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GPR54 and kisspeptin in reproduction

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Kisspeptins, the peptide products of the *KiSS-1* gene, were identified in 2001 as natural ligands of the previously orphan G protein-coupled receptor, GPR54. They include, among others, metastin and kisspeptin-10. The known biological functions of kisspeptins were initially restricted to their ability to suppress tumour metastasis, hence the name of metastin. However, in late 2003, two groups independently reported that loss-of-function mutations of the *GPR54* gene are linked to absence of puberty onset and hypogonadotrophic hypogonadism in humans—a phenotype that was reproduced in GPR54-null mice. Those seminal observations revealed a totally unexpected, fundamental role of the KiSS-1/GPR54 system in control of puberty and reproductive function and boosted an extraordinary interest for the characterization of these novel facets of kisspeptin physiology. Indeed, in the last 2 years, metastin and kisspeptin-10 have been demonstrated as very potent stimulators of the gonadotrophic axis, in a number of species and through different routes of administration. In addition, the hypothalamic KiSS-1/GPR54 system has been proven as an essential gatekeeper of GnRH neurons, involved in their activation at puberty and their regulation by gonadal steroids and (probably) metabolic factors. This review comprehensively examines the experimental evidence obtained to date supporting a pivotal role of kisspeptins and GPR54 in the control of reproduction.

Key words: GnRH/gonadotrophins/GPR54/kisspeptin/puberty

Introduction

In mammals, including humans, reproductive capacity is attained at puberty as the end-point of a complex series of developmental and neuroendocrine events that lead to full activation of the GnRH pulse generator, enhanced gonadotrophin secretion and complete gonadal maturation and function (Ojeda and Skinner, 2005). The hormonal network responsible for the control of reproduction constitutes the so-called gonadotrophic axis, which is mainly composed of three major hierarchical elements: the hypothalamic GnRH, the pituitary gonadotrophins (LH and FSH), and sex steroids and other hormone products of the gonads (Tena-Sempere and Huhtaniemi, 2003). This neuroendocrine system is dynamically regulated by a plethora of central and peripheral signals, which target the hypothalamic GnRH neurons, as ultimate effectors for the control of gonadotrophin secretion. Accordingly, numerous central excitatory and inhibitory inputs to the GnRH axis have been identified during last decades, including excitatory amino acids, noradrenaline, neuropeptide Y (NPY), γ-aminobutyric acid (GABA) and opioid peptides (Ojeda and Skinner, 2005). Among peripheral hormones, the gonadal sex steroids are well-known major regulators of GnRH secretion, through negative and positive feedback loops. In addition, reproductive function is extremely sensitive to the energy status, signalled through the adipose-derived factor leptin, as well as other peripheral hormones and metabolic cues (Casanueva and Dieguez, 1999; Tena-Sempere and Barreiro, 2002). Conspicuously, however, the signals ultimately relaying such peripheral information onto the GnRH system remain to be fully characterized.

In this context, our understanding of the mechanisms responsible for the physiological control of the reproductive axis, and its physiopathological alterations, was recently revolutionized by the identification of the fundamental role of kisspeptins, the peptide products of the *KiSS-1* gene, and their putative receptor, GPR54, in the neuroendocrine regulation of reproduction. Indeed, genetic, physiological, pharmacological and clinical data, accumulated during the last 2 years, strongly indicate that the KiSS-1/GPR54 system is not merely one more element in the cascade of signals controlling the gonadotrophic axis, but an essential gatekeeper of GnRH function, which allows for the integration of central and peripheral inputs, thereby playing a pivotal role in the control of reproductive function. This article will review the experimental evidence obtained to date supporting this contention.

Identification of kisspeptins as natural ligands of GPR54

Despite the indispensable function of kisspeptins and GPR54 in reproduction, discovery of this system took place several years before the reproductive connection was ever suspected. The first element of the system to be identified was the *KiSS-1* gene. By the use of subtractive hybridization in melanoma cell lines with different metastatic capacity, in 1996 the KiSS-1 mRNA was originally reported to be selectively overexpressed in metastasis-suppressed

tumour cells (Lee *et al*., 1996). This original finding was followed by the cloning and chromosomal localization of the *KiSS-1* gene (West *et al*., 1998). However, it was not until 2001 that the peptide products of *KiSS-1* were identified. On the basis of structural similarities, these were globally termed kisspeptins, as they were found to derive from the differential proteolytic processing of a common precursor. In humans, the KiSS-1 precursor contains 145 amino acids, with a putative 19-amino-acid signal sequence, two potential dibasic cleavage sites (at amino acids 57 and 67) and one putative site for terminal cleavage and amidation (at amino acids 121–124) (Kotani *et al*., 2001; Muir *et al*., 2001; Ohtaki *et al*., 2001) (Figure 1). The major peptide product of the *KiSS-1* gene appears to be a 54-amino-acid peptide, largely secreted by the placenta and termed metastin or kisspeptin-54 (Ohtaki *et al*., 2001). In addition, other derivatives of the KiSS-1 precursor, such as kisspeptin-14, kisspeptin-13 and kisspeptin-10, have been identified (Kotani *et al*., 2001). All kisspeptins share the C-terminal region of the metastin molecule, where they harbour an Arg-Phe-NH_2 motif distinctive of the Arg-Phe-amide peptide family. In the rat and mouse, metastin is composed of 52 amino acids, and the terminal RF-amide signature is substituted by an Arg-Tyr-NH_2 motif (Terao *et al*., 2004).

Coincident with the characterization of KiSS-1 peptides, the receptor mediating their biological actions was identified. This turned out to be a G protein-coupled receptor, termed GPR54 (Kotani *et al*., 2001; Muir *et al*., 2001; Ohtaki *et al*., 2001). GPR54 was cloned in the rat in 1999 as an orphan receptor with a significant sequence similarity (>40%) with the transmembrane regions of galanin receptors (Lee *et al*., 1999). Subsequently, the human orthologue of GPR54 was cloned, and named AXOR12 or hOT7T175 (Muir *et al*., 2001; Ohtaki *et al*., 2001). The functional characteristics of GPR54 were dissected out using heterologous cell systems (CHO K1 cells stably expressing GPR54). These studies revealed that all kisspeptins efficiently activate GPR54, with the shorter 10-amino-acid fragment (kisspeptin-10) retaining maximal activity at the receptor level (Kotani *et al*., 2001). Upon ligand–receptor interaction, the major intracellular signalling systems recruited by GPR54 include activation of phospholipase C and PIP_2 hydrolysis, which is followed by accumulation of inositol-(1,4,5)-triphosphate, Ca^{2+} mobilization, arachidonic acid release and phosphorylation of ERK1/2 and p38 MAP kinases (Kotani *et al*., 2001). Of note, however, the signalling cascades used by GPR54 in normal tissues, where this receptor is expressed and where biological effects of kisspeptins have been reported (discussed below), remain to date virtually unexplored.

Pre-reproductive era of the KiSS-1/GPR54 system: expression and functions

For obvious reasons, initial studies on KiSS-1 function were conducted in the context of tumour biology, where its role as metastasis-suppressor gene attracted quite some attention (Lee *et al*., 1996; Lee and Welch, 1997a). The pioneering observations that KiSS-1 suppresses metastasis in melanoma cells were followed by a number of reports, assessing *KiSS-1* gene expression and/or biological actions of kisspeptins in additional tumour specimens and cell lines. Those studies demonstrated that metastin and other kisspeptins provide an anti-metastasis activity in several tumours, such as papillary thyroid carcinoma, breast cancer, melanoma, pancreatic cancer cells and ovarian carcinoma (Lee and Welch, 1997a,b; Ohtaki *et al*., 2001; Matsui *et al*., 2004a; Jiang *et al*., 2005). In addition, loss of *KiSS-1* gene expression was identified as a bad prognosis factor for tumour progression and metastasis in oesophageal squamous cell carcinoma, gastric carcinoma and bladder cancer, among others (Sanchez-Carbayo *et al*., 2003; Ikeguchi *et al*., 2004). Nonetheless, despite molecular and clinical evidence for a potential role of KiSS-1 in metastasis regulation (Harms *et al*., 2003), some conflictive data on the function of KiSS-1 in tumour metastasis have been reported, and the actual relevance of this system in cancer progression remains to be defined.

Besides a role in metastasis regulation, expression analyses of *KiSS-1* and *GPR54* mRNAs, conducted upon cloning of the corresponding genes, strongly suggested that this signalling system was also provided with additional functions in normal tissues. Thus, prominent expression of the *KiSS-1* gene was demonstrated in human placenta and, at lower levels, in the testis and small intestine. KiSS-1 mRNA was also detected in the human brain, with

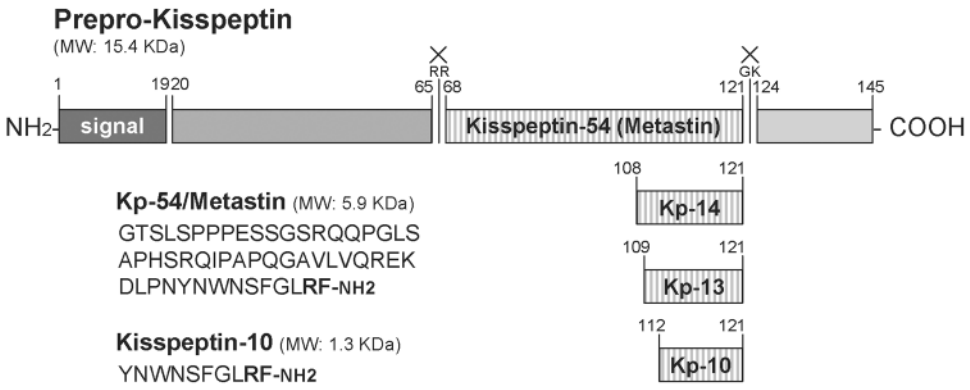

Figure 1. Structural features of human kisspeptins, generated by cleavage form a common precursor, the prepro-kisspeptin. Prepro-kisspeptin, encoded by the *KiSS-1* gene, is a 145-amino-acid protein that contains a 19-amino-acid signal peptide and a central 54-amino-acid region, flanked by two consensus cleavage sites (denoted by *X*), which gives rise to metastin or kisspeptin-54. Further cleavage of metastin generates kisspeptins of lower molecular weight: kisspeptin-14 (Kp-14), Kp-13 and Kp-10. All kisspeptins are able to bind and activate GPR54. Besides general structural organization, the complete amino acid sequences of human metastin and kisspeptin-10 are shown. The consensus C-terminal RF-amide motif, hallmark of this peptide super-family, is indicated in bold.

scattered distribution throughout the central nervous system, including the basal ganglia and the hypothalamus (Muir *et al*., 2001; Ohtaki *et al*., 2001). In addition, *GPR54* gene expression appeared widely distributed, with maximal mRNA levels in placenta, pancreas, pituitary, spinal cord and different brain areas (including hypothalamus, basal ganglia, amygdala, substantia nigra and hippocampus). Altogether, these findings strongly suggested the potential involvement of the KiSS-1/GPR54 system in the physiological control of diverse biological systems.

At central levels, the implication of kisspeptins in diverse brain functions, such as nociception and visceral regulation, was suggested, mostly on the basis of indirect evidence (Brailoiu *et al*., 2005). However, direct assessment of the role of kisspeptins in synaptic transmission in discrete brain areas (such as the hippocampus) has been initiated only very recently (Arai *et al*., 2005). In addition, some preliminary evidence indicated that KiSS-1 may participate in the regulation of specific neuroendocrine systems, such as oxytocin release (Kotani *et al*., 2001). At the periphery, expression of the *KiSS-1* gene was proven to be maximal in human placenta, which likely accounts for the dramatic increase in serum metastin levels during pregnancy (Horikoshi *et al*., 2003), a phenomenon whose endocrine relevance is yet to be determined. In terms of paracrine control, placental KiSS-1 peptides have been suggested to play a role in the physiological regulation of trophoblast invasion (Bilban *et al*., 2004). Although molecular and pharmacological data strongly support this contention, the lack of gross abnormalities in placental formation in GPR54-null mice seems to cast doubts on the physiological relevance of this function (Colledge, 2004). Other potential peripheral functions of the KiSS-1/GPR54, in tissues such as the pancreas and the gonads where the expression of this system has been reported, remain to date unexplored.

KiSS-1/GPR54 system and reproduction: initial evidence

Although placental expressions of KiSS-1 and GPR54 had been reported, and the potential implication of this system in the control of neuroendocrine functions had been anticipated on the basis of expression analyses and preliminary hormonal tests (Kotani *et al*., 2001), compelling evidence for the essential role of kisspeptins and GPR54 in the control of reproductive function emerged only in late 2003. At that time, two research groups independently reported the presence of loss-of-function mutations of the *GPR54* gene in patients suffering idiopathic hypogonadotrophic hypogonadism (IHH) (de Roux *et al*., 2003; Seminara *et al*., 2003). Notably, although its clinical diagnosis is relatively straightforward (Seminara, 2005), the molecular basis of IHH, which appears to be highly heterogenous, begun to be unravelled only in late 1990s, when a number of causative factors, such as inactivating mutations in Kal1, fibroblast growth factor receptor or GnRH receptor genes, were reported (Iovane *et al*., 2004; Seminara, 2005). Interestingly, those conditions only accounted for a subset of familial and sporadic forms of IHH, suggesting the concurrence of other aetiological factors. One of those factors turned out to be the inactivation of GPR54.

To date, a limited number of studies have been published describing sequence variations in the *GPR54* gene responsible for familial or sporadic forms of IHH. These included the originally reported homozygous deletion of 155 nucleotides at the intron 4–exon 5 junction of *GPR54* gene (de Roux *et al*., 2003), the homozygous Leu146Ser substitution and the compound heterozygous mutations Arg331X (which inserted a premature stop codon) and X399Arg (which eliminated the canonical stop codon) (Seminara *et al*., 2003). Thereafter, compound heterozygous missense mutations at Cys223Arg and Arg297Leu (Semple *et al*., 2005), and the homozygous insertion (1001–1002insC) resulting in a shift of the open reading frame of GPR54 (Lanfranco *et al*., 2005), were described as causative factors for IHH. In most cases, testing of the functionality of the mutants *in vivo* has been provided, showing severe to mild receptor inactivation (Seminara *et al*., 2003; Semple *et al*., 2005). Interestingly, mice carrying null mutations of *GPR54* gene were a complete phenocopy of affected humans (Funes *et al*., 2003; Seminara *et al*., 2003), evidencing the highly conserved, indispensable role of GPR54 in mammalian reproduction. Indeed, analyses of those human and mouse phenotypes were extraordinarily illustrative from a physiological standpoint, as they set the basis for the study of the effects and sites of actions of kisspeptin upon the gonadotrophic axis. Thus, despite severe hypogonadotrophism and sexual immaturity, GPR54-null mice showed preserved hypothalamic GnRH content, suggesting that disruption of GPR54 does not impair migration of GnRH neurons from the olfactory placode during development, nor does it disrupt GnRH synthesis (Seminara *et al*., 2003). Moreover, pituitary gonadotrophin responsiveness to exogenous GnRH was conserved (in terms of relative responses) in humans and mice carrying inactivating mutations of GPR54 (Seminara *et al*., 2003). Altogether, these observations pointed out that defective GPR54 signalling results in the shutting-down of the GnRH pulse generator, through mechanisms that remained obscure at that time.

KiSS-1/GPR54 system and the neuroendocrine control of reproduction

As indicated in previous sections, genetic and molecular studies published in late 2003 set the basis for the exhaustive analysis of the role of GPR54 and their ligands, the kisspeptins, in the neuroendocrine control of reproduction. This finding was especially fortunate as the elements (ligands and receptor) of the KiSS-1/GPR54 had been known for several years, thus providing the intellectual basis and the tools for a rapid progress in the field. Even the name of KiSS-1 (originally coined to incorporate the 'SS' of suppresser sequence) turned out to be felicitous, in the face of the unexpected reproductive dimension of the molecule.

The goals of the first studies on the reproductive role of KiSS-1 system were two-fold: to provide evidence for the effects of the peptide products of *KiSS-1* gene upon gonadotrophin secretion and to characterize the pattern of hypothalamic expression of *KiSS-1* and *GPR54* genes at relevant physiological states and experimental models. The latter was based on the proven expression of both genes in the hypothalamus, and inferential data from human and mouse models of GPR54 inactivation suggesting a primary defect in the function of the hypothalamic GnRH pulse generator in the absence of KiSS-1 signalling. Regarding gonadotrophin-releasing effects, between 2004 and early 2005, studies conducted in laboratory animals (mostly, in rat, mouse and macaque) clearly demonstrated that metastin and kisspeptin-10 are extraordinary potent elicitors of LH and FSH release. The ability of kisspeptins to stimulate LH secretion was also confirmed in the

sheep (Messager *et al*., 2005). Those releasing effects were observed both after central (intracerebroventricular) and systemic (intravenous, intraperitoneal and subcutaneous) administration of the peptides (Gottsch *et al*., 2004; Irwig *et al*., 2004; Matsui *et al*., 2004b; Navarro *et al*., 2004a; Thompson *et al*., 2004; Navarro *et al*., 2005a,b; Shahab *et al*., 2005).

Although some minor differences in the threshold doses required to elicit unambiguous LH responses are noticed across the studies, it is very clear that the sensitivity of the gonadotrophic system to the stimulatory effect of kisspeptin is extraordinary high, as illustrated by the fact that doses as low as 100 fmol to 1 pmol injected centrally, and 0.3 nmol/kg BW (equivalent to 0.1 μg) injected systemically, are able to significantly increase serum LH levels in the rat (Gottsch *et al*., 2004; Navarro *et al*., 2005a; Tovar *et al*., 2006). Notably, FSH release *in vivo* appeared to be approximately 100-fold less sensitive to the stimulatory effect of kisspeptin than LH (EC50: approximately 4 and 400 pmol for LH and FSH, respectively). Nonetheless, meta-analyses of kisspeptin results and previously published data on the LH-releasing activity of other neuropeptides and neurotransmitters, such as glutamate and galanin-like peptide, show that the KiSS-1 system is probably the most potent elicitor of the GnRH–gonadotrophin axis known so far. Moreover, the fact that systemic administration of kisspeptins, even at low doses, is able to stimulate consistent LH responses is quite striking, as most of the neuropeptides involved in the central control of gonadotrophin secretion are ineffective when administered systemically. In keeping with the animal data, peripheral administration of kisspeptin-54 was very recently proven to stimulate LH secretion in humans (Dhillo *et al*., 2005). Of note, the LH-releasing effects of kisspeptin-10 were totally abrogated in GPR54-null mice, which retained pituitary responsiveness to GnRH. This suggests that the gonadotrophic effects of kisspeptin are solely mediated via GPR54 (Messager *et al*., 2005).

The sites of action of kisspeptin in the control of the gonadotrophic axis have also been a matter of intense study. Analyses of GPR54 knock-out animals suggested a defective function of the GnRH pulse generator due to absent signalling of an essential upstream regulator. On this basis, specific studies were conducted to demonstrate whether kisspeptins are able to act on GnRH neurons and to elicit GnRH secretion. The first demonstration that mammalian GnRH neurons are potential targets for kisspeptins was provided by double-label *in situ* hybridization (ISH) studies in the rat that showed that >75% of GnRH neurons co-express GPR54 mRNA. Moreover, kisspeptin was able to induce c-fos expression (as an early marker of activation) in >85% of GnRH neurons (Irwig *et al*., 2004). Simultaneously, the ability of kisspeptin to elicit GnRH secretion by rat hypothalamic explants *ex vivo* was reported (Thompson *et al*., 2004; Castellano *et al*., 2005), and kisspeptin was demonstrated to induce the release of GnRH into the cerebrospinal fluid in the sheep (Messager *et al*., 2005). Altogether, these data strongly suggested that the primary site of action of kisspeptins in the control of the gonadotrophic axis is located at hypothalamic GnRH neurons, where kisspeptins elicit GnRH secretion, which in turn stimulates LH and FSH release from the pituitary. This contention was indirectly supported by the fact that blockade of the actions of GnRH (by means of pharmacological antagonists) totally blunted the gonadotrophin-releasing effects of kisspeptins in the rat, mouse and monkey (Gottsch *et al*., 2004; Matsui *et al*., 2004b; Navarro *et al*., 2004b; Navarro *et al*., 2005a,b; Shahab *et al*., 2005). Interestingly, although the potential role of kisspeptins in the control of reproduction in non-mammalian species remains unexplored, expression of *GPR54* gene has been demonstrated in GnRH neurons of tilapia (cichlid fish) (Parhar *et al*., 2004), suggesting a high degree of conservation of the function of kisspeptins as gatekeeper of GnRH neurons during evolution.

KiSS-1/GPR54 system and puberty onset

Besides hormonal tests, expression analyses of KiSS-1 and GPR54 mRNAs at the hypothalamus were immediately conducted upon identification of the putative role of this system in the central control of reproduction. To seek for developmental changes of expression, we monitored the mRNA levels of both targets in rat hypothalamic samples along post-natal maturation. In keeping with the proposed role of the KiSS-1 system in puberty, both male and female rats showed a marked increase in KiSS-1 and GPR54 mRNA levels coinciding with the onset of puberty (Navarro *et al*., 2004a). This phenomenon was later confirmed in primates, where KiSS-1 and GPR54 mRNA levels increased approximately three-fold in the hypothalamus during the transition from the juvenile to the mid-pubertal stage of the female. Likewise, hypothalamic KiSS-1 mRNA levels increased in male monkeys during puberty (Shahab *et al*., 2005). Moreover, functional studies conducted in female rats demonstrated that repeated administration of kisspeptin-10 to immature animals was able to induce precocious vaginal opening (as an external sign of puberty) and early activation of the gonadotrophic axis (Navarro *et al*., 2004b). Similarly, repetitive administration of kisspeptin-10 at the end of the juvenile phase of primate development was able to precociously elicit a sustained train of GnRH discharges, reminiscent of that found during puberty (Plant *et al*., 2006). Altogether, these findings indicate that enhancement of endogenous KiSS-1 tone in the hypothalamus takes place during pubertal maturation and that (pharmacological) activation of GPR54 is apparently sufficient to trigger the neuroendocrine events leading to the onset of puberty. Such a fundamental role of kisspeptins at puberty is in good agreement with the state of sexual immaturity observed in human patients and mouse models of GPR54 inactivation (Funes *et al*., 2003; Seminara *et al*., 2003).

A contentious issue, however, is the precise mechanism whereby kisspeptins efficiently activate GnRH neurons at puberty, as well as the hierarchical role of KiSS-1 in the sequence of events leading to complete pubertal maturation. Very recently, elegant studies using a combination of patch-clamp electrophysiological recordings of GnRH-green fluorescent protein neurons and *in vivo* hormonal tests demonstrated that the developmental activation of the GnRH axis by KiSS-1 at puberty reflects a dual phenomenon involving not only the increase of kisspeptin tone but also the enhancement of its efficiency to activate GnRH neurons, probably through post-transcriptional changes in GPR54 signalling (Han *et al*., 2005). Thus, although only 27% of GnRH neurons were activated by kisspeptin in juvenile mice, >90% of GnRH neurons were depolarized by kisspeptin in adult animals—a switch that cannot be explained by changes in *GPR54* gene expression. Moreover, the sensitivity of GnRH system to kisspeptin was dramatically enhanced in adult versus juvenile mice (Han *et al*., 2005). A similar phenomenon seems to take place also in the rat, where central

doses of 1 pmol kisspeptin-10 elicited maximal LH responses in pubertal males but not in juvenile rats (our unpublished data). Notwithstanding, despite low basal activity of the gonadotrophic axis before puberty, high doses of kisspeptin-10 were able to potently stimulate GnRH release *in vitro* and LH secretion *in vivo* at early stages (neonatal to juvenile) of post-natal development in the rat (our unpublished data). These observations further demonstrate a pivotal role of the KiSS-1 system in timing puberty onset. Yet, the precise location of kisspeptins and GPR54 in the complex neuroendocrine network controlling activation of GnRH neurons at puberty, and their interactions with other elements of this hierarchical system, remain to be completely elucidated. Using a systematic biological approach, it was recently proposed that *KiSS-1* and *GPR54* are subordinate genes, under the control of upstream regulators, whose protein products operate as transsynaptical regulators of GnRH neurons (Ojeda *et al*., 2006). The fact that inactivation of GPR54 switches off the function of the GnRH pulse generator demonstrates the indispensable role of this signalling system in the control of puberty. However, it remains to be established whether KiSS-1 is the trigger of mammalian puberty or the ultimate effector of hierarchically higher regulatory factors.

KiSS-1 and the control of reproduction by feedback signals and metabolic cues

Further evidence for the pivotal role of KiSS-1 system in the dynamic control of the gonadotrophic axis came from expression analyses of *KiSS-1* and *GPR54* genes in the hypothalamus in gonadectomized animals. It is well known that the removal of negative feedback of gonadal steroids brings about an elevation of serum LH and FSH levels. However, hypothalamic GnRH mRNA levels are not consistently elevated following gonadectomy, thus suggesting the involvement of intermediate signals. In male rats, bilateral orchidectomy evoked a consistent increase in KiSS-1 mRNA levels in whole hypothalamic fragments. Likewise, bilateral ovariectomy resulted in enhanced expression in KiSS-1 mRNA at the hypothalamus (Navarro *et al*., 2004a). Interestingly, sex steroid replacement fully reverted these responses, as testosterone supplementation in males and estrogen administration in females totally prevented the increase in KiSS-1 mRNA levels post gonadectomy. Moreover, although no significant fluctuations in relative GnRH levels were observed, hypothalamic KiSS-1 levels closely paralleled the changes in circulating LH concentrations in these models (Navarro *et al*., 2004a). Taken as a whole, these observations strongly suggested that the hypothalamic KiSS-1 system plays an essential role in relaying the negative feedback input of sex steroids onto GnRH neurons.

Although those data were highly illustrative of the physiological role of kisspeptins in negative feedback control of gonadotrophin secretion, original expression analyses suffered from the lack of anatomical resolution, which partially hampered interpretation of their complete physiological relevance. In this sense, ISH assays recently added further refinement to our knowledge of the role of kisspeptin in the feedback control of gonadotrophins. These studies demonstrated that negative regulation of hypothalamic *KiSS-1* gene expression by estrogen appears to be restricted to the arcuate nucleus (Arc), an area classically recognized as pivotal for negative feedback of sex steroids. In contrast, at the anteroventral periventricular nucleus (AVPN), KiSS-1 mRNA decreased after gonadectomy and increased after sex steroid replacement (Smith *et al*., 2005a,b). Considering that the AVPN, which is a sexually dimorphic nucleus with far more KiSS-1-expressing neurons in the female, has been involved in mediating the positive feedback effects of estrogen upon GnRH and LH surges, it is tempting to propose that KiSS-1 neurons (at the AVPN) might be involved also in the generation of the pre-ovulatory gonadotrophin surge via positive regulation of GnRH secretion. Indeed, immunoneutralization of endogenous metastin resulted in blockade of the preovulatory LH surge and disruption of estrous cyclicity (Kinoshita *et al*., 2005). As a whole, expression and functional studies demonstrate the crucial involvement of the KiSS-1 system as an essential downstream element in the negative and (probably) positive feedback loops controlling gonadotrophin secretion (Tena-Sempere, 2005).

Besides feedback control, compelling evidence indicates that hypothalamic KiSS-1 may participate in relaying information regarding the nutritional status of the organism to GnRH neurons, thereby contributing to the link between energy stores and fertility. In this sense, it is well known that proper function of the gonadotrophic axis is gated by metabolic and nutritional factors, although the signals and networks ultimately responsible for such integrated control remain to be fully elucidated. In this network, the adipocyte-derived hormone leptin plays a fundamental role in signalling the magnitude of body energy (fat) stores to the centres controlling reproduction (Casanueva and Dieguez, 1999; Tena-Sempere and Barreiro, 2002). Indirect evidence strongly suggested that the permissive actions of leptin on the reproductive axis are mediated through modulation of GnRH secretion (Cunningham *et al*., 1999). A conspicuous finding, however, is that GnRH neurons do not express leptin receptors (Cunningham *et al*., 1999), evidencing the involvement of intermediate circuits and signals, whose identity has remained ill defined. On the basis of their biological properties, kisspeptins were considered plausible candidates for ultimately conveying metabolic cues (likely signaled via peripheral hormones) onto GnRH neurons. Indeed, our recent studies on the expression and function of the KiSS-1/GPR54 system in conditions of energy insufficiency support this contention. Thus, short-term (72-h) fasting in pubertal animals induced a decrease in the expression of KiSS-1 and an increase in GPR54 mRNAs at the hypothalamus of males and female rats. Moreover, LH responses to kisspeptin *in vivo* and GnRH responses *in vitro* were significantly augmented in fasting conditions. These observations suggest that a decrease in central KiSS-1 tone occurs during negative energy balance, which may in turn cause inhibition of the gonadotrophic axis and sensitization to the effects of exogenous kisspeptin (Castellano *et al*., 2005). More importantly, repeated administration of kisspeptin in a model of under-nutrition of immature female rats, where 30% decrease in daily food intake blunted normal pubertal development, was sufficient to restore vaginal opening (as external index of puberty) in a significant number of animals (60%) and induced robust gonadotrophin and estrogen responses in all rats treated with kisspeptin. Altogether, these findings illustrate the interaction between energy status and the hypothalamic KiSS-1 system, which may constitute a target for suppression of pubertal development and reproductive function in conditions of negative energy balance (Castellano *et al*., 2005). The key role

of leptin in this process has been very recently substantiated, as KiSS-1 neurons were proven to express leptin receptors, and defective *KiSS-1* gene expression at the Arc was demonstrated in leptin-deficient ob/ob mice, a phenomenon that was reverted by leptin treatment (Smith *et al*., 2006). Taken together, these data provide direct evidence for a leptin–kisspeptin pathway in the regulation of the GnRH/gonadotrophin axis. Of note, although body energy stores do have an impact on the expression and function of hypothalamic KiSS-1 system, kisspeptins do not appear to have a specific regulatory effect on feeding (Thompson *et al*., 2004; Castellano *et al*., 2005). This phenomenon further stresses the role of KiSS-1 as a selective downstream regulatory signal for the GnRH–LH axis.

New challenges in kisspeptin physiology

As summarized in previous sections, although the reproductive dimension of the KiSS-1/GPR54 system was disclosed less than 2.5 years ago, our knowledge on its neuroendocrine role in the control of puberty onset and gonadotrophin secretion has rapidly expanded, defining the indispensable function of kisspeptins and GPR54 as essential gatekeepers of GnRH neurons and, hence, of the gonadotrophic axis. Notwithstanding, several key aspects of the physiology of this system remain to be clarified. For instance, the nature and hierarchical position of KiSS-1 neurons within the complex network controlling the GnRH pulse generator are yet to be completely defined. In this context, further work, aimed at characterizing the neuronal inputs and projections of KiSS-1 neurons, is eagerly required. In addition, refined assessment of the functional properties of KiSS-1 neurons should be conducted by means of interference or conditional knock-out of *KiSS-1* (and/or *GPR54*) gene expression in specific hypothalamic cell populations *in vivo*, as well as by the electrophysiological characterization of KiSS-1 neurons *ex vivo*. These studies would allow one to define the potential interactions of kisspeptins and other neurotransmitters involved in the control of GnRH release and to predict the roles, if any, of KiSS-1 in the regulation of other neuroendocrine axes. As an antecedent, pharmacological studies suggested that the kisspeptin system is located distal (or eventually independent) to other central excitatory signals controlling GnRH, such as glutamate and nitric oxide (Navarro *et al*., 2005a,b). In addition, hormonal tests evidenced that central administration of kisspeptin-10 can decrease prolactin secretion in pubertal rats (Navarro *et al*., 2004a). Finally, the major intracellular signals recruited by kisspeptins upon binding to GPR54 in GnRH neurons await to be characterized.

The extraordinary potency of kisspeptins to elicit gonadotrophin secretion, and their high efficiency even after systemic delivery, makes them suitable candidates for pharmacological manipulation of the gonadotrophic axis. In principle, by the use of agonists and antagonists of GPR54 (either natural kisspeptins or their analogues), activation or suppression of the reproductive axis might be achieved. However, to set the basis for the rational use of kisspeptins in gonadotrophin control, further characterization of specific aspects of their physiology is mandatory. For instance, even though pharmacological manipulation of the female reproductive axis is common in clinical practice (from super-ovulation induction to contraceptive suppression), most of the studies on the reproductive roles of the KiSS-1 system have been conducted in the male. Moreover, considering the striking changes in the functionality of the gonadotrophic axis in the reproductive female (ovarian cycle, pregnancy, lactation and senescence), it is anticipated that detailed analyses of this system, in terms of expression and biological functions, at those reproductive states will be of high physiological value. In terms of therapeutic implications, another important aspect to be elucidated is to what extent desensitization to the gonadotrophin-releasing effects of kisspeptins takes place after continuous administration. In this sense, it is well known that the GnRH–LH axis is exquisitely sensitive to the pattern of stimulation, and continuous exposure to high doses of GnRH induces desensitization of LH responses (Belchetz *et al*., 1978). Very recent evidence suggests that, as is the case for GnRH, the pattern of kisspeptin stimulation is determinant for the occurrence of desensitization events. Thus, continuous infusion of kisspeptin-10 desensitized GnRH release in juvenile rhesus monkeys and induced a significant drop in circulating LH levels despite persistent exposure to kisspeptin, which was reverted after cessation of kisspeptin infusion (Seminara *et al*., 2006). In contrast, a protocol of repeated administration of four boluses of kisspeptin-10 in male rats elicited a sustained pattern of LH pulses, without decrement in terms of peak amplitude, duration and secretory mass. Absence of down-regulation of pituitary responsiveness to GnRH with this protocol of administration was further demonstrated by the conserved LH responses in terminal GnRH provocative tests in animals pretreated with four boluses of kisspeptin (Tovar *et al*., 2006). Although species (monkey versus rat) and age (juvenile versus adult) differences are noticed between those studies, the above observations strongly suggest that depending on the pattern of GPR54 activation, maximal activation or reversible suppression of the gonadotrophic axis can be achieved by means of kisspeptin administration—a phenomenon that may pose interesting therapeutic implications.

Another contentious issue regarding KiSS-1 function in the control of gonadotrophin secretion is the potential direct effects of kisspeptins at the pituitary level. In this sense, prominent expression of *GPR54* gene has been demonstrated in human pituitary; yet, the role of KiSS-1 signalling at this site has received less attention. Functional studies assessing direct stimulatory effects of kisspeptins on gonadotrophin secretion at the pituitary have yielded conflicting results. Thus, either no effects or moderate stimulatory actions of kisspeptin upon LH secretion *in vitro* have been reported (Matsui *et al*., 2004b; Thompson *et al*., 2004; Navarro *et al*., 2005a). Of note, some of these discrepancies might derive from differences in the *in vitro* settings used (cell culture versus static incubation) and developmental stages tested (adult versus pubertal). Yet, the available data strongly suggest that the pituitary is not the major primary site of action for the potent gonadotrophin-releasing effect of kisspeptins. Nonetheless, the possibility that GPR54 signalling at the pituitary may play a physiological role in the modulation of gonadotrophin secretion and/or might be involved in additional endocrine or non-endocrine functions cannot be excluded and warrant additional experimentation. Indeed, recent studies in the sheep evidenced that GnRH and kisspeptin co-localize in a subset of neurons within the ovine hypothalamus projecting to the median eminence (Pompolo *et al*., 2006), suggesting that kisspeptin may

function as hypophysiotropic neuropeptide. Similarly, autocrine or paracrine roles of locally produced kisspeptins at the pituitary cannot be ruled out, as prominent expression of *KiSS-1* gene has been detected in rat pituitary at specific physiologic states (our unpublished data).

Finally, within the spectrum of potential reproductive roles of KiSS-1, the possibility of peripheral effects of kisspeptins has remained so far largely neglected. This is partially caused by the fact that GnRH replacement appeared sufficient to rescue the profound hypogonadotrophic state of GPR54-null mice and humans (Seminara *et al*., 2003). However, detectable expression of *GPR54* and/or *KiSS-1* gene has been demonstrated in human and rodent gonads (Kotani *et al*., 2001; Ohtaki *et al*., 2001; Funes *et al*., 2003; Terao *et al*., 2004). Indeed, our recent evidence suggests prominent expression of KiSS-1 mRNA in the rat ovary coinciding with the pre-ovulatory period, and under the control of the LH surge (our unpublished data)—a finding whose physiological relevance is yet to be defined. Similarly, the potential role of KiSS-1 in the testis, where prominent expression of its mRNA has been described in the human (Ohtaki *et al*., 2001), needs to be evaluated. In addition, another conspicuous issue is the function(s), if any, of circulating kisspeptin, whose levels are generally low to negligible but dramatically increase in human pregnancy (Horikoshi *et al*., 2003). Interestingly, LH levels in pregnancy tend to be decreased, despite the reported elevation in plasma metastin and its proven capacity to elicit gonadotrophin secretion even after systemic administration. Recent studies conducted by our group in the rat evidenced that the GPR54 system is not desensitized during gestation, as maximal responsiveness and sensitivity to kisspeptin are preserved at mid- and late-pregnancies (Roa *et al*., 2006). This leaves unsolved the underlying mechanism for the apparent contradiction of low LH levels in the face of (presumably) elevated circulating kisspeptin at pregnancy. Overall, definition of the potential impact of circulating kisspeptins upon the reproductive axis might be relevant not only in terms of physiology but also for prediction of clinical manifestations and overall outcome of tumours and other conditions overexpressing KiSS-1 (Aparicio, 2005).

Concluding remarks

In the last 2 years, the KiSS-1/GPR54 system has been recognized as an indispensable regulator of the reproductive axis, whose function is essential for normal puberty onset and proper secretion of GnRH and gonadotrophins. Although physiological characterization of this system is still incomplete, the data so far available allow one to hypothesize that KiSS-1 neurons bear a central, downstream position in the cascade of signals and circuits controlling the GnRH system (Dungan *et al*., 2006), as tentatively depicted in Figure 2. Although such a scheme is an oversimplification of a complex neuroendocrine network, it illustrates the proposed master role of the KiSS-1 system, as integrator of peripheral signals and, possibly, upstream central factors regulating GnRH neurons, i.e. the ultimate effectors for gonadotrophin release. Further characterization of the fundamental role of kisspeptins and GPR54 as gatekeepers of reproduction promises to be an exciting area of research in the near future.

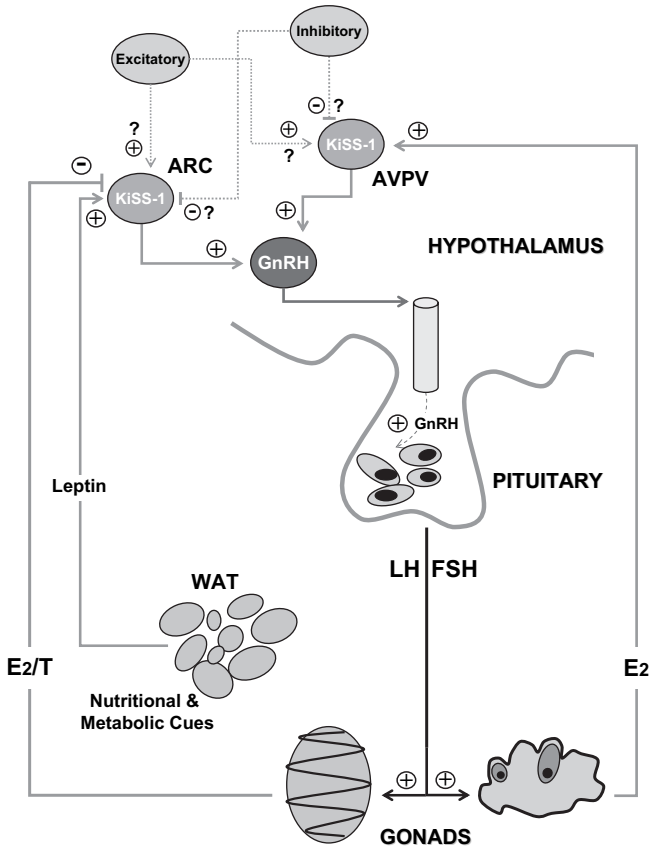

Figure 2. Theoretical model for the role of KiSS-1 neurons as key integrators in the central networks controlling GnRH secretion and, hence, the gonadotrophic axis. On the basis of expression analyses and functional testing, the hypothalamic KiSS-1 system is through to be located downstream in the hierarchy of central regulators of GnRH neurons, which express GPR54 thus being targets for kisspeptins. However, the potential interplay of KiSS-1 neurons with the plethora of central excitatory and inhibitory circuits classically involved in the control of GnRH secretion is yet to be defined. In addition, pivotal peripheral regulators of the gonadotrophic axis appear to operate through modulation of the KiSS-1 system. These include gonadal steroids, as estrogen (E_2) and testosterone (T) have been proven to regulate the expression of *KiSS-1* gene at the hypothalamus. Interestingly, steroid regulation of *KiSS-1* expression seems to be nucleus specific, because androgen and estrogen suppress KiSS-1 mRNA levels at the ARC, although estrogen enhances *KiSS-1* gene expression at the AVPV, thus providing the potential basis for the negative and positive feedback control of gonadotrophin secretion. In addition, the nutritional status modulates the expression and function of KiSS-1 system at the hypothalamus, probably through leptin (produced by the adipose tissue WAT) and/or other metabolic cues, whose nature is yet to be defined. It is highly plausible that other peripheral regulators and central signals, with ability to modulate the gonadotrophic axis, operate though the regulation of the KiSS-1 system. Overall, the emergent model postulates that kisspeptins and GPR54 at the hypothalamus are essential gatekeepers of reproductive function.

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