BRIEF REPORT

Inactivating *KISS1* Mutation and Hypogonadotropic Hypogonadism

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SUMMARY

Gonadotropin-releasing hormone (GnRH) is the central regulator of gonadotropins, which stimulate gonadal function. Hypothalamic neurons that produce kisspeptin and neurokinin B stimulate GnRH release. Inactivating mutations in the genes encoding the human kisspeptin receptor (*KISS1R*, formerly called *GPR54*), neurokinin B (*TAC3*), and the neurokinin B receptor (*TACR3*) result in pubertal failure. However, human kisspeptin loss-of-function mutations have not been described, and contradictory findings have been reported in *Kiss1*-knockout mice. We describe an inactivating mutation in *KISS1* in a large consanguineous family that results in failure of pubertal progression, indicating that functional kisspeptin is important for puberty and reproduction in humans. (Funded by the Scientific and Technological Research Council of Turkey [TÜBİTAK] and others.)

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IT IS STILL UNKNOWN HOW PUBERTY IN HUMANS, OCCURRING DURING THE early years of the second decade of life, is initiated.[1] The hallmark of puberty is increased secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which act in concert to stimulate the gonads to drive sex-hormone secretion and gametogenesis. The production of gonadotropins from pituitary gonadotropic cells is controlled by the pulsatile delivery of GnRH. Inactivating mutations in the genes encoding GNRH1[2] or the GNRH receptor (GNRHR)[3] give rise to normosmic idiopathic hypogonadotropic hypogonadism in humans.[4] However, GnRH neurons lack sex-steroid receptors. This suggests the existence of GnRH-regulating neurons, which would mediate this effect.

A major breakthrough in identifying such candidate neurons was the finding that inactivating mutations in genes encoding the human kisspeptin receptor (*KISS1R*, formerly called *GPR54*), the cognate receptor for a hypothalamic peptide, kisspeptin, resulted in pubertal failure.[4,5] More recently, mutations in *TAC3* or *TACR3* (encoding neurokinin B and its receptor, respectively) were shown to result in the same phenotype.[6] Kisspeptin and neurokinin B are coexpressed, along with dynorphin, in sex-hormone–responsive neurons in the arcuate nucleus (infundibular nucleus in primates), and their coordinated activity appears to regulate GnRH secretion.[7] Gene defects associated with normosmic idiopathic hypogonadotropic hypogonadism have been described in all the neuropeptides and receptors identified as stimulators of GnRH except for the kisspeptin gene (*KISS1*).

Although *Kiss1*- and *Kiss1r*-knockout mouse models largely produce phenocopies (i.e., affected noncarriers) of human normosmic idiopathic hypogonadotropic hypogonadism resulting from inactivating mutations of *KISS1R*, there is evidence of remarkable residual activity of the hypothalamic–pituitary–gonadal axis.[4,8-10] More-

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over, a recent report challenged the requirement of kisspeptin signaling for pubertal maturation and fertility in female mice.[11]

Here, we describe a large consanguineous family in which normosmic idiopathic hypogonadotropic hypogonadism was associated with a loss-of-function mutation of *KISS1* in four affected sisters with pubertal failure. The findings indicate that kisspeptin is required for the initiation of puberty in humans.

CASE REPORTS

The proband (Patient II-8) had grown and developed normally until her early-to-mid-teen years. At 14.9 years of age, breast development was absent and the bone age was 13 years. Pelvic ultrasonography revealed a hypoplastic uterus and ovaries lacking follicles. The proband's affected sisters (Patients II-1, II-7, and II-10) also had no spontaneous breast development. Clinical features including measures of growth, pubertal indexes, and circulating hormone concentrations in the proband and her affected sisters are shown in Table 1.

The pedigree of the family is shown in Figure 1A. All four affected sisters were otherwise healthy and had a normal sense of smell. The patients had normal anterior pituitary function other than the failure to undergo puberty. The parents were healthy paternal cousins of Kurdish origin. The mother underwent menarche at 12 years of age, and the father began having facial-hair growth at 14 years of age. The proband's unaffected, sexually mature sisters (II-2, II-4, II-6, and II-9) under-

Table 1. Clinical and Hormonal Characteristics of the Proband and Her Affected Sisters.*

Characteristic	Patient II-1†	Patient II-7	Patient II-8 (Proband)	Patient II-10	Normal Range for Women and Girls
Age (yr)	30	16	14	12	
Height (cm)	168.1	166.1	152.1	140.0	
Body-mass index‡	20.6	19.4	17.4	18.5	
Tanner stage§					
Breast development	5	1	1	1	
Pubic-hair development	5	3	2	1	
FSH (mIU/ml)	1.7	0.7	0.2	0.4	2.5–10.2
LH (mIU/ml)	0.1	0.2	0.0	0.2	1.9–12.5
Estradiol (ng/dl)	0.1	0.2	0.1	0.0	6.3–16.5
GnRH stimulation test					
Maximal FSH (mIU/ml)	NA	NA	7.4	NA	
Maximal LH (mIU/ml)	NA	NA	3.3	NA	
Prolactin (pg/ml)	NA	NA	5.0	NA	2.8–29.2
Thyrotropin (mIU/ml)	NA	1.8	1.4	0.6	0.3–4.2
Thyroxine (ng/dl)	NA	1.0	1.1	1.1	0.9–1.8
Cortisol (μg/dl)	NA	17.7	10.7	NA	3.0–25.0
Dehydroepiandrosterone sulfate (μg/dl)	NA	NA	77	NA	35–430
Insulin-like growth factor 1 (ng/ml)	NA	NA	271	NA	87–368

* The coefficients of variation within and between hormonal assays were less than 5%. To convert the values for estradiol to picomoles per liter, multiply by 36.71. To convert the values for thyroxine to picomoles per liter, multiply by 12.87. To convert the values for cortisol to nanomoles per liter, multiply by 27.59. To convert the values for dehydroepiandrosterone sulfate to nanomoles per liter, multiply by 27.21. FSH denotes follicle-stimulating hormone, GnRH gonadotropin-releasing hormone, LH luteinizing hormone, and NA not available.

† Patient II-1 had received estrogen-replacement therapy, resulting in Tanner stage 5 breast development. However, her FSH, LH, and estradiol levels were measured while she was not receiving therapy.

‡ The body-mass index is the weight in kilograms divided by the square of the height in meters.

§ The Tanner stage is used to describe breast and pubic-hair development, with 1 representing prepubertal status and 5 representing maturity.

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went menarche around 12 years of age and had regular menses. The two adult siblings heterozygous for *KISS1*, a sister (II-2) and a brother (II-3), both had children without receiving any medical assistance. (For the complete case reports, see the Supplementary Appendix, available with the full text of this article at NEJM.org.)

METHODS

STUDY CONDUCT

The Mersin 1st Clinical Research Ethics Committee approved this study, and written informed consent was obtained from each adult participant and the parents of the participating children.

LABORATORY STUDIES

Plasma corticotropin and serum FSH, estradiol, dehydroepiandrosterone sulfate, cortisol, and testosterone levels were analyzed with the use of commercial kits based on a solid-phase, two-site sequential or competitive chemiluminescent immunometric assay. Serum LH levels were determined with the use of a two-step immunometric sandwich assay and a luminometric technique

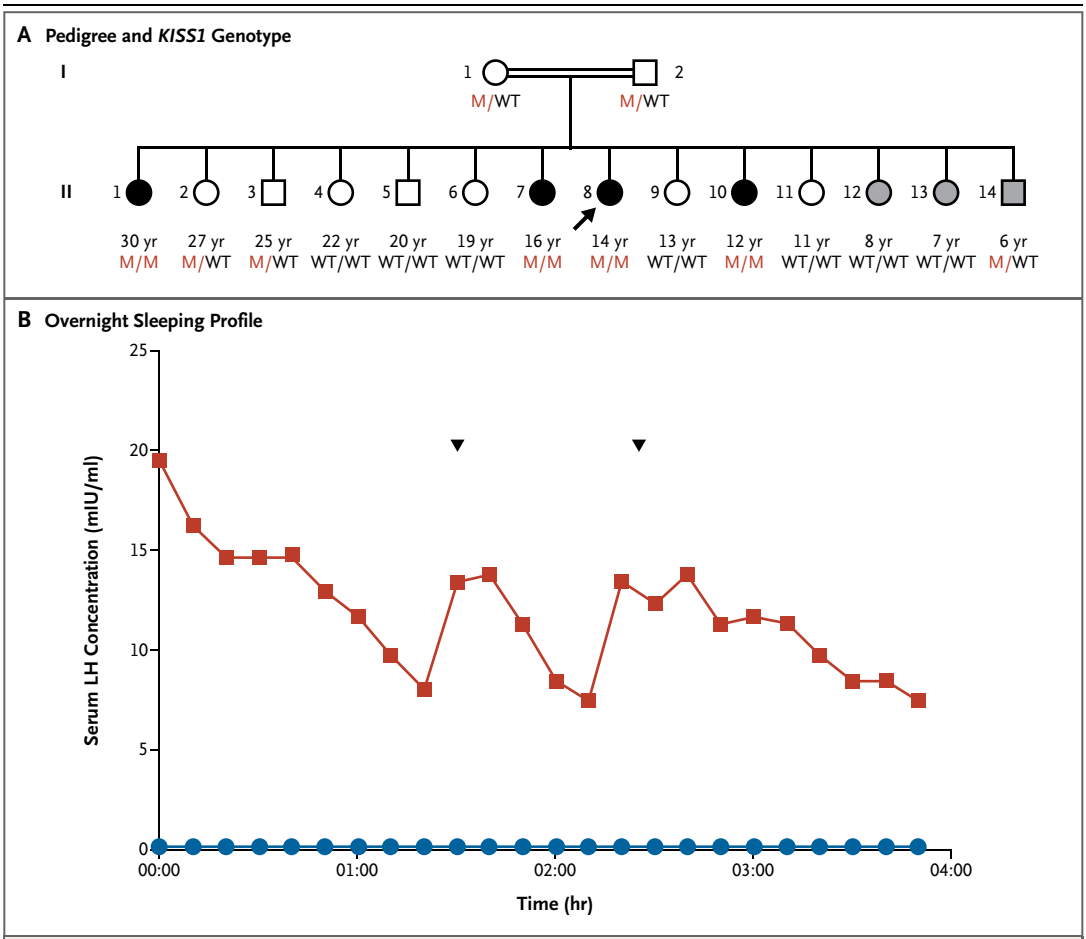

Figure 1. Pedigree and Luteinizing Hormone (LH) Profiles of the Proband's Family.

Panel A shows the pedigree of the proband (arrow) and her family. Solid circles indicate affected girls or women, open symbols unaffected family members, gray symbols family members of uncertain status (prepubertal), squares male family members, circles female family members, and the double line consanguinity. The age is shown under each symbol in generation II. Under every symbol is the *KISS1* genotype, with M denoting mutant, and WT wild type. Panel B shows the overnight sleep LH profiles of the proband (Patient II-8, blue circles) and a healthy sister (Patient II-6, red squares), a *KISS1* WT homozygote, in the midfollicular phase. The two arrowheads show the healthy sister's LH peaks. The proband's LH readings are all below the lower limit of detection of the assay, 0.2 mIU per milliliter.

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(Beckman Coulter). The intraassay and interassay coefficients of variation are less than 6% and less than 7%, respectively. The lower limit of detection with a 95% confidence interval is 0.2 mIU per milliliter.

We conducted a GnRH-stimulation test in the proband (Patient II-8) by injecting 0.1 mg of GnRH intravenously and obtaining blood samples at 0, 20, 40, and 60 minutes after injection for measurement of FSH and LH. Profiling of overnight LH levels during sleep was carried out during the midfollicular phase in the proband and a healthy sister (Patient II-6) who had a homozygous wild-type genotype for the *KISS1* mutation (see below). Blood samples were obtained every 10 minutes for the 4 hours from midnight to 4 a.m. The pattern of LH secretion was analyzed according to a modified Santen and Bardin method.[12]

GENOTYPING

We performed automated sequencing of blood specimens from the proband for variants in known genes or strong candidate genes for idiopathic hypogonadotropic hypogonadism, including *GNRHR*, *GNRH1*, *KISS1R*, *KISS1*, the Kallmann syndrome 1 sequence gene (*KAL1*), the prokineticin 2 gene (*PROK2*) and PROK2 receptor gene (*PROK2R*), and the fibroblast growth factor receptor 1 gene (*FGFR1*). We performed genomewide analysis of single-nucleotide polymorphisms (SNPs) on microarrays (250K *Nsp*I SNP microarrays, Affymetrix) and analyzed the data using AutoSNPa software.[13] This helps identify autozygous regions in affected persons, which are based on inheritance of the same ancestral mutant allele from both parents.

We also used whole-exome sequencing (selective sequencing of coding regions of the human genome) to identify the causative mutations. For exome sequencing, samples were prepared as an Illumina sequencing library, and in the second step, the sequencing libraries were enriched for the desired target according to the Illumina Exome Enrichment protocol. The captured libraries were sequenced (Illumina HiSeq 2000 Sequencer, Macrogen). The reads were mapped against the UC Santa Cruz Genome Browser (hg19 assembly). Identified mutations were checked in familial and sporadic cases of normosmic hypogonadotropic hypogonadism as well as in ethnically matched healthy adult controls in our study cohort.

INOSITOL PHOSPHATE PRODUCTION

Human kisspeptin-10 (amino acid sequence Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2) was purchased from Sigma. Mutant human kisspeptin-10 (amino acid sequence Tyr-Asn-Trp-Lys-Ser-Phe-Gly-Leu-Arg-Phe-NH_2) was custom-synthesized at a purity of at least 95% (EZBiolabs). Mutant and wild-type kisspeptin-10 were compared for their ability to stimulate inositol phosphate production in COS-7 cells transfected with KISS1R (see the Supplementary Appendix for details).

RESULTS

A GnRH stimulation test revealed a blunted response with maximum LH and FSH concentrations of 3.3 and 7.4 mIU per milliliter, respectively. LH profiling during sleep (Fig. 1B) in the proband (Patient II-8) showed readings that were all below the lower limit of detection (0.2 mIU per milliliter). The LH readings in the healthy sister (Patient II-6), on the other hand, showed a normal adult female profile with two LH peaks.

Genomewide SNP analysis identified two regions of homozygosity common to all affected patients but not found in any unaffected family member. One of the regions, a 5.5-Mb segment (from 201.6 Mb to 207.1 Mb) on chromosome 1, includes the *KISS1* gene, which was a candidate for causing normosmic idiopathic hypogonadotropic hypogonadism.

We found homozygous nonsynonymous mutations in the coding sequence of *KISS1* (Human Genome Organisation's Gene Nomenclature Committee [HGNC] number 6341) in all affected subjects (Fig. 2A). All affected family members were homozygous for a change from cytosine to guanine at complementary DNA nucleotide 345 (National Center for Biotechnology Informatics [NCBI] reference sequence NM_002256.3:c.345C→G), leading to the substitution of asparagine by lysine at residue 115 (NCBI reference sequence NP_002247.3:p.115N→K). This residue is conserved among all species with this gene (Fig. 2B). The Sorting Intolerant From Tolerant (SIFT) score for whether this amino acid substitution affects protein function is 0.00, which corresponds to a very damaging effect (possible range, 0 to 1, with scores ≤0.05 predicting damage to protein function; http://sift.jcvi.org). This mutation was not found in 100 ethnically matched healthy adult

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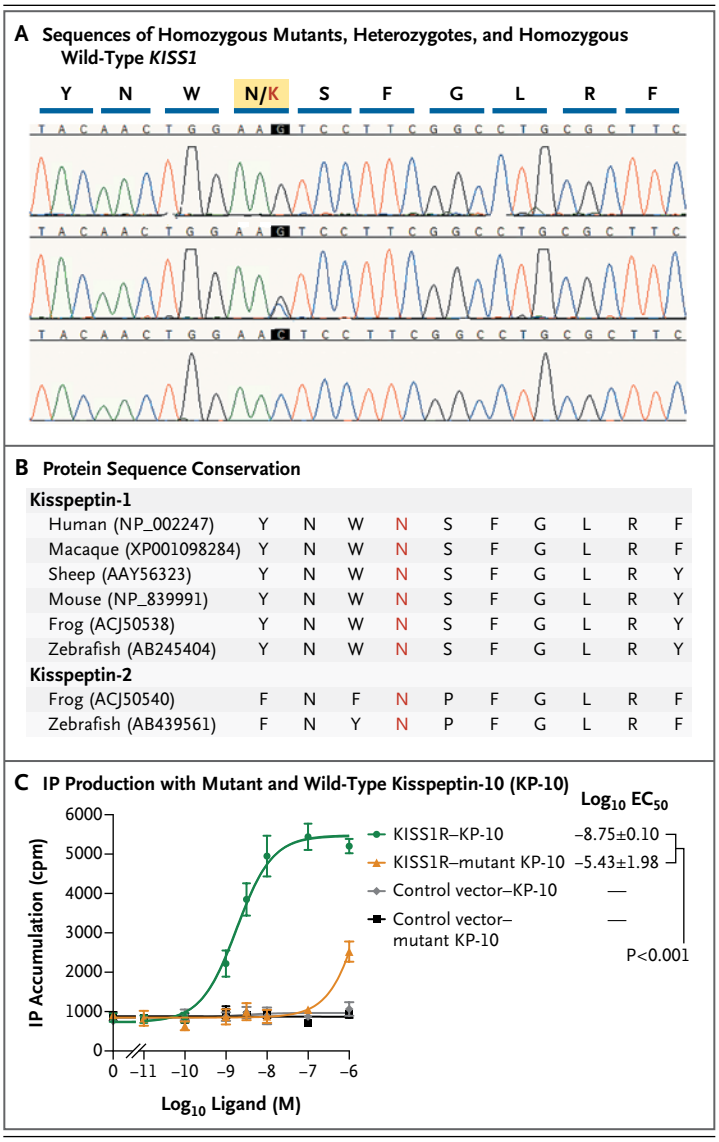

Figure 2. Genetic Profile of the Proband's Family.

Panel A shows the results of genotype sequencing of the kisspeptin-1 gene (*KISS1*) in affected and unaffected members of the proband's family. Amino acids are labeled with the one-letter symbols at the top of the chromatograms, with the corresponding double-stranded nucleotide sequences below: the top line shows the homozygous mutant genotype, the middle line shows the heterozygous genotype, and the bottom line shows the homozygous wild-type genotype. We found a p.115N→K mutation that resulted in a nucleotide change from C to G (highlighted) at complementary DNA nucleotide 345 (National Center for Biotechnology Informatics [NCBI] reference sequence NM_002256.3:c.345C→G) and the replacement of asparagine by lysine at amino acid residue 115 (NCBI sequence NP_002247.3:p.115N→K). Panel B shows that the mutated amino acid residue N115 is conserved among all species possessing orthologues or paralogues of *KISS1*. The reference sequence identifier is given in parentheses. Panel C shows the results of in vitro analysis of the functional consequence of the mutant kisspeptin-10 variant. *Myo*-[2-^{3}H]inositol phosphate (IP) accumulation in COS-7 cells expressing human KISS1 receptor (KISS1R) was induced with graded concentrations of kisspeptin-10 (KP-10) for 1 hour. The intracellular ^{3}H-IP concentrations are shown as scintillation counts per minute (cpm). The I bars indicate means (±SE) for triplicate samples from three or more independent experiments. KP-10 concentrations stimulating an IP response halfway between baseline and maximum (EC_{50}) were derived from the means and standard errors and were $\log_{10}$ transformed. The P value was calculated with the use of Student's t-test.

controls (data not shown). No *KISS1* mutations were found in 12 other families with more than 1 affected member or in 90 sporadic cases of normosmic idiopathic hypogonadotropic hypogonadism.

Analysis of whole-exome sequencing data, with particular attention to the autozygous regions identified in autozygosity mapping and other candidate genes, on the basis of studies in animals revealed that the only mutation to account for the phenotypes of the proband and her affected sisters is the *KISS1* mutation.

Functional studies (Fig. 2C) showed that human KISS1R had significantly reduced sensitivity to the mutant kisspeptin-10 variant (i.e., the concentration leading to a response halfway between baseline and maximum [EC_{50}] value of 3.7 μM) as compared with wild-type kisspeptin-10 (EC_{50} value of 1.8 nM) (P<0.001). In addition, the mutant kisspeptin-10 variant was unable to stimulate a maximal inositol phosphate response at any of the concentrations tested, indicating significantly reduced efficacy as well as potency. Empty-vector–transfected COS-7 cells showed no inositol phosphate response to wild-type or mutant kisspeptin-10.

DISCUSSION

This large consanguineous family has four members with complete normosmic idiopathic hypogonadotropic hypogonadism, all of whom were found to have a *KISS1* mutation. All affected family members were homozygous for the mutation, whereas their unaffected parents were heterozygous, and their unaffected siblings were either

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heterozygous or wild-type homozygous. The disorder was thus transmitted as an autosomal recessive trait, which indicates that one copy of *KISS1* is sufficient for normal function of the gonadotropic axis, thus ruling out haploinsufficiency. Given the rarity of oligogenic inheritance in normosmic idiopathic hypogonadotropic hypogonadism,[14] we ensured that the *KISS1* mutation is the sole variant to account for the normosmic idiopathic hypogonadotropic hypogonadism phenotype in our patients. This was accomplished by combining a variety of methods, including candidate-gene screening, autozygosity mapping, and whole-exome sequencing.

In humans, kisspeptins derive from a common 145–amino acid precursor protein, which is proteolytically processed to peptides of 54, 14, 13, and 10 amino acids in length; all share the common carboxy-terminal decapeptide sequence YNWNSFGLRF-NH_2, comprising kisspeptin-10, which is critical for biologic activity at the kisspeptin receptor.[15,16] This decapeptide sequence is highly conserved during evolution (Fig. 2B). In addition, asparagine 4 (Asn4), which is mutated to lysine in our patients, is preserved in all known orthologues and paralogues.

Several groups have investigated the effects of structural modification of kisspeptin molecules. The five carboxy-terminal amino acids of kisspeptin-10, in particular residues 6, 8, 9, and 10, appear critical for agonist activity at the kisspeptin receptor.[17-21] The next most important residue is Asn4; an alanine substitution for Asn4 in human kisspeptin-10 was approximately 25% as potent as the parent kisspeptin-10 in vivo.[17] Nuclear magnetic resonance studies of kisspeptin have shown a helical structure spanning Asn4 to phenylalanine 10; it appears that the helical structure plays an important role in receptor binding or activation.[19] Thus, substitution of Asn4 with lysine in our patients may disrupt this helical structure and account for the severely impaired ability to activate the kisspeptin receptor. Our data on in vitro receptor activation support this conclusion, since mutant kisspeptin was markedly less potent than the wild type. Studies of the development of antagonists have shown that substitutions for Asn4 decrease binding affinity and may make contact with the receptor,[20,22] both of which may contribute to the loss of activity of the mutant ligand.

Thus, the predicted consequence of the homozygous *KISS1* mutation in the affected sisters in our study is a defective endogenous kisspeptin that is deficient in activating the kisspeptin receptor on the GnRH neurons and would result in the inability to elicit GnRH secretion — thereby accounting for the complete gonadotropin deficiency we have observed.

In the past 8 years, 26 patients with normosmic idiopathic hypogonadotropic hypogonadism have been reported to have loss-of-function mutations in *KISS1R*.[23] *Kiss1r*- and *Kiss1*-knockout mouse models show a variable phenocopy of human *KISS1R*–inactivating mutations. A *Kiss1*-knockout mouse duplicated the human KISS1R mutant phenotype.[8] However, a subgroup of another *Kiss1*-knockout mouse model had a phenotype similar to the wild-type mouse.[9] Chan and colleagues[10] observed that nearly all *Kiss1*-knockout mice eventually underwent estrus. In general, the phenotype of the *Kiss1*-knockout model is less severe than that of the *Kiss1r*-knockout model.[9] Likewise, a potent kisspeptin antagonist inhibits the rise in LH after castration in rodents and lowers LH-pulse amplitude after castration in sheep, but does not reduce basal LH concentrations; similarly, it inhibits GnRH pulsatility but does not lower the basal GnRH level in female pubertal monkeys.[20,22] Taken together, these findings suggest that GnRH secretion may be more dependent on kisspeptin signaling in humans than in animal models.

Recently, Mayer and Boehm[11] reported that ablation of *KISS1*-expressing cells in mice did not prevent progression of puberty or adult reproductive function. This observation contrasts with the impaired reproductive function reported in *Kiss1*-knockout mice[5,10-13] and the complete absence of reproductive function in our female patients with an inactivating *KISS1* mutation. Given the stochastic nature of Cre recombinase expression during development in the *Kiss1*-Cre mice studied by Mayer and Boehm, it is possible that ablation of the *Kiss1* neuron was incomplete, thus explaining the contrast in findings. Since only a small percentage of GnRH neurons is required for full reproductive function,[24] the same explanation may apply to the inconsistency of findings regarding kisspeptin neurons.

In conclusion, we found that an inactivating mutation of *KISS1* causes complete normosmic idiopathic hypogonadotropic hypogonadism in humans and would argue that kisspeptin signaling

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is a critical element in the human hypothalamic–pituitary–gonadal axis.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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