposed role of the AVPV as major hypothalamic center for conveying the positive feedback effects of estradiol of LH secretion, raised the intriguing possibility that the KiSS-1 system may also play an important role in the generation of the pre-ovulatory surge of gonadotropins [45]. This contention has been recently substantiated by experimental studies showing that KiSS-1 mRNA levels significantly increase at the AVPV during the evening of proestrus in the cyclic rat [47]. In sum, these data evidence that the hypothalamic KiSS-1 system participates in relaying not only negative, but also positive feedback inputs of sex steroids onto the centers governing the HPG axis.

Finally, compelling evidence has been recently presented showing that, besides gonadal steroids, other key regulators of the gonadotropic axis convey their modulatory influences via the hypothalamic KiSS-1 system. These include the metabolic status of the organism [48, 49], and critical environmental cues, such as dark–light cycles in seasonal breeders [32]. Some of these will be briefly summarized in following sections, in the context of the analysis of the role of hypothalamic KiSS-1 as mediator for the influence of internal and external factors on puberty onset.

5 Kisspeptin and puberty onset: Roles and mechanisms

Based on its salient biological profile, one of the reproductive facets of the KiSS-1 system that was first explored was its role in puberty onset. Indeed, the state of sexual immaturity (impuberism) in humans and mice carrying inactivating mutations of GPR54, as reported by Seminara *et al.* [18], already led to the formulation that GPR54 and its ligands play an essential role in puberty. This contention has been fully substantiated during the last 2 years by a combination of expression analysis and functional studies, which have not only demonstrated the pivotal role of kisspeptin in the pubertal activation of the gonadotropic axis, but have initiated also the characterization of their potential mechanisms of action.

Experimental studies on the role of KiSS-1 system in puberty onset initially involved pharmacological tests in prepubertal male and female rats, which demonstrated that intracerebral injection of kisspeptin-10 is capable to elicit robust LH and FSH responses [35]. In addition, expression analyses of KiSS-1 and GPR54 genes in rat hypothalamus along postnatal development demonstrated a significant increase in KiSS-1 and GPR54 mRNA levels coinciding with the onset of puberty, both in male and female animals, which was preceded by low expression levels during the prepubertal period (see Fig. 1). These observations were

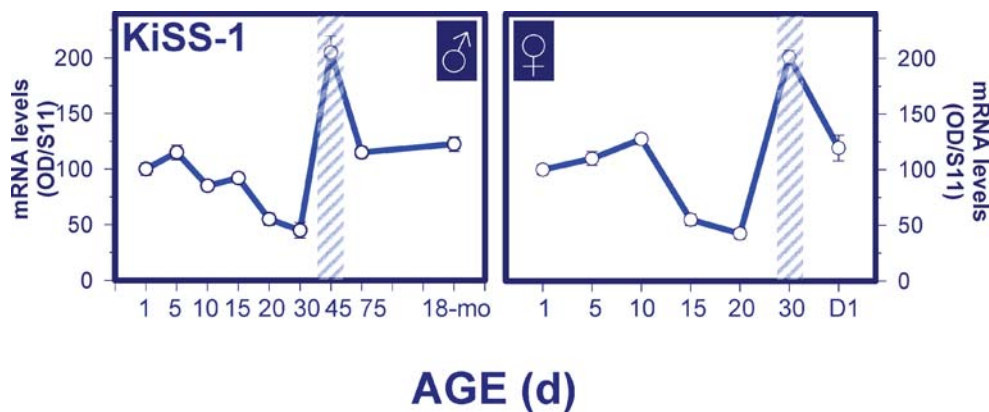


Fig. 1 Changes in relative expression levels of KiSS-1 mRNA in rat hypothalamus from male and female rats along postnatal maturation. Despite persistent expression, significant fluctuations were noticed in KiSS-1 mRNA levels across the different stages, with minimum expression during the prepubertal period and maximum levels

coinciding with puberty onset (*shaded area*), as denoted by preputial separation in males and vaginal opening in females. Ages are expressed in days (*d*) postnatally. In addition, data from 18-month-old male rats and 60-day-old cyclic female rats at diestrus-1 (D1) are shown. Composed from data of [35], with modifications

subsequently confirmed in primates, where central injection of kisspeptin evoked robust LH responses, and hypothalamic levels of KiSS-1 and GPR54 mRNAs were found to increase during the transition from the juvenile to the mid-pubertal stage of the female, while in male puberty a significant rise was only detected for KiSS-1 mRNA levels [38]. Altogether, these findings suggested that an increase in endogenous KiSS-1 signaling (caused by enhanced expression of KiSS-1, and possibly GPR54) takes place along the pubertal transition both in rodents and primates.

To assess the physiological consequences of such a phenomenon, functional studies, involving repeated administration of kisspeptin to immature animals, were conducted in those species. In the rat, chronic intermittent injections of kisspeptin-10 to immature females induced the precocious activation of the gonadotropic axis, as evidenced by advancement in the age of vaginal opening (as external sign of puberty) and consistent hormonal (increased LH and estradiol levels) and trophic (increase uterus weight) responses [50]. Likewise, repetitive administration of kisspeptin-10 at the end of the juvenile phase of primate development evoked a sustained train of GnRH discharges, similar of that found at puberty [43]. Those functional data evidenced that activation of GPR54 is apparently sufficient to trigger the neuroendocrine events leading to puberty onset.

The mechanisms whereby kisspeptin contributes to the pubertal activation of the HPG axis have been extensively analyzed recently. From a general stand-point, studies in rodents and sheep have demonstrated that the primary site of action of KiSS-1 in the control of the gonadotropic axis is the GnRH neuron. This contention is based on the following findings: (1) GnRH neurons express the GPR54 gene [44]; (2) central kisspeptin administration is able to activate GnRH neurons, as estimated by induction of c-fos expression [44]; and (3) kisspeptin is able to elicit GnRH release *in vivo* [37],

and to stimulate, in a dose-dependent manner, GnRH secretion by hypothalamic fragments *ex vivo* [36, 48]. Considering that activation of the gonadotropic axis at puberty is ultimately driven by the increase in the neurosecretory activity of the GnRH pulse generator, it was tempting to hypothesize that the rise of KiSS-1 tone, as deduced from expression analyses in the rat and monkey, is a major excitatory afferent to GnRH neurons at the time of puberty.

In addition, recent studies have shown that, besides the enhancement of KiSS-1 input, a significant increase in the sensitivity of GnRH system to kisspeptin activation does take place during pubertal maturation in the rat and mouse [25, 51]. Thus, by the use of electrophysiological recordings, it was recently demonstrated that while in juvenile mice only 27% of GnRH neurons are activated by kisspeptin, >90% of GnRH neurons are depolarized by kisspeptin in adult animals [51]. In addition, enhanced sensitivity to low doses of kisspeptin (in terms of LH responses) have been demonstrated *in vivo* in rats and mice at the time of puberty [25, 51]. The basis for this phenomenon remains unclear, as the magnitude of such a sensitization apparently exceeds the changes in GPR54 mRNA expression in GnRH neurons. Thus, it has been proposed that an increase in the efficiency of GPR54 coupling to its intracellular effectors takes place along puberty [51]. Overall, the combined enhancement in KiSS-1 expression and GPR54 signaling is likely to contribute to the state of activation of the GnRH pulse generator at puberty.

Finally, an additional level of complexity in the maturational activation of the KiSS-1 system at puberty has been recently disclosed by immunohistochemical analyses in mouse hypothalamus showing that kisspeptin neurons selectively at the AVPV show a striking pattern of postnatal development, with virtually no immunoreactive (IR) neurons during the infantile period, and a consistent increase in kisspeptin-IR from

the prepubertal stage to puberty onset [52]. Moreover, a significant increase in the appositions between kisspeptin fibers and GnRH cell bodies was demonstrated along pubertal transition [52]. These observations point out that, besides changes in gene expression and GPR54 signaling efficiency, regional differences exist in the activation of the hypothalamic KiSS-1 system, which are likely relevant for puberty onset. Moreover, the tuning of kisspeptin action at puberty seems to rely also on striking developmental changes in the pattern of appositions between KiSS-1 and GnRH neurons, which increase along puberty transition. The latter could be mechanistically important for the precise timing of puberty, as GnRH/LH responsiveness to kisspeptin is present well in advance (e.g. neonatal/infantile period) the onset of puberty [25]. Overall, such a sophisticated, multi-step regulatory process illustrates the crucial role of KiSS-1 and GPR54 as pivotal excitatory signal for the activation of the HPG axis during pubertal maturation.

6 Kisspeptin and the metabolic control of puberty onset

As described in previous sections, puberty is the result of a complex interaction of genetic factors and a plethora of regulators of endogenous and environmental origin. Among those, it is well recognized that one of the most relevant factors in the regulation of puberty onset is the state of energy reserves of the organism; conditions of energy insufficiency or defective nutritional states being invariably coupled to disturbed (delayed or absent) pubertal development [2]. Our knowledge on the neuroendocrine networks responsible for linking energy homeostasis and reproductive function in general, and puberty onset in particular, has increased dramatically over the last decade with the identification of the fundamental role of the adipocyte-derived hormone, leptin, in the metabolic control of fertility [10–13]. However, while leptin and other peripheral hormones (such as insulin, ghrelin, and PYY3-36) are now recognized as potential modulators of the HPG axis [10], the central pathways whereby this metabolic information is ultimately conveyed onto the centers governing the gonadotropic system (i.e., GnRH neurons) remain to be fully characterized.

Considering the prominent role of kisspeptin neurons as central processors for the regulation of GnRH/LH secretion, we hypothesized that the KiSS-1 system might also operate as central conduit for the metabolic regulation of puberty. Expression analyses and functional tests recently conducted at our laboratory strongly suggest that this is very likely the case. Thus, conditions of energy insufficiency (such as short-term fasting) in pubertal male and female rats not only induced the anticipated suppression of basal gonado-

tropin secretion, but caused also a significant decrease in the expression of KiSS-1 mRNA at the hypothalamus. Of note, a concomitant increase in hypothalamic GPR54 mRNA levels was also observed in fasted animals [48]. This might reflect a state of sensitization to kisspeptin actions due to a primary decrease in endogenous KiSS-1 tone. In fact, evidence for such a sensitization has been obtained by our group in fasted rats, both *in vivo* (LH responses to intracerebral administration of kisspeptin) and *ex vivo* (GnRH release by hypothalamic fragments challenged with increasing doses of kisspeptin).

In addition, in order to provide further proof for the relevance of changes in hypothalamic KiSS-1 system in conditions of negative energy balance, functional tests, involving repeated central administration of kisspeptin, were conducted in immature female rats subjected to a protocol of undernutrition [48]. In this setting, restriction of daily food intake in 30% (from postnatal d-23 onwards) was able to totally suppress the activation of the gonadotropic axis at puberty, as evidenced by absent vaginal opening and significantly decreased gonadotropin and estradiol levels in food-deprived animals. In this model, chronic injection of kisspeptin was sufficient to restore vaginal opening in a significant number of cases (60%), and

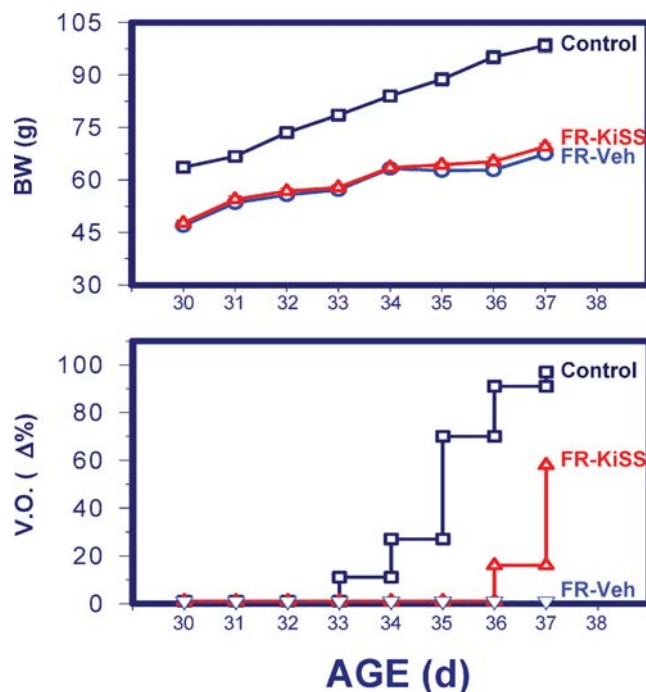


Fig. 2 Body weight (BW) data and accumulated percentages of vaginal opening (VO) in peripubertal female rats, either fed *ad libitum* (Controls) or subjected to 30% restriction in daily food intake (FR) from postnatal d-23 onwards. FR animals underwent a protocol of repeated central injections of vehicle (Veh) or kisspeptin-10 (KiSS-1), between d-30 and d-37. In addition to VO, hormonal responses to the treatments were evaluated in terminal blood samples, taken 1-h after the last injection of vehicle or KiSS-1 (*data not shown*; for details see text). Composed from data of [48], with modifications

to induce robust gonadotropin and estrogen responses in all rats treated with kisspeptin, despite the absence of significant changes in body weight vs. pair-fed animals (see Fig. 2; and [48]). These observations demonstrate that restoration of endogenous kisspeptin tone can rescue, at least partially, puberty onset and defective gonadotropin secretion in conditions of energy insufficiency, as undernutrition. Interestingly, we have recently obtained evidence for similar alterations in the expression and function of the hypothalamic KiSS-1 system in other models of hypogonadotropism associated to disturbed metabolic state, such as experimental diabetes [49]. Although the latter studies have been so far conducted in adult animals only, our results jointly suggest that kisspeptin neurons are likely to operate as sensors for energy balance and the metabolic status; the decrease in KiSS-1 tone being mechanistically relevant for the suppression of pubertal development and reproductive function in conditions of energy deficit.

7 Leptin as major metabolic regulator of hypothalamic KiSS-1 system

The data summarized above clearly demonstrate the impact of the nutritional and metabolic state of the organism on the expression and function of the KiSS-1 system, as potential mechanism whereby energy reserves, puberty and fertility are functionally coupled. However, these results rise the question of which are the peripheral signals and eventual neuropeptide mediators responsible for the metabolic control of hypothalamic KiSS-1. This is an especially relevant issue as, except for gonadal steroids, the nature of the major regulators of kisspeptin neurons remained mostly unknown.

An obvious candidate for the metabolic regulation of KiSS-1 neurons at puberty was leptin [48, 50]. This assumption was based on the known pivotal role of leptin in signaling the magnitude of energy reserves to GnRH neurons, and its indispensable, permissive role in puberty onset [10]. Interestingly, following the initial observations on the positive actions of leptin on the gonadotropic axis, it was deduced that such trophic effects were conducted mostly at the hypothalamus, through regulation of GnRH neurons [10]. However, despite initial reports showing expression of leptin receptors in immortalized GnRH cell lines, it has been globally accepted that GnRH neurons do not physiologically express leptin receptors, suggesting the involvement of intermediary signals. Experimental evidence, reported mostly during the last year, strongly suggest that kisspeptin operates as central effector for leptin modulation of GnRH system. These data include identification of leptin receptors in a significant (>40%) proportion of KiSS-1 neurons at the arcuate nucleus, as well as the demonstration that leptin deficient ob/ob mice show

decreased hypothalamic KiSS-1 mRNA levels, which can be (partially) rescued by leptin treatment [53]. Moreover, using another model of hypoleptinemia, such as the uncontrolled diabetic rat, we have recently shown that chronic intracerebral infusion of leptin is capable to normalize defective hypothalamic KiSS-1 mRNA levels, as well as LH and testosterone concentrations, in hypogonadotropic diabetic male rats [49]. Although those studies were conducted in adult animals [49, 53], the above evidence strongly suggests that the mechanism whereby leptin positively modulates puberty onset may include the regulation of KiSS-1 expression at the hypothalamus. However, whether leptin is solely acting onto KiSS-1 neurons for the central control of GnRH, or whether additional peripheral regulators co-operate with leptin in the control of KiSS-1 for the integration of energy balance and puberty onset, remain to be elucidated.

8 KiSS-1 as central conduit for regulation of puberty by environmental factors

Besides endogenous conditions (such as energy status), different environmental cues play an essential role in the tuning of puberty onset in different species, including humans [2]. Among those, exposure to endocrine disrupting compounds, present in the environment and provided (mostly) with estrogenic activities, has been claimed as mechanistically relevant for alterations in the timing of human puberty [2]. Moreover, data from animal studies have demonstrated that inappropriate exposure to estrogen during critical periods of development (e.g. neonatal estrogenization in the rat) induces the disturbance of the normal process of brain sexual differentiation, which manifests in a plethora of reproductive defects later on life, including disruption of puberty [54]. However, the mechanisms whereby this action is conducted remain to be elucidated. Yet, a conspicuous feature of the models of perinatal estrogenization is that, despite evidence for suppressed function of the GnRH system, GnRH neurons themselves appear to retain the ability to express the neuropeptide and to respond to conventional stimuli, suggesting a primary defect located at the level of up-stream regulatory elements of GnRH secretion.

On the basis of the proven ability of estrogen to transiently regulate the hypothalamic expression of KiSS-1 gene in adult animals, we hypothesized the estrogen might also play a role in the organization of KiSS-1 system during critical periods of development. Our initial data demonstrated that, indeed, neonatal exposure to high doses of estrogen induces a persistent decrease in the hypothalamic expression of KiSS-1 mRNA in young adult male rats, which might contribute to the observed decrease in serum LH and

testosterone levels (as well as disturbed puberty) in this model [35]. Further studies are in progress in our laboratory to define the threshold doses, as well as the functional consequences, of such a disturbed pattern of KiSS-1 expression at puberty. The importance of these data might be two-fold: (1) they will contribute to the set the physiologic relevance of the organizing effects of estrogen on the hypothalamic KiSS-1 system and, hence, on the central networks controlling the HPG axis; and (2) they will help to identify potential mechanisms for the reported effects of early exposure to (environmental) estrogenic compounds on the timing of puberty.

9 Conclusions and future perspectives

As reviewed in previous sections, puberty is a critical multifaceted, developmental event, whose neuroendocrine dimension is the result of the orchestrated interaction of a wide spectrum of central regulators of GnRH neurons, under the modulatory influence of diverse peripheral and external signals [2]. In this complex network, the hypothalamic KiSS-1 system has recently emerged as essential gatekeeper of GnRH secretion and puberty onset. Indeed, during the last 2 years, our knowledge on the roles, mechanisms of action and major regulatory elements of KiSS-1 system in the control of puberty has rapidly increased. Thus, it has become clear that, rather than another element among the myriad of central modulators of the reproductive axis, kisspeptin neurons operate as crucial central processors in the precise regulation of GnRH secretion at puberty.

However, despite significant advancements in the field, several aspects of kisspeptin function as major regulator of the HPG axis are yet to be fully elucidated. For instance, the actual position of KiSS-1 neurons in the cascade of factors involved in the central control of the GnRH pulse generator is yet to be fully disclosed. Indirect pharmacological evidence initially suggested that KiSS-1 seems to be located distal to (or eventually independent of) other transsynaptic regulators of GnRH neurons, such as glutamate neurotransmission [40, 41]. Recent immunohistochemical analyses have provided the first morphological evidence for the direct apposition between kisspeptin and GnRH neurons [52]. Mapping of the afferents to and the projections of KiSS-1 neurons in different hypothalamic areas will help to define their actual roles in the regulation of several aspects of reproductive function. These analyses could also provide valuable clues about the major central regulators of KiSS-1 expression at the hypothalamus, which remain to date virtually unknown. Moreover, those studies would determine to what extent kisspeptin operates as ultimate effector for the modulatory

actions of other neuropeptides and transmitters involved in the control of GnRH release. As example, melatonin has been recently suggested to regulate kisspeptin expression as means to conduct its role in the photoperiodic control of reproduction in seasonal species [32]. Yet, complete characterization of the neuronal network responsible for such a phenomenon is still pending.

Regarding the specific roles of KiSS-1 in the control of puberty, a key issue that remains to be solved is whether kisspeptin is the primary trigger for puberty onset, or it rather operates as effector of up-stream regulatory factors. The demonstration of the potent stimulatory effects of kisspeptin on GnRH release, likely acting on GnRH neurons, and the proven ability of different modulators of GnRH system, such as sex steroids and leptin, to conduct their effects via regulation of KiSS-1, strongly suggest that kisspeptin neurons play a fundamental role in the integration of different afferents to the GnRH system and might ultimately drive the central activation of the HPG axis at puberty. In this tentative model, the ‘center of gravity’ for the dynamic control of puberty onset would lie on the KiSS-1 neuron, on which the information conveyed by different central and peripheral regulators would converge and eventually integrate. Conversely, the GnRH neuron would play a more ‘passive’ role, acting as final transducer of the information relayed by the KiSS-1 system. Although this hypothetical model is yet to be fully confirmed experimentally, it is striking that up to 3 years ago the reproductive dimension of kisspeptin and GPR54 had remained totally unnoticed. Expectedly, our knowledge on the physiologic roles of the KiSS-1 system in the control of reproduction in general, and puberty onset in particular, will continue to enlarge significantly in the coming years, in what promises to be one of the most active areas of contemporary Neuroendocrinology.

Acknowledgments The authors are indebted with C. Dieguez, L. Pinilla, E. Aguilar, and other members of the research team at the Physiology Section of the University of Cordoba, for continuous collaboration and support in studies on the neuroendocrine aspects of kisspeptin physiology, and helpful discussions during preparation of this manuscript. The experimental work from the authors’ laboratory summarized in this review has been supported by grants BFI 2002-00176 and BFU 2005-07446 from Ministerio de Educación y Ciencia, Spain, funds from Instituto de Salud Carlos III (Red de Centros RCMN C03/08, Project PI042082 and CIBER Physiopathology of Obesity and Nutrition; Ministerio de Sanidad, Spain), and EU research contract EDEN QLK4-CT-2002-00603.

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