Published in final edited form as:
J Neuroendocrinol. 2008 June ; 20(6): 727–731. doi:10.1111/j.1365-2826.2008.01731.x.

Kisspeptin and GPR54: Discovery of a Novel Pathway in Reproduction

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Abstract

In order to find novel modulators of gonadotrophin-releasing hormone (GnRH) secretion, genetic tools were employed in patients with idiopathic hypogonadotrophic hypogonadism (IHH). Mutations in a G-protein coupled receptor, GPR54, were identified, making this receptor a genetic determinant and indisputable gatekeeper of normal reproductive function. This article places these investigations into historical context and reviews some of the new findings relevant to this pathway.

Keywords

kisspeptin; GPR54; metastin; genetics; hypogonadotrophic hypogonadism

The question

What triggers human puberty is one of the central mysteries of reproductive biology. What factors are responsible for the amplification of the pulsatile gonadotrophin-releasing hormone (GnRH) signal intrinsic to GnRH neurones (1) at the expected time of sexual maturation? What factors modulate the amplitude and frequency of GnRH throughout reproductive life, particularly during the female menstrual cycle? Our research team initially approached the identification of such modulators by employing linkage analysis in an inbred family with a clinical syndrome characterised by endogenous GnRH deficiency, i.e. normosmic idiopathic hypogonadotrophic hypogonadism (nIHH). IHH is characterised by abnormal/absent GnRH-induced luteinising hormone (LH) pulsations in which patients present with delayed or absent pubertal development and other associated somatic abnormalities. Although rare, nIHH is a disease caused by defects in the synthesis, secretion and action of GnRH and, therefore, has long been considered an important entrée to understanding the modulators of GnRH in humans.

Genetic precedents

Up until that point, only a few genes had been implicated in IHH, the first being *KAL1* in 1991 (2,3). Hypogonadotrophism caused by mutations in *KAL1* was shown to be caused by failed embryonic migration of GnRH neurones along the olfactory nerve pathway. Another gene was soon implicated in nIHH that encodes the GnRH receptor, *GNRHR* (4,5). Less informative at the hypothalamic level, GNRHR mutations result in ligand binding and receptor activation defects, yielding several new insights into the structure–activity relationships of this pituitary receptor. Mutations in a third gene, *NROB1* (formerly known as *DAX1*), have been identified

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in patients with adrenal hypoplasia congenita (IHH and adrenal insufficiency) (6). Mutations in this gene cause hypogonadism at multiple levels of the hypothalamic–pitutiary–gonadal axis, including pituitary resistance to GnRH and abnormal testicular development. The physiology revealed by the discovery of each of these genes is both unique and important. That said, none of these biological stories spoke directly to the functionality of the GnRH neuronal network but rather to its migration and/or impact upon the gonadotroph.

Challenges in the scientific approach

The time was therefore ripe to discover a gene or pathway that more directly affected GnRH neuronal function. However, the approach that we chose, linkage analysis, was a challenge to execute. Normosmic GnRH deficiency is rare, affects fertility, and hence reduces family size. Consequently, the large families with multiple affected individuals so essential to many genetic approaches are rare. Although establishing the diagnosis of GnRH deficiency is relatively straightforward, its phenotypic expression is delayed until the time of puberty. Therefore, it is difficult to assign affected/unaffected status to pre-pubertal children and hence truly quantify the disease burden in a family. Although the genetics of nIHH is now increasingly appreciated to be oligogenic (7,8), at the time we initiated our genetic studies, Mendelian rules of inheritance were the accepted standard for the disease. However, even within a straightforward Mendelian framework, IHH was long appreciated to be genetically heterogeneous with autosomal dominant, recessive and X-linked modes of inheritance. This heterogeneity precluded the combination of families for genetic studies. Finally, on a more technical note, while the human genome sequence has now been available for years, overcoming gaps in DNA sequencing was still a formidable problem at the time these studies were initiated. Therefore, linkage analaysis in IHH was fraught with challenges.

Discovery of the mutations in G-protein coupled receptor GPR54

Consequently, as most of our families were too small to enable linkage to be pursued, we were limited in the number of families that were suitable for genetic studies. However, we identified a family from Saudi Arabia with three first-cousin marriages and five affected individuals with IHH with sufficiently robust statistical power to achieve linkage, and a genome-wide scan was performed. Linkage over a 1.06-Mb interval on chromosome 19p13.3 was established with a maximal two-point logarithm of odds (LOD) score of 5.17 (9). The candidate region contained 23 genes and 49 UniGene clusters. Using sequencing, base pair changes were identified in a then little-known seven transmembrane domain receptor, GPR54. (10). In the index family, a homozygous variant (443T → C) in exon 3 substituted a serine for the normal leucine at position 148 (L148S) in the second intracellular loop and this mutation segregated perfectly within the family. In an unrelated African American proband, a heterozygous C to T transition at nucleotide 991 in exon 5 [991C → T (R331X)] was identified as well as a heterozygous T to A transversion at nucleotide 1195 in exon 5 [1195T → A (X399R)]. These base pair changes were not identified in single nucleotide polymorphism (SNP) databases nor in hundreds of ethnically matched controls. Mutant constructs representing each nucleotide change were assembled and their deleterious effects on receptor function were demonstrated by *in vitro* functional assays.

Fortunately, the ligand for GPR54 had been identified (7,9,10). Kisspeptin (encoded by KISS1) is a 154-amino acid protein which is proteolytically processed to a 54-amino acid peptide with an amidated carboxy terminus [critical for the biological function of most central nervous system (CNS)-active peptides] called metastin. This peptide was so named because of its ability to inhibit tumour metastasis (kisspeptin 68–121 = metastin 1–54). Although we could not know it at the time, two other groups had observed that the kisspeptin/GPR54 pathway was key to sexual maturation in humans. One group, led by Nicolas de Roux, also used a linkage approach

in an inbred family to uncover a large deletion in GPR54 (11). In addition, a small biotech company called Paradigm Therapeutics (Cambridge, UK) had created GPR54$^{-/-}$ mice. We combined data sets with this company to establish that the kisspeptin/GPR54 system was a gatekeeper of puberty in both mice and men (10). The GPR54$^{-/-}$ mice were phenocopies of the human with abnormal sexual maturation, small gonads and hypogonadotrophic hypogonadism. Notably, the GPR54$^{-/-}$ mice had a normal content of GnRH in their hypothalami, the first indication that mutations in GPR54 do not affect GnRH neuronal migration, or GnRH synthesis, but rather GnRH release.

Genetics of GPR54: lessons from patients with IHH

Despite the fundamental importance of the kisspeptin/GPR54 pathway in modulating GnRH secretion, mutations in GPR54 are not a common cause of GnRH deficiency in humans. While frustrating from a structure/function point of view, the relative rarity of mutations in GPR54 may speak to the importance of this pathway in reproduction. It is possible that mutations in GPR54 may be so deleterious to reproductive function that they may not be propagated in the population, i.e. they act much like lethal mutations. There are no clear mutational hot spots within the gene and the mutation subtypes include deletion, frameshift, nonsense, non-stop and missense changes. Although the functional studies on most of these mutations suggest that their functional sequelae are severe, patients with them exhibit considerable phenotypic variability. Some exhibit complete absence of pubertal development whereas others have only partial, incomplete or delayed puberty. Despite only a handful of case reports of patients with mutations in GPR54, several important observations have emerged:

1. Mutations in GPR54 suggest that this receptor/ligand pair may play an important role in the mini-puberty of infancy. A patient with compound heterozygote mutations had abnormally low levels of sex steroids and gonadotrophins documented during this neonatal window (12). The first 6 months to 2 years of life is normally characterised by robust activity of the hypothalamic–pituitary– gonadal cascade such that dampened gonadotrophin and sex steroid levels at this time are clearly abnormal and a probable harbinger of future hypogonadotrophism at the expected time of sexual maturation.

2. Frequent blood sampling (every 10 min) in multiple probands with GPR54 mutations demonstrates the existence of low-amplitude LH pulsations, i.e. the persistence of a present albeit dampened ‘GnRH pulse generator’ (10,13). This finding has been confirmed in Sprague Dawley rats, where a marked increase in LH release in response to kisspeptin-10 (100 nmol) administration was not accompanied by any change in multiunit electrical activity volley frequency recorded from electrodes implanted in the arcuate nucleus (14). Taken together, these observations suggest that GPR54/metastin are key for augmenting an already-present intrinsic pulsatility of GnRH neurones.

3. Patients with multiple different mutations in GPR54 have demonstrated the ability to undergo folliculogenesis and spermatogenesis, and achieve fertility in response to the pulsatile administration of exogenous GnRH and gonadotrophins. These data suggest two facets of GPR54 physiology (15): first, that there is no intrinsic defect in the GPR54-deficient patients at the level of the anterior pituitary; secondly, that, while there may be effects of either kisspeptin or GPR54 at the level of the gonad, such effects must be relatively modest, as patients are clearly able to demonstrate intact gonadal function.

4. In addition to achieving fertility, one female patient with homozygous mutations in GPR54 had (i) multiple conceptions, (ii) two uncomplicated pregnancies and deliveries of healthy children, (iii) spontaneous initiation of uterine contractions, and

(iv) lactation for several months post-partum (15). The ligand for GPR54, kisspeptin, is thought to play a role in placental development, but successful pregnancies suggest that, whatever such a putative role is, there must be redundancies in placental development.

5. An African American proband with nonsense/non-stop mutations in GPR54 underwent a dose–response investigation of exogenous GnRH. His dose–response curve was shifted significantly leftward compared with six men with nIHH who did not harbour GPR54 mutations (10). It is unclear why this patient was more sensitive to exogenous GnRH. It is possible that he may have had some degree of endogenous GnRH from the hypothalamus (as was suggested by his baseline pattern of LH pulsations) that somehow primed his pituitary to be more responsive to exogenous GnRH.

Lessons from patients with precocious puberty

As discussed earlier, loss-of-function mutations in GPR54 cause the normosmic form of hypogonadotrophic hypogonadism (10,16). It now appears that the opposite is also true: a gain-of-function mutation in GPR54 appears to lead to central precocious puberty. We have described a GPR54 mutation (Arg386Pro) that was identified in an adopted girl with idiopathic central precocious puberty (17). *In vitro*, her mutation leads to prolonged activation of intracellular signalling pathways in response to kisspeptin. The decreased GPR54 desensitisation seen for this Arg386Pro mutant may increase the stimulatory effects of endogenous kisspeptin on GnRH secretion, thus accelerating the maturation of the reproductive axis. Therefore, kisspeptin is an indisputable gatekeeper of pubertal function in humans, as demonstrated by both loss- and gain-of-function mutations.

Expression of kisspeptin

The past 4 years have witnessed an extraordinary surge of interest in kisspeptin from both molecular biologists and physiologic investigators. In the rodent, kisspeptin-expressing neurones are present in the arcuate nucleus, the periventricular nucleus and the anteroventral periventricular nucleus (AVPV) (18). Kisspeptin expression in the AVPV is sexually dimorphic with much higher expression in females. Both male and female rats undergo considerable augmentation of hypothalamic Kiss1 mRNA during the transition from juvenile to adult life (19). Beyond these changes in expression during sexual maturation, kisspeptin appears to undergo differential expression in distinct hypothalamic nuclei. Gonadectomy increases Kiss1 expression in the arcuate nucleus, and sex steroid replacement reduces expression in this nucleus back to that of intact animals (18). These observations suggest that, in the arcuate nucleus, kisspeptin may modulate the negative feedback on GnRH secretion exerted by sex steroids. By contrast, gonadectomy decreases Kiss1 expression in the AVPV and sex steroid replacement restores it. This observation suggests that, in the AVPV, kisspeptin-mediated positive feedback occurs in the midcycle LH surge (18).

Single boluses of kisspeptin stimulate GnRH release *in vivo*

Kisspeptin peptides are the most powerful stimulators of gonadotrophin secretion that have ever been studied in mammalian species, including rodents on a molar basis (19–24), sheep (25) and monkeys (26). Intracerebral doses as low as 1 fmol evoke significant LH responses (20). The effects of kisspeptin on LH can be completely abrogated by the co-administration of a GnRH antagonist, demonstrating that this protein is acting through the GnRH receptor to stimulate LH release (i.e. hypothalamic effect) (20). Kisspeptin is unable to stimulate LH release when given to GPR54 knockout mice, suggesting that the stimulatory effects of this peptide are mediated only through its cognate receptor (27).

A continuous intravenous infusion (as opposed to a single dose) of kisspeptin (100 μg / hour × 4 days) was given to juvenile, agonadal male monkeys (28). After the initiation of kisspeptin infusion, LH levels rose significantly but then began to drop, returning to their baseline levels by the conclusion of the first day of the infusion. LH levels remained low for the subsequent 3 days of the infusion. On the last day of the infusion, the animals received injections of NMDA, GnRH and kisspeptin. After NMDA and GnRH injections, LH levels rose, demonstrating that the animals were still able to secrete GnRH and mount a gonadotrophin response via the GnRH receptor. However, when a single bolus of metastin (10 μg) was added to the continuous infusion, LH levels did not rise. This observation confirmed that the continuous metastin infusion had decreased LH levels through desensitisation or downregulation of GPR54. Within 21 h of stopping the infusion, the LH responsiveness to a single bolus of kisspeptin was restored. If exogenous GnRH can stimulate the pituitary and GnRH analogues/antagonists can down-regulate or block GnRH receptors, kisspeptin (or analogous compounds) may act in a similar fashion on GPR54. These findings suggest another avenue that might be explored to achieve therapeutic decreases in sex steroids for patients with prostate cancer, endometriosis, infertility and abnormalities of pubertal timing.

Targeted deletion of Kiss1 and GPR54 in mice

At least four GPR54 mutant mouse models have been generated by separate groups, and the phenotypes of two Kiss1 mutant mouse models have been reported (10,27,29–31). In general, the phenotypes of both GPR54 and Kiss1 mice parallel the phenotypes of patients with mutations in GPR54 with abnormal sexual maturation at a variety of developmental time-points.

1. Knockout mice for both ligand and receptor show the effects of neonatal deficiency of sex steroids with decreased anogenital distance at P21. During development, the anogential distance in knockout males remains significantly smaller than that in wild-type mice (10,27,30).
2. Female mice have delayed vaginal opening and do not manifest normal oestrous cycles (10,27).
3. Laboratory studies are consistent with hypogonadotrophic hypogonadism (10,27, 30).
4. Testicular size is significantly reduced and spermatogenesis is impaired, but not completely abrogated (27,30).
5. In one report, female Kiss1 knockouts were found to have a bimodal distribution of ovarian weights, with some ovaries significantly reduced in size and but other ovaries equivalent to those of wild-type animals (27). Animals with larger ovaries had oestrous changes on vaginal smears, suggesting some degree of oestrogenisation. However, follicle development was arrested and no corpora lutea were present, suggesting an ovulatory abnormality.
6. When knockout mice are placed into mating cages with animals of prior proven paternity/maternity, neither knockout males nor females demonstrate fertility (27).
7. GPR54 knockout mice have abnormal sexual behaviour which can be corrected by the administration of testosterone (32).
8. Kiss1 knockout mice are responsive to the administration of exogenous kisspeptin, whereas GPR54 mice are not, suggesting that kisspeptin does not act through another receptor (27).

Conclusions

Despite the initial challenges of a linkage approach in humans with GnRH deficiency and nIHH, kisspeptin and GPR54 have now catapulted to the forefront of the neuroendocrine control of the gonadotrophin axis. Mutations in both mice and men show that kisspeptin and its receptor play key roles in establishing the onset, tempo and pace of sexual development. Kisspeptin is a powerful stimulus for the release of GnRH and may serve as the connecting link between peripheral sex steroids and GnRH secretion.

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