

Review

The Role of Kisspeptin/GPR54 in the Reproductive System

E. PAPAICONOMOU, P. MSAOUEL, A. MAKRI, E. DIAMANTI-KANDARAKIS and M. KOUTSILIERIS

*Department of Experimental Physiology, Medical School,
National and Kapodistrian University of Athens, Goudi-Athens, Greece*

Abstract. *The Kiss-1 gene encodes a secreted protein that is proteolytically cleaved to produce a number of structurally related peptides, with high interspecies conservation, globally termed kisspeptins. The original niche for the role of kisspeptin in human physiology is derived from cancer biology, with the loss of Kiss-1 expression being associated with poor prognosis in several malignancies. However, kisspeptin has recently emerged as a fundamental player in the field of reproductive biology. Genetic analysis of large consanguineous pedigrees by two independent groups led to the association of inactivating mutations of GPR54, the receptor which mediates kisspeptin action, with idiopathic hypogonadotropic hypogonadism. In the present paper the most salient aspects of the multifaceted role of kisspeptin in the reproductive system are reviewed, including the association of kisspeptin with the gonadal steroid feedback loop and the triggering of puberty onset.*

Kiss-1 was originally identified as a metastasis suppressor gene in melanoma and breast cancer (1, 2). The Kiss-1 gene encodes a secreted protein that is proteolytically processed to produce a series of overlapping peptides, designated as kisspeptins, which range from 10 to 54 amino acids length, and all share a common, and conserved among species, C-terminal decapeptide (3, 4). Three biologically active cleavage peptides of the full-length kisspeptin have been identified in the human placenta, kisspeptin-54 (KP-54), kisspeptin-13 (KP-13) and kisspeptin-10 (KP-10) (5-8).

Correspondence to: Michael Koutsilieris, MD, PhD, Professor and Chairman, Department of Experimental Physiology, Medical School, National and Kapodistrian University of Athens, Micras Asias 75, Goudi-Athens, 11527, Athens, Greece. Telephone: +30 2107462597 Fax: +30 2107462571, e-mail: mkoutsil@med.uoa.gr

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Kisspeptins mediate their effect by binding to at least one known endogenous receptor which has approximately 45% homology with galanin receptors (9). This Gq-protein coupled receptor is now commonly known as GPR54, but the designations AXOR12 and hOT7T175 have also been used in the literature (10, 11).

Reproductive regulation is achieved through a meticulous and tightly orchestrated communication between the hypothalamus, the pituitary gland, and the gonads. The Luteinizing-hormone-releasing hormone (LHRH) neurons represent the final cellular conduit where a plethora of excitatory and inhibitory inputs of central and peripheral origin are integrated and interpreted, with high degree of sophistication, in order to dictate the release of the pituitary gonadotropins. However, the neuronal pathways and molecular mechanisms that govern the neurosecretory activity of the LHRH pulse generator remain enigmatic. The discovery of point mutations or targeted deletions of GPR54 in patients suffering familiar forms of isolated hypogonadotropic hypogonadism (HH) (12, 13), as well as the generation of GPR54 knockout (KO) mouse models, which present a phenocopy of human idiopathic HH, has shed light on the potential role of kisspeptin as a molecular switch for puberty and a transsynaptic modulator relaying sex steroid and circadian signals to LHRH neurons (12, 14).

Localization of Kiss-1 and GPR54 in the brain

In rodents, Kiss-1 mRNA has been detected by either Real Time Polymerase Chain Reaction (RT-PCR) or in situ hybridization in discrete sections of the forebrain, with more robust expression in the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (PeN), and the arcuate nucleus (ARC), as well as more subtle expression in the anterodorsal preoptic nucleus (APN), the bed nucleus of the stria terminalis, and the amygdale (15-20).

In sheep brains, Kiss-1 mRNA expression is located predominantly in the ARC, along with a milder expression in the preoptic area (POA), whereas no expression has been

identified in the AVPV (21-23). A similar pattern is observed in primates, including humans, with high Kiss-1 mRNA expression in the infundibular nucleus, which is homologous to the ARC nucleus, and weaker expression in the POA (24-26).

The Role of Kisspeptin in the Steroidal Feedback Loop

The kisspeptin system may play a role in the two important negative and positive feedback loops of the hypothalamic-pituitary-gonadal axis. Pulsatile release of LHRH into the hypophysial portal blood stimulates the synthesis of the gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH), which are subsequently secreted in the peripheral circulation and stimulate sex steroid secretion from the gonads. Gonadal sex steroids, in turn, inhibit the secretion of LHRH, completing the negative feedback loop. However, in females during the preovulatory period, sex steroids stimulate, rather than inhibit, the secretion of LHRH which induces the preovulatory rise in FSH and LH, thus closing the positive feedback loop (27).

In rodents, sheep, and primates, Kiss-1 neurons in the ARC/infundibular nucleus seem to constitute the cellular element that mediate the sex steroid negative feedback effect. On the other hand, Kiss-1 cells in the AVPV of rodents most likely supply the neuronal circuit that orchestrate the positive feedback regulation. This conclusion is based on the observations that Kiss-1 expression in the aforementioned nuclei is regulated by the gonadal steroid milieu, Kiss-1 neurons express estrogen receptor alpha (ER α) and exogenous kisspeptin can stimulate gonadotropin release.

A) Regulation of Kiss-1 expression by sex steroids. Bilateral orchidectomy or ovariectomy, which eliminates the adulthood gonadal steroids, enhances Kiss-1 expression in the ARC/infundibular nucleus of rodents, sheep, and primates, and suppresses Kiss-1 expression in the rodent AVPV (Figure 1). Sex steroid replacement prevents the post gonadectomy alteration of Kiss-1 expression, and restores Kiss-1 mRNA to control levels (16-17, 22, 24, 28-33).

In rodents the positive steroidal feedback loop is conveyed by Kiss-1 neurons in the AVPV (16-17, 30), whereas non-rodent species seem to diverge from this model. In sheep, a plausible candidate for mediating the positive feedback effect is the Kiss-1 population located in the ARC (21). Indeed, in the ewe, it is the mediobasal hypothalamus rather than the AVPV that is known to comprise the neuronal element necessary for transmitting the positive feedback effect. Furthermore, the population of Kiss-1 neurons in the POA is rather small and is not activated by sex steroids at the time of the cyclic surge, while the ovine ARC contains a larger population of Kiss-1 expressing cells, and the ARC Kiss-1

mRNA is up-regulated before and during the LH surge (21).

While kisspeptin neurons throughout the entire ARC participate in the negative feedback (23), only Kiss-1 cells in the caudal portion of the ARC are responsive during the preovulatory period, although a supplementary population in the rostral ARC seems to be conscripted as well (21). Thus, it is likely that in the ovine ARC the negative and positive feedback is conveyed by distinct subpopulations of Kiss-1 expressing cells.

The distribution of Kiss-1 expressing cells in higher primates, including humans, is considerably similar to that of sheep, with kisspeptin cells located predominantly in the mediobasal hypothalamus and in the human ARC synonymous infundibular nucleus (24, 25). Nevertheless, the possible involvement of the infundibular system in the positive steroidal feedback loop has yet to be determined.

In both monkeys and human women, menopause is accompanied by cellular hypertrophy and increased Kiss-1 mRNA content of kisspeptin expressing cells in the infundibular nucleus (24, 34-35). This data is in agreement with results obtained from gonadectomized cynomolgus monkeys (24, 34) suggesting that the postmenopausal increase in Kiss-1 mRNA expression is due to ovarian failure which, similar to ovariectomy, eliminates the negative feedback control from the follicles. Notably, although it could be argued that the Kiss-1 increase in postmenopausal monkeys is due to neuronal aging per se, this hypothesis appears to be unlikely as no similar age-related changes in Kiss-1 expression have been observed in the POA (34).

B) Stimulation of LH/FSH release by kisspeptin. Several studies have demonstrated that KP-10 and KP-54 are able to stimulate LH release *in vivo* in a variety of species, such as rodents (15, 19, 36-43), sheep (43), cows (44), and primates, including humans (25, 45-47). This may be achieved after systemic (intravenous, intraperitoneal, subcutaneous) or intracerebroventricular (ICV) administration, at different periods of postnatal development (prepubertal, pubertal, or adult) (20, 25, 48-49), and in a dose dependent manner. The rise in serum LH levels can be observed in as early as 10 min and is very durable, often lasting more than 3 hours following kisspeptin administration (20, 37-38, 42).

Kisspeptins have emerged as extraordinarily potent agonists of LH secretion, since low doses delivered centrally or peripherally are able to evoke robust LH discharges (15, 37-38, 42, 47). Even KP-54 doses as low as 1 fmol ICV are adequate in mice (15), a dose that is three to five orders of magnitude lower than the threshold doses of N-methyl-D-Aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and galanin-like peptide (GALP) (50, 51), proclaiming kisspeptin as the most potent secretagogue of gonadotropins known to date, other than LHRH itself.

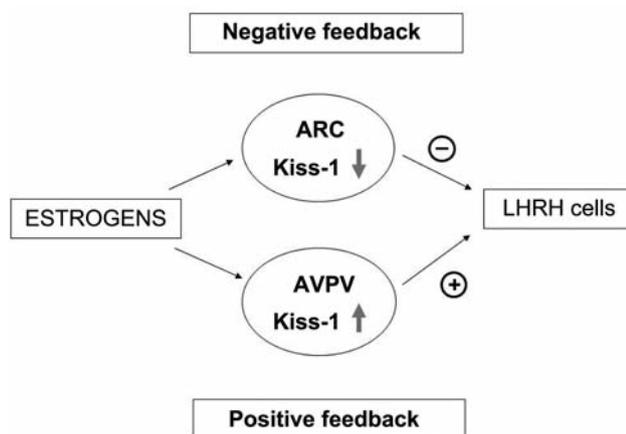


Figure 1. Sex steroids mediate negative feedback by inhibiting *Kiss-1* expression in the arcuate nucleus (ARC) and positive feedback by stimulating *Kiss-1* expression in the anteroventral periventricular nucleus (AVPV).

Similar effects of kisspeptin on FSH levels have also been reported in rodents (15, 36-37, 52-54), sheep (55), and humans (45), although some notable differences are observed. For example, in adult male rats, FSH response to ICV KP-10 administration was delayed (up until 30-min onwards) compared to the more rapid LH secretion (within 5-15-min) (52). In addition, threshold KP-10 ICV doses for FSH stimulation were significantly higher than those for LH, and FSH secretion appeared to be approximately 100-fold less sensitive compared to LH, as the effective dose 50 (ED₅₀) value was estimated to be 400 pmol for FSH and 4 pmol for LH secretion. It remains to be determined whether this dissociation of LH and FSH releases may be due to an effect of kisspeptin on the profile of LHRH release, including changes in the pattern of the frequency and amplitude of LHRH pulses, as it is known that high frequency of LHRH pulses preferentially elicits LH secretion while lower frequency favors FSH secretion. In ewes, however, IV injection of 6.2 nmol KP-10 produced half the maximum response for both LH and FSH, and both gonadotropins were effectively stimulated within 15-min injection, a disparity that may mirror differences between the species or the routes of administration employed (55).

It should be noted that central and systemic kisspeptin administration are equivalent in terms of maximum LH and FSH response (45, 52, 55), and that very low doses of IV KP-10 are able to evoke unambiguous LH release (42). These observations may be explained by the fact that systemically delivered kisspeptin is able to access the LHRH nerve terminals at the median eminence-arcuate nucleus complex, as this region is for the most part positioned outside the blood-brain barrier (56).

Although a single injection of kisspeptin stimulates LH secretion, and repetitive injection generates LH pulses that

can be similar in magnitude to that produced by the LHRH priming infusion (42, 47), continuous kisspeptin delivery disrupts the LH and FSH release in rodents, ewes, and primates (43, 54, 57-59). Gonadotropins are initially increased, but subsequently begin to fall, and finally return to control levels. This desensitization has been ascribed to desensitization of the GPR54 receptor, since NMDA or LHRH challenge is still able to increase the LH secretion during the continuous kisspeptin infusion, while the response to acute kisspeptin administration is truncated or abolished until the receptor recovers some time after the infusion is stopped. Notably, it has been demonstrated in female rats that only LH release can be disrupted by continuous kisspeptin administration, without a simultaneous decrease of FSH secretion (54).

In adult monkeys, continuous kisspeptin treatment may desensitize not only GPR54 but the LHRH receptor as well, given that the LH response to NMDA and LHRH was found to be diminished (57). The contribution of gonadotroph refractoriness in the phenomenon of desensitization has also been reported in ewes, where during the course of ICV kisspeptin infusion plasma LH levels finally decline while LHRH levels in the cerebrospinal fluid are persistently elevated (43). Direct testicular desensitization to kisspeptin administration may also occur, as subcutaneous administration of KP-54 for 13 days in rats led to striking testicular degeneration without significant changes in circulating LH or FSH levels (37). In addition, it should be noted that in pre-pubertal animals desensitization may not always occur, indicating a modification in GPR54 sensitivity during puberty (54).

C) Kisspeptin and sex steroid receptors. Since LHRH neurons do not express the receptor subtypes which are considered to mediate the positive and negative steroidal feedback loop, namely ER α and androgen receptor (AR) (60), it was accordingly speculated that other steroid-sensitive interneurons, located “upstream” of LHRH neurons, accept and convey the sex steroid inputs to the reproductive axis (61). In the ARC of male mice, 87% of all *Kiss-1* mRNA expressing cells have been shown to co-express ER α mRNA, while 64% co-express AR mRNA (17). In female mice 99.8%, 98.7 %, and 97.9% of all *Kiss-1* mRNA expressing neurons in the ARC, AVPV, and PeN, respectively, also express ER α mRNA, while ER β is also co-expressed in 25.0%, 30.5%, and 43.3% of *Kiss-1* cells in these nuclei respectively (16). In female mice, 67% of kisspeptin neurons in the AVPV express progesterone receptors (PR) as well (62).

In rats, 62% of *Kiss-1* neurons in the AVPV and 70% in the ARC express ER α mRNA, while a smaller component of *Kiss-1* expressing neurons in the ARC (11%) and AVPV (21%) also express ER β mRNA (32). In the ewe, ER α

immunoreactivity is detected in 93% of kisspeptin-10 cells in the caudal ARC, but only in about 50% in the POA (13). Approximately 86% of kisspeptin immunoreactive cells in the ARC of ewes are reported to co-localize with PR as well (23). In postmenopausal women, Kiss-1 mRNA has been detected in the larger fraction of the hypertrophied infundibular nucleus (24), with the distribution and morphology of kisspeptin neurons matching that of ER α and neurokinin B (NKB) neurons (63-64). The above observations indirectly indicate that kisspeptin, NKB, and ER α are co-localized.

In female mice, the absence of ER α fully blunts both negative and positive Kiss-1 regulation by estradiol (E2) in ARC and AVPV, while ER β KO mice show no discernible impairments in feedback regulation and LHRH/LH secretion compared to the wild type animals (16). In male mice, the effect of testosterone (T) in Kiss-1 expression in the ARC is mimicked fully by estradiol and partially by dihydrotestosterone (DHT), which is a non-aromatizable androgen, indicating that T effect may be coupled to both ER α and AR (15). This finding is supported by the fact that testosterone retains its effect in mice that lack the ER α (ER α KO mice), as well as in mice that bear a hypomorphic AR allele (AR^{invflox(ex1)-neo} mice), and is furthermore confirmed by the presence of either ER α or AR coexpression in the majority of identifiable KiSS-1-positive neurons in the ARC (17).

The primary mechanism of T action probably involves the ER after aromatization to estradiol, as estradiol fully imitates T effect while DHT only partially mimics it. In the AVPV and PEN, the effect of testosterone on Kiss-1 regulation appears to be conveyed mainly by ER α , since DHT has been shown to have no distinct effect on Kiss-1 mRNA expression. However ER β may also be involved, since T is still able to act in ER α KO mice (17).

In female rats, selective blockade of ER α and ER β demonstrated that ER α plays a major positive role in the regulation of endogenous preovulatory LH surge and in the LH responsiveness to kisspeptin, whereas ER β probably does not participate in the endogenous LH surge but may operate as an inhibitory modulator of the LH response to kisspeptin (65). As far as the FSH surge is concerned, blocking experiments showed that ER α and PR are both equally involved in the generation of the primary and secondary FSH surge, as well as in the FSH response to kisspeptin during the preovulatory period, and that ER β does not affect the endogenous FSH surges but serves as a weak positive modifier of the preovulatory FSH responsiveness to kisspeptin (53). The dual role of ER β in LH/FSH responsiveness suggests that estrogens transmit through ER β opposite regulatory signals targeting LH and FSH secretion, and may in this way contribute to the phenomenon of LH/FSH dissociation. Finally, in female rats, the ability of estradiol replacement to inhibit the increase of the

hypothalamic Kiss-1 levels following ovariectomy (OVX) can be mimicked by the selective ligand of ER α propyl pyrazole triol (PPT), but not by the selective agonist of ER β diaryl propyl nitrite (DPN), signifying the contribution of ER α pathways in this phenomenon (20).

Kisspeptin Stimulates the LHRH Neurons Directly

There is accumulating evidence that the kisspeptin system triggers the surge mechanism directly at the level of LHRH neurons: a) Close appositions between kisspeptin fibers and LHRH neurons have been persistently detected; b) GPR54 is expressed in LHRH neurons; c) Administration of LHRH antagonists or infusion of antibody against kisspeptin into the POA hinders the cyclic surge; d) Kisspeptin evokes c-Fos expression and membrane depolarization of LHRH neurons.

Dual immunofluorescence experiments in mice and female rats showed close appositions between kisspeptin fibers and LHRH cell bodies in the POA (18, 40). In mice, the Kiss-1 neurons that derive from the AVPV/PeN project to the LHRH cell bodies in the POA, whereas the Kiss-1 neurons that derive from the ARC seem to project to the LHRH nerve terminals, which are located in the median eminence (18). In adult female mice, approximately 40%, 12%, and 10% of the LHRH neuron somata and proximal dendrites in the rostral portion of the POA (rPOA), the horizontal and vertical dimps of the diagonal band of Broca respectively, were presented with kisspeptin-immunoreactive fiber appositions. Moreover, in adult male mice, appositions were only found in the rPOA and in 10% of the LHRH neurons, a considerably reduced percentage compared to females. The kisspeptin appositions to the LHRH neurons are believed to emerge from the AVPV/PeN nucleus, because the appearance of kisspeptin appositions to LHRH neurons during postnatal development of mice is concurrent with the appearance of kisspeptin neurons in the AVPV/PeN, and both kisspeptin appositions and AVPV/PeN kisspeptin neurons are sexually dimorphic (18). This hypothesis is also in agreement with recent studies demonstrating that the AVPV cells project to the POA LHRH neurons in female rats (66).

In mice, mediobasal hypothalamus (MBH) explants containing the median eminence (ME) region, where the LHRH nerve terminals are located, but lacking the POA, where the LHRH cell bodies are sited, respond by releasing LHRH when challenged with KP-10 and in a GPR54-dependent manner. This can occur even at the presence of tetrodotoxin which blocks any possible effect from the few remaining LHRH cell bodies left (67). Thus, kisspeptin may act directly at the LHRH nerve terminal of the MBH.

In the monkey hypothalamus, in the agonadal condition, kisspeptin-beaded axonal projections have been discovered throughout the mediobasal hypothalamus and, to a lesser degree, in the POA. At the ARC nucleus, axo-somatic, axo-

Table I. Molecular, laboratory, and clinical characteristics of loss-of-function or gain-of-function GPR54 mutations*.

Mutation	Receptor impairment	<i>In vitro</i> receptor activity	Phenotype	Reference
c.443T>C (L148S)	Impaired signaling	Significantly reduced	IHH	(12)
c.991C>T (R331X)	Protein elongation	Significantly reduced	IHH	(12)
c.1195T>A (X399R)	Protein elongation	Significantly reduced	IHH	(12)
g.2937_3091del	Protein truncation	Not assessed	IHH	(13)
c.667T>C (C223R)	Impaired signaling	Significantly reduced	IHH	(75)
c.891G>T (R297L)	Impaired signaling	Slightly reduced	IHH	(75)
c.1001_1002insC	Protein elongation	Not assessed	IHH	(76)
c.305T>C (L102P)	Impaired signaling	Significantly reduced	IHH	(77)
c.1157 G>C (R386P)	Prolonged signaling	Significantly prolonged	CPP	(81)

*The nomenclature system proposed by den Dunnen and Antonarakis (2000) is used for the description of GPR54 mutations (98). CPP: Central precocious puberty, IHH: isolated hypogonadotropic hypogonadism.

dendritic and axo-axonic contacts of kisspeptin on LHRH neurons are infrequent, but the kisspeptin perikarya may occasionally be contacted by LHRH-beaded axons, highlighting the possibility of reciprocal control between kisspeptin and LHRH. In the median eminence, kisspeptin and LHRH axons appear to be extensively and intimately associated, allowing speculation for non synaptic kisspeptin pathways at this level (57).

In sheep, numerous kisspeptin immunoreactive fibers have been found in the POA and the median eminence, with maximum density located in the internal zone (22). In pony mares numerous close appositions have been observed in the median eminence, and fewer have been detected in the ARC (68). In the rat brain, 77% of the LHRH neurons express GPR54 (29), whereas in mice this expression rate varies from 55% (43) to 90% (49). The stimulatory effect of both centrally and peripherally administered kisspeptin on LH/FSH secretion, in different species, is blocked with co-treatment with LHRH receptor antagonists such as acyline, cetrorelix, and Org 30276 (15, 19, 25, 47, 69). Furthermore, administration of monoclonal antibodies against kisspeptin into the POA blocks the preovulatory surge in female rats (30, 40).

In rodents, kisspeptin administration elicits c-Fos (an early response gene) expression in LHRH neurons (20, 62, 70). Furthermore, kisspeptin neurons in the AVPV also express c-Fos during the LH surge (30, 32, 40, 62, 71), and in strong correlation with the c-Fos expression in the LHRH cells, indicating that the kisspeptin cells in the AVPV are activated during the positive feedback loop. In contrast, the percentage of c-Fos-expressing Kiss-1 cells in the ARC is low and does not rise during the LH surge. Multiple studies have also shown that kisspeptin exerts a remarkably strong and durable stimulatory effect on the electrical excitability of the LHRH cells. In adult mice, for example, kisspeptin evokes a prolonged membrane depolarization of approximately 90% of the LHRH cells (49).

Kisspeptin Acts as a Molecular Switch for Puberty

The paramount importance of kisspeptin as a regulator of the neuroendocrine reproductive axis and the puberty onset was first brought to light in 2003, by Seminara *et al.* (12) and by de Roux *et al.* (13) independently, based on clinical studies of patients who carried loss-of-function GPR54 mutations and fulfilled the established diagnostic criteria for isolated hypogonadotropic hypogonadism (IHH). Subsequently, more GPR54 mutations have been described in patients with IHH (Table I). Furthermore, transgenic knockout technology provided Kiss-1 and GPR54 null mice, which exhibit striking defects in pubertal development and essentially represent phenocopies of human hypogonadotropic hypogonadism syndromes (12, 14, 70, 72-73).

A) Kisspeptin association with IHH syndromes and CPP. IHH is defined by low levels of sex steroids coupled with low levels of FSH and LH and normal levels of the other pituitary hormones, and is further separated into two subgroups based on the presence (Kallmann syndrome) or absence (idiopathic IHH) of anosmia. About 50% of familial cases of idiopathic IHH are attributable to loss-of-function mutations of the LHRH receptor (74), whereas GPR54 mutations seem to be responsible for a small part of the remaining 50% (12-13, 75-77).

Seminara *et al.*, described a Saudi Arabian family, members of which were carrying a homozygous single-nucleotide variant (c.443T>C) in exon 3 of GPR54 (12). This mutation substitutes serine for the normal and highly conserved among species leucine (L148S) in the second intracellular loop of the receptor, and alters the polarity of the amino acid residue from hydrophobic to neutral. They also reported an unrelated patient with sporadic IHH, who was a compound heterozygote for the GPR54 mutations R331X and X399R. The R331X is a nonsense mutation that involves a c.991C>T substitution in

exon 5 of GPR54, and results in the replacement of arginine at residue 331 with a premature stop codon. The X399R is a nonstop mutation that involves a c.1195T>A substitution, in exon 5, which replaces the stop codon at amino acid residue 399 with an arginine, thus leading to the continuance of the open reading frame up to the polyA signal and to the production of an elongated receptor protein. However, this nonstop transcript was only produced in an inconsequential quantity in the heterozygote patient. Transfection of COS-7 cells with L148S and R331X constructs resulted in a considerable decrease of maximal inositol phosphate response to the c-terminal kisspeptin decapeptide kisspeptin 112-121. It is also worth noting that the patient harboring the compound heterozygous mutations showed decreased LH secretion following LHRH administration compared with IHH patients without GPR54 mutations (12).

Furthermore, Wacker *et al.* demonstrated in 2008 that the L148S mutation does not influence the expression, localization, or ligand binding properties of GPR54, but affects functional coupling of the receptor, since the mutant GPR54 failed to set off G-protein dissociation or to stimulate phospholipase C production or ERK1/2 activation (78). De Roux *et al.*, reported a large consanguineous family comprising of five siblings with IHH, who carried a homozygous deletion of 155 nucleotides that removes the splicing acceptor site of the intron 4–exon 5 junction, as well as part of exon 5, resulting in deletion of the regular protein sequence downstream of amino-acid residue 247 (13). The mutant receptor is truncated within the third intracellular loop, and therefore lacks transmembrane domains 6 and 7. Such receptors are known to be incapable of activating the G-protein-coupled signal transduction pathways, although it was not determined whether the truncated protein was indeed produced or there was no protein synthesis at all in these patients.

Semple *et al.* described an individual of mixed Turkish-Cypriot and Afro-Caribbean descent, who was a compound heterozygote for two rare sequence variants (75). The first involved a c.667T>C point mutation in exon 4, leading in the substitution of the highly conserved cysteine with arginine at the fifth transmembrane helix (C223R), and the second involved a c.891G>T mutation in exon 5, resulting in the substitution of arginine with leucine in the third extracellular loop (R297L). Calcium mobilization assays demonstrated that the C223R mutation causes a dramatic impairment of GPR54 signaling capability, whereas R297L has a milder effect, which is consistent with the fact that R297L is sited in a less conserved region. Even though the patient had not reached the age of puberty, he had clinical and biochemical indications of IHH, such as bilateral cryptorchidism and micropenis at birth, undetectable gonadotropins at 2 months of age, and poor response to LHRH stimulation at the age of 10. At 10 years of age his testosterone response to hCG stimulation was also low, suggesting direct impairment of testicular function (75).

Lanfranco *et al.* reported a case of an insertional mutation (c.1001_1002insC) in a patient with sporadic IHH and consanguineous parents that resulted in open reading frame shift and stretch of 43 amino acids of the seventh transmembrane segment, in the intracellular domain of the receptor (76). There was a remarkable contribution of proline residues to the protein elongation that conferred high hydrophobicity, compatible with impaired signal transduction. It should be noted that the patient received pulsatile LHRH therapy and reached sperm maturation adequate to induce pregnancy with assisted fertilization (76).

Tenenbaum-Rakover *et al.* reported a family with five affected patients bearing a homozygous mutation that involved the substitution of thymidine with cytosine at position 305, and resulted in a leucine substitution with proline at amino-acid residue 102 (c.305T>C [L102P]) within the first extracellular loop (77). The substitution of a hydrophobic residue like leucine with proline impedes the normal conformational change of the receptor during activation, and completely blunts its ability to activate the phospholipase C (PLC) pathway. The binding affinity of the mutant receptor was normal, although there was a small decrease in its cell-surface expression. Analysis of LH pulsatility, following LHRH stimulation, showed peaks with low amplitude but normal and homogeneous frequency. It is also of note that there was variable expressivity of the mutation in the same family, with some members presenting partial and others complete IHH. In fact, an affected homozygous patient demonstrated, between the ages of 12 and 21 years, a progressive increase in pituitary responsiveness to LHRH stimulation, from an early pubertal to a nearly full pubertal pattern, with shift from an FSH-predominant to an LH-predominant response, which is similar, although at a slower rate, to what occurs in normal puberty. Therefore, this mutation may retard rather than completely inhibit the onset of puberty.

Three GPR54 variants have also been reported by Cerrato *et al.*, two of which were detected in the non-coding sequence, whereas the remaining variant, (c.1079A>T [H360L]), was found in a heterozygous form (79). Since loss-of-function mutations of GPR54 are related to IHH, it is plausible that gain-of-function mutations could be associated with central precocious puberty (CPP). Indeed, in rodents, chronic intracerebral treatment of immature female rats with kisspeptin, between 26 and 31 day postpartum, resulted in precocious activation of the hypothalamic pituitary gonadal axis (HPG), as confirmed by the elevated levels of gonadotropins and sex steroids, as well as the increased uterus weight (69).

In 2007, Luan *et al.* studied a cohort of Chinese girls with CPP and discovered a novel nonsynonymous single nucleotide polymorphism (SNP) of the kisspeptin ligand that substitutes proline for threonine at the 110th amino acid

residue (c.328C>A [P110T]), and was statistically related to CPP (80). Moreover, Teles *et al.* reported an autosomal dominant gain-of-function mutation of GPR54 (c.1157 G>C [R386P]) in an adopted girl with CPP, that involved the substitution of a guanine with cytosine at nucleotide position 1157 in exon 5, and resulted in the substitution of proline for arginine at codon 386 (81). The R386P variant led to a reduced rate of receptor desensitization that resulted in a prolonged activation of the intracellular signaling pathways. Indeed, while for both wild-type and R386P receptor inositol phosphate levels peaked at 2 h, the decline in IP accumulation was slower with R386P, and significantly higher levels of IP were observed 18 hours after kisspeptin stimulation. Strikingly, the patient exhibited premature breast development at the neonatal period, an observation which may indicate neonatal activity of the kisspeptin-GPR54 signaling or may result from the activity of maternal steroids to the fetus during gestation. In addition, De Vries *et al.* have measured significantly higher plasma kisspeptin levels in girls with CPP compared with controls (82).

B) GPR54 and Kiss-1 knockout mouse. Both genders of adult GPR54 knockout (KO) mice demonstrate striking reproductive abnormalities, including underdevelopment of gonads and accessory reproductive organs, diminished levels of gonadotropins and steroid hormones, impaired gametogenesis, and absence of estrous cycling. GPR54 KO females have smaller ovaries and uteri than their wild type (WT) littermates, delayed vaginal opening, scant folliculogenesis, and acyclicity. GPR54 KO males display decreased mean body weight, smaller testes and genitalia, reduced sperm count, lack of preputial separation, and shorter anogenital distance than WT males (12, 14, 70, 72-73). Baseline gonadotropin levels in male or female GPR54 KO mice are low, and do not demonstrate any increment after kisspeptin stimulation. However, GPR54 KO mice have been reported to respond to estradiol with LH surge, which is also temporally associated with c-Fos expression in LHRH cells (73).

The observation that male and female WT and KO embryos have no histological differences at 16.5 days post-conception, along with the fact that GPR54 expression is low in WT mice at that stage, suggests that the impact of the GPR54 deletion is most prominent postnatally, prior to puberty (14). However, a divergence in anogenital distance between KO and WT males becomes clear at 3 weeks of age (70), although a postweaning difference that does not reach statistical significance has been observed as well (72). Therefore, GPR54 may also act during the fetal and/or the juvenile period, which is consistent with the fact that a patient with a GPR54 mutation presented microphallus and cryptorchidism at birth, as mentioned above (75).

Kiss-1 knockout mice also have impaired sexual maturation (72, 83), though they display a milder phenotype

compared to their receptor KO counterparts, and retain responsiveness to kisspeptin stimulation (72). Lapatto *et al.* also showed that the female Kiss-1 KO mice they generated exhibited a bimodal phenotypic variability (72). One subgroup in particular had larger ovaries, larger vaginal openings, and vaginal smears that showed persistent vaginal cornification, which is indicative of the presence of higher estrogen levels, while the other subgroup had a similar phenotype to that of the GPR54 KO mice. This latest subgroup showed a more robust response to kisspeptin administration than the former.

The fact that GPR54 and Kiss-1 KO mice have LHRH neurons that are correctly anatomically located, project to the median eminence, and have normal morphology and LHRH mRNA content, substantiates the hypothesis that the hypogonadism is not due to abnormal maturation or migration of the LHRH neurons, as it is the case in the X-linked form of Kallmann's syndrome, but is derived from decreased LHRH secretion by hypothalamic LHRH neurons (12, 43, 73).

Sexual Differentiation of the Kiss-1 System

In rodents, the expression of Kiss-1 in the AVPV/PeN nucleus is sexually differentiated, with adult females exhibiting up to 25 times more Kiss-1 expressing neurons and Kiss-1 mRNA cellular content than adult males, under identical hormonal conditions (intact or gonadectomized, with or without replacement treatment) (18, 28, 30). Furthermore, in rats, Kiss-1 neurons are more densely located in the medial portion of the AVPV in females compared to males (30), although in mice the overall distribution of kisspeptin immunoreactivity is almost identical between males and females (18).

The Kiss-1 sex difference in the rat AVPV is not attributable to dissimilarities in the circulating levels of gonadal steroids in adulthood, since adult gonadectomized males and females receiving similar hormonal replacement still display robust gender Kiss-1 expression differences (28). In contrast, it appears to be the sex steroid milieu during a "critical window" of perinatal development that influences the Kiss-1 expression in the adult AVPV, because neonatally androgenized females exhibit male-typical pattern (18), while male rats castrated as newborns exhibit female-like pattern of Kiss1 expression in the AVPV/PeN as adults (83).

In contrast to AVPV, no gender based difference has been observed in the ARC with regards to the distribution pattern and density of Kiss-1 expression, independently of the hormone levels during development or adulthood (18, 28, 30). It is also notable that in the rat AVPV the sexually dimorphic Kiss-1 and tyrosine hydroxylase (dopaminergic) neuron systems represent two separate and discrete populations, because although there may be some slight

overlapping in their anatomical distribution, and a modest degree of co-labeling by *in situ* hybridization, the amount of tyrosine hydroxylase mRNA in the double-labeled Kiss-1 cells is low (28). Quite the opposite is observed in mice, where practically all Kiss-1 neurons in the AVPV co-express tyrosine hydroxylase mRNA (84). In summary, it is of note that there is an inverse regulation of tyrosine hydroxylase and kiss-1 neurons by sex steroids in rats, a fact that could mirror a collaborative functioning between the two populations in generating the LH surge *via* the simultaneous inhibition of dopaminergic cells and activation of the kiss-1 system which may result in increased net stimulation of LHRH neurons (28).

Kisspeptin Influences the Development of Sexual Differentiation

In mice, GPR54 signaling in perinatal life is indispensable for the appropriate male-like development of a number of sexually dimorphic traits in adulthood, most likely by regulating the perinatal LHRH-induced gonadal hormone secretion (70). This inference is based on the fact that GPR54 KO males display deficient masculinization/defeminization of several sexually dimorphic characteristics. One of the sexually dimorphic parameters inhibited is the olfactory partner preference. GPR54 KO T-treated or untreated males consume an equal proportion of their investigatory time with males and females, while their WT counterparts show a 70% increased olfactory partner preference for females over males. This female-like preference does not appear to be caused by anosmia, since GPR54 KO mice performed normally at a “hidden cookie test”. In addition, while WT males exhibit low numbers of tyrosine hydroxylase neurons in the AVPV and high numbers of motoneurons in the spino-bulbocavernosus nucleus compared to females, GPR54 KO males display feminized patterns of tyrosine hydroxylase neurons and motoneuron expression in the spind nucleus of the bulbocavernosus, even with proper adult testosterone replacement. On the other hand, perinatal GPR54 signaling does not seem to affect the gender-specific sexual behavior of properly hormone-replaced adult male and female mice. For instance, GPR54 KO adult males may not exhibit any male sexual behavior at all, but when they are treated with T they display robust male copulatory behavior, confirming that the previous impaired sexual behavior was due to shortage of activational adult T (70).

Postnatal Development of the Kiss-1 and GPR54 System

In the hypothalamus of both male and female rats, Kiss-1 and GPR54 mRNA is persistently expressed throughout

postnatal development, with moderate levels found at birth, decreased levels in prepubertal animals, and maximum levels at puberty (20). In female rats Kiss-1 mRNA levels in the AVPV increase at day 26, decline at day 31, and rise again at day 36/41, whereas in the ARC, Kiss-1 mRNA expression increases at the time of the vaginal opening. Moreover, estrogen replacement of OVX rats is able to suppress the enhanced Kiss-1 expression in the ARC, along with the detected LH pulses, only during the prepubertal stage, suggesting that ARC Kiss-1 expression and LH pulses are negatively regulated by estradiol more strongly during the prepubertal period compared to adulthood (85). Kisspeptin immunoreactive (kisspeptin-IR) cells have also been reported during puberty onset in the ARC, PeN, and POA of female rats. In postpuberty, the number of kisspeptin-IR cells is reduced in the ARC and PeN and increased in POA, while no kisspeptin-IR cells are detected in pre-pubertal animals (86).

Kisspeptin is able to evoke robust secretory LHRH and LH response, both *in vivo* and *ex vivo*, during all periods of postnatal maturation (48). In prepubertal rats, ICV kisspeptin administration stimulates a clear increase in serum LH, from low prepubertal values to levels comparable to those of adult animals (20). Furthermore, in male rats, there is a considerable enhancement of LH responsiveness to sub-maximal doses of kisspeptin during pubertal transition (48). LHRH mRNA values in the rat hypothalamus do not synchronize with changes in Kiss-1 and GPR54 mRNA, supporting the conjecture that kisspeptin does not act through transcriptional regulation of the LHRH gene but *via* the stimulation of LHRH release (20).

In male and female mice no kisspeptin neurons are identified in the AVPV/PeN at postnatal day 10 (juvenile stage), while increased numbers are found at postnatal day 25 (prepubertal stage) and adult levels are achieved at the time of puberty onset. This pattern of postnatal development is not observed in the ARC or dorsomedial hypothalamus. Close appositions between kisspeptin fibers and LHRH neuron somata are virtually absent before postnatal day 25, and begin to develop afterwards (18, 62).

In rhesus monkeys, hypothalamic Kiss-1 mRNA levels increase along puberty in both agonadal males and ovary-intact females, while GPR54 mRNA levels display a 3-fold increase from juvenile to midpubertal stage only in females (25). In females, an increase in KP-54 release in the stalk-median eminence (S-ME) across puberty has also been reported, in concert with the pubertal increase of LHRH release. The KP-54 release was pulsatile, with approximately 75% of kisspeptin pulses correlating with LHRH pulses. Furthermore, a nocturnal increase in KP-54 release was detected at the prepubertal stage, although the nocturnal increase in LHRH release occurs in early pubertal and midpubertal but not prepubertal animals (87).

Local Kisspeptin Effects on Ovaries and Testes

GPR54 expression has been detected in testes (6, 8, 14, 57) (6, 8, 12, 54) and ovaries (88-90), and the available data suggest that kisspeptin may have an additional direct effect on the gonads, in addition to the anticipated indirect effects through the modulation of the HPG axis. In adult male rats, long-term subcutaneous administration of 50 nmol of KP-54 for 13 days resulted in a significant reduction in testicular weight, accompanied by degeneration of the seminiferous tubules with loss of both germ and Sertoli cells as well as a significant reduction of the circulating levels of the testes-derived hormone inhibin B. This testicular degeneration was similar to what is observed after continuous administration of LHRH agonists (91-93), which are known to exert extrapituitary direct testicular effects (92, 94), supporting the hypothesis that kisspeptin can mediate direct testicular effects (37). Furthermore, continuous infusion of human kisspeptin in adult male monkeys significantly alters the quantitative relationship between serum LH and T levels, with plasma testosterone concentration for a certain LH stimulus being invariably higher during the high-dose kisspeptin infusion compared to vehicle infusion, an observation that is also suggestive of a direct testicular effect by kisspeptin (57). In addition, the patient harboring a c.667T>C (C223R) mutation described by Semple *et al*. had low T response to hCG at age of 10, which is also indicative of direct testicular impairment (66).

Kiss-1 expression in rodent ovaries and oviducts show cycle-dependent changes, with peak levels in proestrus and estrus and lower levels at metestrus and diestrus (88-90, 95). The proestrus rise in Kiss-1 expression in rat ovary is blunted by administration of LHRH antagonists, and restored by hCG treatment (88). In turn, the stimulatory effect of hCG is partially hampered by indomethacin (90). In the rat ovary, kisspeptin-IR has been detected in the corpus luteum and in the cytoplasm of steroidogenic cells of theca and granulosa origin, indicating the potential involvement of kisspeptin in tissue remodeling during the formation and/or regression of corpora lutea, as well as in hormone secretion of the steroidogenic luteal cells (88).

Kiss-1 expression in the rat oviduct follows a discrete distribution pattern, with higher expression in the isthmus, fainter in the proximal ampulla, and undetectable expression in the fimbriated infundibulum and the interstitial portion (95). This distribution pattern, along with the fact that kisspeptins are physiological regulators of implantation, partly *via* the down-regulation of matrix metalloproteinases (MMPs) (5, 96), indicate the potential role of kisspeptin in the coordination of embryo-epithelial interactions in rats which may contribute to the extremely low frequency of ectopic tubal pregnancies in laboratory rats compared to humans (97).

Conclusion

Since the first report demonstrating that GPR54 loss-of-function mutations are correlated with the IHH phenotype (12), an increasing number of studies in several mammalian species, mainly rodents, sheep, and primates, is supplying mounting evidence regarding the role of kisspeptin/ GPR54 signaling in the normal regulation of the reproductive system. Kiss-1 is an estrogen-dependent LHRH neuron regulatory system employed during late postnatal maturation in order to trigger puberty onset, and then serves as a vital part of the neural circuitry that modulates the positive and negative steroidal feedback effect at the hypothalamus, and stimulates the preovulatory LHRH/LH surge in females. Recent data further suggest that Kiss-1 may also act directly on peripheral reproductive organs (ovaries/testes).

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