Supplementary Methods

Hormone Assays

Serum LH and FSH concentrations were determined by radioimmunoassay (RIA) up to 1991 or immunofluorometric assays (IFMA, AutoDELFIA hLH Spec and AutoDELFIA hFSH, Wallac Oy, Turku, Finland) after 1991. For IFMA, the sensitivity was set at 0.6 IU/L for LH and 1.0 IU/L for FSH. Serum testosterone and estradiol concentrations were measured by commercial solid phase fluoroimmunoassay (FIA) (AutoDELFIA Testosterone and AutoDELFIA Estradiol, PerkinElmer, Turku, Finland), with sensitivity of 13.6 pg/mL (47 pmol/liter) for estradiol and 14 ng/dL (0.6 nmol/liter) for testosterone. The coefficient of interassay variation was 5% or less for all assays.

For the acute GnRH stimulation test, serum LH and FSH were measured at –15, 0, 15, 30, 45, and 60 min after intravenous (i.v.) administration of 100 μg GnRH. For RIA, LH peak after GnRH >15 U/L in girls and >25 U/L in boys was considered a pubertal response (30). For IFMA, basal LH >0.6 U/L was considered a pubertal level in both sexes and a GnRH-stimulated LH peak >6.9 U/L in girls and >9.6 U/L in boys was considered a pubertal response (30). Basal testosterone >30 ng/dL (RIA) or >19 ng/dL (FIA) was considered as a pubertal level (30). Reference range for testosterone in adult males was 271-965 ng/dL (30).

Genetic Analysis

For PCR amplification of the *KISS1* gene fhe following intronic primers pairs were used: 1-f 5'GGGCTTTATAAAAGGGATGTG-3'; 1-r 5'- CTTAGAACGGATTCCCTG3'; 2-f 5'-CAGATCCTGTGCCTGACCT-3'; 2-r 5'-TTGCAACAACCCACTTGCT-3', 3-f 5'-GTGTTGCAAAGCCATCTTTC-3'; 3-r 5'-TCTTTTATTGCCTCGGGTTG-3'. The *KISS1* promoter region, including 1.1 kb conserved sequence upstream to the transcription start site, was amplified in the CPP cases using the following primer pairs: Kisspro1-f 5'-ACCTGGAAGTCCCCTC TCTG -3'; Kisspro1-r 5'-AGCCTCTGAGGTGACGAGAC-

3'.Kisspro2-f 5'-GAGCGTCCTGTCTGAGGGTA-3'; Kisspro2-r 5'-AGAGAGGGGACTTCCAG GTG-3'. Amplification reactions were performed in a final volume of 25 µl containing 10 pmol of each primer, 150 ng template DNA, 200 µmol/L dNTPs, 2.5 U *Taq polymerase,* 2.5 µl 10x buffer containing 1.5 mM $MgCl_2$ and carried out for 35 cycles: denaturation at 94°C for 30 sec, annealing at 55-57 °C for 30 sec, extension at 72° C for 1 min, followed by a final extension for 10 min at 72° C. All PCR products were separated on 1% agarose gel electrophoresis and automatically sequenced using the ABI Prism ™ Big Dye terminator Kit (*Perkin-Elmer, Foster City, CA, USA*) in an ABI Prism Genetic Analyzer 3100 automatic DNA sequencer (*Perkin- Elmer, Foster City, CA, USA*).

Studies of KISS1R Signaling

Wild-type (kp54-wt) and mutant (kp54-P74S and kp54-H90D) kisspeptin-54 were chemically synthesized (Tufts University Peptide Synthesis Core Facility, Boston, MA). A stable KISS1R-expressing Chinese hamster ovary (CHO) cell line (CHO-KISS1R), previously established in the Kaiser laboratory, was maintained in Dulbecco's modified Eagle medium (DMEM)/F12 growth medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), glutamine, nonessential amino acids and Neomycin 800 ug/ml, at 37° C in 5% CO_2. Inositol phosphate assays were performed as previously described (30). In brief, CHO-KISS1R cells were plated in 6-well plates and 24 h later, medium was replaced with inositol-free DMEM for 2 h at 37°C, after which 2 uCi/ml myo-[2-^{3}H]-inositol (Perkin Elmer, Waltham, MA) was added, followed by the addition of 10 mM LiCl 15 min later. After overnight incubation, CHO-KISS1R cells were stimulated with varying concentrations of kp54-wt, kp54-P74S or kp54-H90D for two hours. Subsequent to the stimulation, the cells were lysed with 20 mM formic acid. Supernatants were loaded onto previously equilibrated AG-X8 resin anion-exchange columns (Fisher Scientific, Pittsburgh, PA) and inositol phosphates were extracted. Total inositol phosphate accumulation was measured and corrected for total protein content. All assay points were performed in triplicate, and each experiment was repeated at least three times to ensure accuracy. In some experiments, kp54-wt, kp54-P74S or kp54-H90D were incubated

with 50% human serum (Atlanta Biologicals, Lawrenceville, GA) for 2 hours at 37°C prior to addition to the CHO-KISS1R cells, to assess the stability of the wild-type and mutant kisspeptin-54's.

Supplementary tables

Table 1. Clinical and hormonal features of three unrelated patients with CPP and *KISS1* mutations

Case	Sex	CA* (yr)	BA (yr)	FSH (U/L)	LH (U/L)	LH Peak (U/L)	Sex steroids T	Sex steroids E2	Mutation
1	M	1.4	3	8.3#	11.5[#]	47.2[#]	600[#]	_	p.P74S
2	F	8.2	11.5	5.3	1.5	_	_	46	p.H90D
3	F	7.4	11	4.8	2.1	_	_	16	p.H90D

M, male; F, female; BA, bone age; T, testosterone (ng/dL); E2, estradiol (pg/mL). *CA, chronological age at first visit. Pre pubertal reference range: [#] RIA peak LH <25 IU/L; IFMA basal LH <0.6 U/L, peak LH <6.9 U/L, E2 <13 pg/mL (IFMA), T <30 ng/dL (RIA).

Table 2. Frequency of the allelic variants identified in the *KISS1* gene in CPP, IHH and controls

Location	*Nucleotide*	*Amino acid change*	*Controls (n=200) %*	*CPP (n=83) %*	*IHH (n=61) %*
Promoter	g.-936 delC	_	C 92 _8	C 91 _9	_
Promoter	g.-454 C>T	_	C 93 T 7	C 93 T 7	_
Promoter	g.-415 G>A	_	G 45 A 55	G 45 A 55	_
Promoter	g.-353 T>C	_	T 94 C 6	T 95 C 5	_
Promoter	g.-60 G>A	_	G 98 A 2	G 98A 2	_
Exon1 (5' UTR)	c.18 or 19 delT	_	T 64 _36	T 55 _45	T 62 _38
Exon1 (5' UTR)	c.75 G>A	_	G 92 A 8	G 91 A	G 95 A 5
Exon2	c.221 G>A	E20K	G 98 A 2	G 98 A 2	G 97 A 2.5
Exon3	c.270 A>G	Q36R	A 95 G 4	A 93 G 7	A 93 G 7
Exon3	c.405 C>G	P81R	C 67 G 33	C 71 G 29	C 70 G 29
Exon3	c.579_580insA	Omits 7 aa	A 77 _23	A 76 _24	A 79 _20
Exon3 (3' UTR)	c.583-584insT	_	T 0 _ 200	T 1 _ 82	T 1 _ 60

ref seq CONTIG NT-004487.3 (www.ncbi.nlm.nih.gov/SNP)