Molecular analysis of the neuropeptide Y1 receptor gene in human idiopathic gonadotropin-dependent precocious puberty and isolated hypogonadotropic hypogonadism

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Objective: To investigate the role of mutations or polymorphisms in the *NPY-Y1R* gene in human idiopathic central pubertal disorders.
Design: Molecular studies.
Setting: University hospital.
Patient(s): Thirty-three patients with gonadotropin-dependent precocious puberty, 22 with hypogonadotropic hypogonadism, and 50 controls.
Intervention(s): Genomic DNA extraction, *NPY-Y1R* gene sequence analysis, cell-surface expression, and functional activity of an identified receptor variant.
Main Outcome Measure(s): Results of sequencing, cell-surface receptor expression, and receptor function.
Result(s): A heterozygous substitution of lysine (K) by threonine (T) at position 374 in the carboxyl terminal region of NPY-Y1R was identified in a girl with familial GDPP. Her mother, who had pubertal developmental at appropriate age, carried the same genetic variant. Introduction of the K374T variant into an expression vector containing the human *NPY-Y1R* complementary DNA led to a partial reduction in cell-surface expression of NPY-Y1R in transiently transfected HEK293 cells. This mutation did not lead to a significant reduction in NPY-stimulated activity of the receptor in this heterologous expression system. No other allelic variants of the *NPY-Y1R* gene were identified in patients or controls.
Conclusion(s): We have identified an inherited heterozygous variant of the *NPY-Y1R* gene in a girl with precocious puberty; however, this most likely did not contribute to her phenotype. Mutations of the highly conserved *NPY-Y1R* gene do not appear to represent a frequent mechanism underlying human idiopathic central pubertal disorders. (Fertil Steril® 2007;87:627–34. ©2007 by American Society for Reproductive Medicine.)

Key Words: GnRH secretion, neuropeptide Y (NPY), precocious puberty, hypogonadotropic hypogonadism

Normal human puberty begins at the end of the first decade of life and is characterized by the development of secondary sexual characteristics, growth acceleration, and the achievement of reproductive function (1). The activation of the ovary and testis during puberty leads to a dramatic increase in gonadal steroid production and completion of gametogenesis (1).

Received September 18, 2005; revised and accepted July 22, 2006.
Supported in part by the grants from Fundação de Amparo a Pesquisa do Estado de São Paulo-FAPESP (to KCMF, process number: 02/09222-1) and the National Institutes of Health (to DLS, grant number HD22196) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq (to ACL and BBM grant numbers: 303192/2002-0 and 301246/1995-5, respectively).
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The pulsatile secretion of GnRH by a network of hypothalamic neurons is critical for pituitary gonadotropin secretion and, consequently, to gonadal steroid production during normal puberty (2, 3). The ability of hypothalamic GnRH neurons to generate a pulsatile discharge of GnRH originates during early fetal development and is subsequently restrained during childhood by several neurotransmitters (3, 4).

Neuropeptide Y (NPY) is considered one of the major components in the inhibition of the pulsatile GnRH secretion during the prepubertal period in nonhuman primates (5). It has been shown, for example, that the central administration of NPY to adult female monkeys causes inhibition of pulsatile GnRH release (6). More recently, El Majdoubi et al. (7) reported that the postnatal pattern of GnRH pulse generator activity was inversely related to the NPY gene and protein expression in the mediobasal hypothalamus of male rhesus monkeys. These findings suggest that NPY is a funda-

mental component of the neurobiological brake-restraining pubertal onset in primates.

NPY mediates its effects through the activation of at least six different receptor subtypes (8, 9). The Y1 subtype, an inhibitory G-protein–coupled receptor, has been the subtype most frequently implicated in the effects of NPY on GnRH secretion (10). However, the role of NPY and its receptors in human puberty remains unclear.

Idiopathic forms of precocious and delayed puberty are not infrequent in humans, and genetic defects may be responsible for a significant proportion of these conditions. In the present study, we hypothesize that mutations of the human *NPY-Y1R* gene may be associated with idiopathic central pubertal disorders such as idiopathic gonadotropin-dependent precocious puberty (GDPP) and hypogonadotropic hypogonadism.

MATERIALS AND METHODS

Patients

We selected 55 Brazilian patients belonging to 52 families with idiopathic pubertal disorders who were willing to participate in this study. Thirty-three of these patients had GDPP, and 22 had permanent isolated hypogonadotropic hypogonadism (HH) without olfactory abnormalities.

Written informed consent was obtained from the subjects and/or their parents, depending on the subject's age. Patient recruitment and DNA sequencing protocols were approved by the Ethical Committee of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil.

The sense of smell was tested in all patients with HH by using the Smell Identification Test, which was developed at the University of Pennsylvania (www.smelltest.com). Other forms of HH, such as transitory gonadotropin deficiency or HH caused by chronic or functional diseases were excluded from this study. GnRH receptor mutations had previously been ruled out in these patients (11). Fifty unrelated Brazilian subjects (age ranged from 18–40 years, 28 females and 22 males) who experienced normal pubertal development at the appropriate chronological age and normal fertility were used as controls. Chronic diseases were ruled out in these subjects.

Pertinent demographic, clinical, and family history data on each of the subjects were gathered. Family history revealed 6 patients with familial GDPP and 6 with familial HH. Basal and/or GnRH-stimulated gonadotropin levels and estradiol or testosterone levels were obtained for all patients with GDPP and HH.

Clinical and hormonal data from the patients are shown in Tables 1 and 2. Thirty-one patients with GDPP were females, and two were males. Chronological age at the onset of pubertal development ranged from 0.66–7.0 years, and age at first evaluation ranged from 1.6–10.2 years. Basal and/or GnRH-stimulated gonadotropin levels were within the pubertal range for all GDPP patients (basal LH $>$0.6 U/L and/or after GnRH-stimulation test $\geq$6.9 UI/L in girls and $\geq$9.6 UI/L in boys by immunofluorometric assays [IFMA]), and a satisfactory response to GnRH analog therapy was shown by all of these patients.

There were 14 males and 8 females among the subjects with HH. The chronological age in this group ranged from 18–30 years at first evaluation. All of them were followed up for a long period (range from 5–10 years). Basal and GnRH-stimulated gonadotropin levels were within the prepubertal or normal range in all patients. In the male subjects with HH, testosterone ranged from 14–54 ng/dL (486–1,875 pmol/L), whereas estradiol levels ranged from $<$13–15 pg/mL ($<$47–55 pmol/L) in the females with HH. All patients had a normal magnetic resonance imaging of the central nervous system, indicating an idiopathic cause of their pubertal disorders.

Hormonal Assays

Serum LH, FSH, estradiol, and testosterone were measured by immunofluorometric assays (Delfia; Wallac, Inc., Turku, Finland). The coefficient of variation was 5% or less for all assays. The lower limit of detection was 0.6 IU/L for LH, 1.0 IU/L for FSH, 13 pg/mL (47 pmol/L) for estradiol, and 14 ng/dL (0.6 nmol/L) for testosterone (12). Serum LH and FSH were also measured at $-$15, 0, 15, 30, 45, and 60 minutes after 100 μg IV of GnRH. The results were compared with normal values established in our population and previously published (12, 13).

DNA Analysis

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures. The *NPY-Y1R* gene, located at chromosome 4q(31.3-32), is composed of 3 exons (www.ncbi.nml.nhi.gov, NM 000909). Exons 2 and 3, the translated region of this gene, were amplified by using 30 pmol of each PCR primer, 200 μmol of each deoxynucleotide triphosphate, 2.5 U *Taq* polymerase (Pharmacia, Upsala, Sweden), 10 mmol/L Tris-hydrochloride (Tric-HCl) (pH 8.3), and 200 ng genomic DNA. The amplified 1,240-bp fragment included exon 2 (698 bp), intron 2 (97 bp), and exon 3 (445 bp).

The primers used for amplification of exons 2 and 3 were as follows: 5′-GCTGAACAGTTGACCTGCTTTG-3′ and 5′-GGAGAACAGGT AATCAAAGTATGTTGCAGG-3′, whereas an additional inner primer, 5′-GTGAGGCGATGTGTAAG-3′, was used for sequencing. A negative control was used in each PCR.

Amplification was performed in a Gene Amp 9600 system (Perkin-Elmer Corp., Foster City, CA) using 40 cycles. DNA was denatured at 95°C for 5 minutes in the first cycle and for 30 seconds in all subsequent cycles. Annealing was per-

TABLE 1

Clinical data of 33 patients with idiopathic gonadotropin-dependent precocious puberty.

Patient	Sex	CA at first pubertal sign (y)	CA[a] (y)	BA (y)	Height (cm)	SD (Ht/CA)	Weight (kg)	BMI (kg/m^2)	SD (BMI/CA)	Pubertal stage (Tanner)
1	F	6.0	7.3	9.0	129.5	1.3	38.2	22.8	2.6	B3P3
2	F	4.0	5.2	6.8	115.7	1.8	19.6	14.6	0.6	B3P1
3	F	6.0	8.6	12.0	141.5	2.3	33.2	16.6	0.2	B4P4
4	F	6.2	6.6	10.0	124.0	1.4	25.7	16.7	0.6	B2P1
5	F	6.5	8.1	12.0	136.8	2.0	33.6	18.0	0.9	B3P3
6	F	1.2	2.6	6.8	100.5	2.8	18.3	18.1	1.4	B4P4
7	F	0.6	1.6	2.0	83.0	0	12.4	18.0	0.9	B4P3
8	F	3.1	3.4	6.8	106.0	2.2	19.1	17.0	0.8	B4P1
9	F	6.4	7.8	11.0	141.5	3.0	32.8	16.4	0.2	B3P4
10	F	2.3	7.2	11.0	136.4	3.1	37.7	20.3	1.9	B3P2
11	F	6.0	6.4	8.8	123.1	0.7	26.6	17.6	1.1	B2P2
12	F	2.3	4.7	8.8	118.8	2.2	24.7	17.5	1.2	B4P1
13	F	3.1	5.2	12.0	129.4	3.5	30.0	17.9	1.4	B4P3
14	F	7.0	7.8	11.0	136.5	2.2	38.9	20.9	2.0	B3P1
15	F	5.8	9.6	12.0	134.0	0	38.8	21.6	1.7	B4P3
16	F	3.1	6.5	8.8	136.0	3.7	30.4	16.4	0.5	B4P3
17	F	6.6	7.3	10.0	125.5	0.8	35.4	22.5	2.5	B3P2
18	F	6.8	7.8	8.8	125.5	−0.1	27.9	17.7	0.9	B3P3
19	F	4.0	6.8	9.5	133.5	3.2	31.3	17.6	1.0	B4P3
20	F	2.3	7.4	11.0	140.0	2.6	34.4	17.6	0.9	B2P4
21	F	0.3	8.5	11.0	131.5	0.6	26.7	15.4	−0.4	B4P2
22	F	7.0	8.7	11.5	135.4	0.9	32.6	17.8	0.7	B3P3
23	F	6.0	8.0	11.0	128.0	0.4	32.0	19.5	1.5	B3P3
24	F	2.0	4.8	8.0	115.0	2.4	23.3	17.6	1.3	B3P1
25	F	6.0	8.2	12.0	128.6	0.3	30.7	18.6	1.0	B3P2
26[b]	F	6.5	10.2	11.0	131.7	−0.1	37.0	21.3	1.5	B4P5
27[b,c]	F	7.0	9.8	11.5	141.4	0.7	34.8	17.4	0.3	B4P4
28[b]	F	5.5	8.0	11.0	132.0	1.2	28.2	16.2	0.1	B3P4
29[b]	F	5.0	8.0	11.0	136.3	1.9	36.2	19.5	1.5	B4P1
30[b]	F	4.0	6.4	7.0	125.5	1.7	26.5	16.8	0.7	B3P1
31[b]	F	5.8	6.6	11.0	129.5	2.4	30.0	17.9	1.2	B4P2
32	M	3.0	5.0	13.0	129.0	4.3	27.9	16.8	1.0	P4
33	M	1.0	1.4	2.6	85.0	0.5	14.3	19.8	1.9	P2

Note: CA = chronological age; BA = bone age; SD = score deviation; BMI = body mass index; F = female; M = male; B = breast; P = public hair.

[a] At first interview.

[b] Patients with familial PPDG.

[c] Patient with K374T variant.

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formed at 53°C for 30 seconds. Elongation was performed at 72°C for 30 seconds, with a final elongation of 10 minutes.

All amplified fragments were examined on 1% agarose gel electrophoresis. The PCR products were pretreated with an enzymatic combination of exonuclease I and shrimp alkaline phosphatase (United States Biochemical Corp., Cleveland, OH) and directly sequenced by using the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) in an ABI PRISM 3100 automatic sequencer (Perkin Elmer Cetus, Hercules, CA). The genetic variants identified were confirmed by automated sequencing by using bidirectional oligonucleotide primers of three distinct PCR products.

Analyses of biological paternity were performed with highly polymorphic VNTR loci (AmpFSTR Profiler PCR amplification, PE Applied Biosystems) in a family by using

TABLE 2

Clinical date of 22 patients with hypogonadotropic hypogonadism.

Patient	Sex	CA (year)	Height (cm)	SD (Ht/CA)	Weight (Kg)	BMI (kg/m^2)	BD/GC (Tanner)	RT (cm)	LT (cm)
1[a]	F	16	157.0	−0.8	40.8	16.5	T1	—	—
2	F	28	158.7	−0.5	40.8	16.5	T4	—	—
3[a]	F	30	170.5	+1.4	72.0	24.9	T5	—	—
4	F	20	159.8	−0.4	58.0	22.9	T1	—	—
5[a]	F	21	146.0	−2.7	37.5	17.5	T1	—	—
6	F	17	154.9	−1.2	60.7	25.5	T2	—	—
7[a]c	F	14	144.3	−2.4	37.0	17.8	T1	—	—
8	F	17	160.2	−0.3	72.3	28.4	T3	—	—
09	M	27	173.5	−0.1	54.6	18.2	—	CR	CR
10	M	25	152.0	−2.7	93.5	40.4	T2	3.0	CR
11	M	20	167.2	−1.2	52.4	18.5	T3	2.3	2.3
12	M	19	170.2	−0.7	91.0	31.4	T2	2.8	3.1
13[a]c	M	30	167.4	−1.1	56.5	20.0	—	2.0	2.0
14c	M	19	167.6	−1.1	60.5	21.0	—	3.0	3.0
15	M	16	153.5	−2.6	45.0	19.0	—	3.5	3.5
16	M	29	166.0	−1.3	53.3	19.0	—	3.5	2.8
17[a]	M	18	169.0	−2.1	81.2	19.0	—	CR	<2.0
18	M	23	183.5	+1.3	109.0	31.5	T3	CR	3.0[b]
19	M	27	181.0	+0.9	73.5	22.5	—	2.4	2.4
20	M	22	178.6	+0.5	59.6	18.8	T2	2.5	2.0
21	M	19	176.0	+0.1	56.2	18.0	—	<2.0	<2.0
22	M	22	169.6	−0.7	80.4	28.0	T3	<2.0	<2.0

Note: CA = chronological age; SD = score deviation; BMI = body mass index; RT = right testis; LT = left testis; F = female; M = male; BD = breast development; GC = gynecomastia; c = consanguinity; CR = cryptorchidism.

[a] Familial.

[b] After surgery.

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1–2 ng of genomic DNA from the identified patient, her sister, and her parents.

In Vitro Mutagenesis of NPY-Y1R

The K374T point mutation was introduced into the wild-type (wt) *NPY-Y1R* complementary DNA (cDNA) (provided by the cDNA Resource Center at the University of Missouri-Rolla, Rolla, MO, www.cdna.org) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotide primers that introduce the variant K374T displaced sequences in the wt double-stranded plasmid prepared in pBlueScript vector (Stratagene). By using *pfu* Turbo DNA polymerase, the PCR cycles consisted of initial denaturation at 95°C for 30 seconds, followed by 18 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 12 minutes. Automated DNA sequencing (performed by the DNA Core Facility at the University of Iowa Carver College of Medicine) was performed to ensure that the mutation was correct. The coding region was then released from pBlueScript and inserted back into pcDNA 3.1 (Invitrogen, Carlsbad, CA). The plasmid was prepared by using the Qiagen maxiprep kit (Qiagen, Valencia, CA), and the entire coding sequence was subjected to automated DNA sequencing to ensure that the mutation was correct and that no inadvertent errors had been introduced during the PCR or ligation.

Wild-type NPY-Y1R cDNA with a 3HA tag at the N-terminus (hereafter referred to as HA-NPY-Y1R) in pcDNA 3.1 vector was also obtained from the cDNA Resource Center at the University of Missouri-Rolla. The enzymes *ApaI* and *PpuMI* were used to digest the untagged NPY-Y1R(K374T) plasmid and the HA-NPY-Y1R(wt) plasmid, and an overnight ligation was performed. Transformation was performed by using high-efficiency JM109 bacteria. Again, automated DNA sequencing was performed to ensure that the intended mutation was achieved in the tagged plasmid. Finally, automated sequencing of the entire coding region of the plasmid was performed to ensure the correctness of the sequence.

Cells and Transfections

Human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection (Manassas, VA)

and cultured at 5% CO_2 in Dulbecco's modified Eagle's medium containing 50 μg/mL gentamicin, 10 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and 10% newborn calf serum. For transfections, cells were plated on gelatin-coated, 35-mm, 6-well plastic plates from Corning (Corning, NY). Cells were transfected by using the calcium precipitation method (14) and 4 μg plasmid in 2 mL media per 35-mm well. Cells cotransfected with the wt and mutant NPY-Y1R received 2 μg of each plasmid. Approximately 48 hours after transfection, cells were used for flow cytometry studies of cell-surface expression and for radioimmunoassay of NPY attenuation of forskolin-stimulated cyclic adenosine 3′:5′ monophosphate (cAMP) production.

Flow Cytometry for Evaluation of Cell-Surface Expression

HEK293 cells were plated and transfected as described previously. Forty-eight hours after transfection, cells were washed once with filtered phosphate-buffered saline for immunohistochemistry (phosphate-buffered saline–intralipid + heparin [IH], 137 mmol/L NaCl, 2.7 mmol/L KCl, 1.4 mmol/L KH_2PO_4, 4.3 mmol/L Na_2HPO_4, and pH 7.4). Cells were then detached by washing with phosphate-buffered saline–IH and centrifuged at 500 × g to precipitate the cells. The supernatant was then removed, and the cells were resuspended in phosphate-buffered saline–IH and incubated for 1 hour at 4°C with or without fluorescein isothiocyanate conjugate–labeled monoclonal antibody HA.11 (Covance Research Products, Berkeley, CA) diluted 1:100 with buffer.

A Becton Dickinson (Devon, UK) fluorescence-activated cell sorter DiVa with a 488-nm wavelength laser was then used to quantify cell-surface expression in 10,000 cells from each transfection. Control gating was set by using cells transfected with pcDNA 3.1 empty vector and stained with HA.11. The arbitrary fluorescence units describing total cell-surface fluorescence was determined as the product of the percent cells gated and the geometric mean fluorescence of the sample minus the respective product in the control group.

Attenuation of Intracellular cAMP Production After NPY Treatment

HEK293 cells were plated and transfected as described earlier. Forty-eight hours after transfection, cells were washed twice with warm Waymouth's MB752/1 media modified to contain 50 μg/mL gentamicin and 1 mg/mL bovine serum albumin. One milliliter of Waymouth's MB752/1 media containing 50 μg/mL gentamicin and 1 mg/mL bovine serum albumin as well as 0.5 mmol/L isobutylmethylxanthine (Sigma-Aldrich, St. Louis, MO) was then added to each well, and the cells were incubated at 37°C for 15 minutes. After this incubation, buffer alone, 10 μmol/L forskolin (Sigma-Aldrich) alone, or 10 μmol/L forskolin and 100 nmol/L NPY (Bachem, Torrance, CA) were added to the wells, and the incubation was continued for another 15 minutes at 37°C. All assays were performed in triplicate. Plates were then set on ice, media was aspirated, and 0.5 N perchloric acid containing 108 μg/mL theophylline was added to extract cAMP. Intracellular cAMP levels were measured by RIA with all measurements performed in duplicate. The cAMP RIA was performed by using reagents generated by one of the laboratories (D.L. Segaloff) rather than a commercial kit and has been the method used for cAMP assays in all published studies by this laboratory.

Statistical Analyses

One-way ANOVA and unpaired *t*-tests were used for statistical analyses, with a *P* value set at .05. Statistical analyses were performed by using InStat (GraphPad Software, San Diego, CA).

RESULTS

Identification of NPY-Y1R Mutation K374T

Automated sequencing of the *NPY-Y1R* gene revealed a heterozygous substitution of adenine for cytosine at nucleotide 1330 in exon 3 in one of the patients with GDPP (patient 27, Table 1). This resulted in the substitution of lysine (K) by threonine (T) at codon 374 (K374T) in the carboxyl terminal region of the receptor. This patient's mother carried the same heterozygous variant but had normal pubertal development, with menarche at age 12. The patient's younger sister was homozygous for the wt sequence of the *NPY-Y1R* gene yet had also been diagnosed with idiopathic GDPP. Microsatellite analysis confirmed that samples were attributed to the appropriate subjects and that biological maternity and paternity were correct.

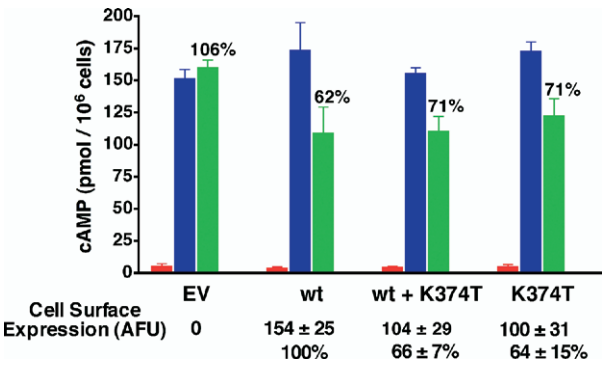
No other allelic variants of the *NPY-Y1R* gene were identified in the remaining subjects with GDPP or HH. Furthermore, sequencing analysis of 100 alleles from the 50 Brazilian controls with normal puberty did not reveal the K374T mutation of the NPY-Y1R.

Functional Analysis of K374T NPY-Y1R

To test the functional activity of the NPY-Y1R(K374T), this variant was introduced into a recombinant hemagglutinin epitope (HA)-tagged NPY-Y1R. HEK293 cells transiently transfected with wt HA-NPY-Y1R were compared with cells transfected with the K374T mutant of HA-NPY-Y1R (reflecting a homozygous expression of the mutant) or cells cotransfected with equal parts wt and mutant HA-NPY-Y1R (reflecting a heterozygous expression of the mutant as in the proband). In the same experiments, cells were assayed for cell-surface expression of receptor by flow cytometry by using an antibody against the HA epitope and were assayed for NPY-induced attenuation of forskolin-stimulated cAMP by using concentrations of NPY previously shown to be maximally effective (15). Preliminary studies showed that the HA tag on the N-terminus of the NPY-Y1R did not affect its signaling as determined by NPY-induced attenuation of forskolin-stimulated cAMP production in 293 cells (data not shown).

FIGURE 1

Attenuation of forskolin-stimulated cAMP production by NPY is not significantly impaired in HEK293 cells expressing the K374T variant of the NPY-Y1R. HEK293 cells were transiently transfected with empty vector (pcDNA3.1), HA-NPY-Y1R(wt) only, HA-NPY-Y1R(wt) plus HA-NPY-Y1R(K374T), or HA-NPY-Y1R(K374T) only. cAMP production was measured in cells incubated with no additions (red), forskolin only (blue), or foskolin plus NPY (green) as described in the Materials and Methods section. The numbers above the bars depict the percent cAMP accumulation in the presence of NPY plus forskolin as compared with forskolin only for each cell group; 100 minus each number would be the percent attenuation by NPY. The percent attenuation by NPY in the wt plus K374T cells or the K374 cells was not statistically different than that in the wt cells. Cell surface expression was determined in the same experiments by flow cytometry in intact cells by using an antibody to the HA epitope tag, as described in Materials and Methods. The arbitrary fluorescence units (AFU), therefore, are proportional to the total amount of NPY-Y1R (wt and mutant) on the cell surface of each group of cells. Cell-surface expression of the K374T cells and the wt plus K374T cells are also shown as percent control relative to the wt cells. Results are presented as the mean ± SEM of three independent experiments.



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As shown in Figure 1, cell-surface expression of the cotransfected wt and mutant or mutant alone was 66% and 64% of wt expression, respectively, in the same 3 experiments. Despite the decreased level of expression of the mutant NPY-Y1R, HEK293 cells expressing the mutant only or mutant plus wt receptor did not exhibit a significantly diminished attenuation of cAMP in response to NPY as compared to cells expressing wt receptor only. Thus, as shown in Figure 1, cells transfected with empty vector were not affected by the addition of NPY. In contrast, the addition of NPY to cells expressing the wt HA-NPY-Y1R caused a marked attenuation of forskolin-stimulated cAMP production. The decrease to 62% of control (i.e., forskolin only) represents a 38% attenuation and is similar to that reported by others (15). Cells cotransfected with equal concentrations of wt HA-NPY-Y1R and HA-NPY-Y1R(K374T), representative of a heterozygous expression of the mutant receptor, also showed a NPY-induced attenuation of forskolin-stimulated cAMP. In this case, the NPY-mediated reduction of cAMP levels to 71% of control represented an attenuation of 29%. Finally, cells expressing only HA-NPY-Y1R(K374T) showed an identical NPY-induced attenuation (29%) of forskolin-stimulated cAMP. Statistically, the percent NPY-induced attenuation of forskolin-stimulated cAMP was not significantly different between the cells transfected with wt receptor only, wt plus K374T, or the K374T mutant only (P=.496).

DISCUSSION

The exact systems that regulate the timing of human puberty remain elusive (3). Multiple factors are implicated in the regulation of the reproductive axis, including environmental, metabolic, and genetic influences (16). A genetic component has become increasingly evident through the study of patients with isolated congenital hypogonadotropic hypogonadism for instance (11, 17–24).

More direct evidence for the genetic regulation of puberty is provided by data showing a correlation between the ages at which a mother and her offspring attain puberty, by the variation in onset of puberty between racial groups, and by the greater concordance in the timing of puberty between monozygotic versus dizygotic twins (25). The identification of functional gene mutations or sequence variants that lead to abnormalities in the reproductive axis can further aid in understanding the regulation of pubertal timing. Because it has been shown that NPY is a fundamental component in the inhibition of the pulsatile GnRH secretion in the nonhuman primate, it is a promising candidate for study in human puberty.

In the present study, we identified a missense variant at codon 374 in 1 girl with idiopathic GDPP, resulting in the substitution of the amino acid lysine for threonine within the carboxyl terminal region of the NPY-Y1R. Segregation analysis in this family showed that the proband's sister had precocious puberty despite the absence of the K374T variant, whereas the proband's mother had normal pubertal development despite carrying the K374T mutant allele. Therefore, linkage between this mutant allele and clinically relevant abnormalities appears to be absent.

The codon 374, however, is highly conserved in NPY-Y1R among different species, including the Guinea pig, mouse, rat, and monkey. To date, few polymorphisms have been identified in the entire coding region of the human *NPY-Y1R* gene. Halushka et al. (26) investigated the pattern

and frequency of single-nucleotide polymorphisms in 75 candidate genes for blood pressure homeostasis in humans, including the *NPY-Y1R*. Their analysis of 2,272 bp of the *NPY-Y1R* gene revealed only 2 polymorphisms, including the K374T substitution, and these were deposited in NCBI (www.ncbi.nml.nhi.gov/SNPs, rs5578). Details of the pubertal development of the subjects affected by this mutation have not been reported. Recently, a genotype analysis of the *NPY-Y1R* and *NPY-Y5R* genes in 14 girls and 1 boy with GDPP, along with 72 controls, was reported (27). No sequence variants of the *NPY-Y1R* were identified in any of the patients or control subjects. Furthermore, in our current study, we identified only 1 nucleotide substitution out of 210 alleles that were sequenced.

The genes encoding the NPY-Y1R and NPY-Y5R receptor subtypes are transcribed in opposite directions from a common promoter in a region of chromosome 4q31-32 and most likely evolved from a gene duplication event (28). Thus, a mutation in one of these receptors genes may have consequences on expression of the other gene. However, the K374T variant identified in this study appears to be located in the 5′ untranslated region of the NPY-Y5R gene and, therefore, would not affect the coding sequence of the NPY-Y5R protein.

Considering the high interspecies conservation and low frequency of polymorphisms, it appears that human NPY-Y1R has been highly conserved throughout evolution. This also suggests a high functional preservation of the corresponding NPY-Y1R protein. Therefore, despite the lack of segregation of the K374T variant among affected and unaffected members of this studied Brazilian family, we investigated the functional properties of this mutant in transiently transfected HEK293 cells.

By using immunological means to quantify the cell-surface expression of wt or mutant HA-NPY-Y1R, we determined that cells expressing the K374T mutant only (per homozygous expression) or expressing equal parts K374T mutant and wt receptor (per heterozygous expression) possessed ~35% decreased levels of cell-surface receptor as compared with cells expressing the wt receptor only. We presume that this reduction in cell-surface expression is because of the partial retention of mutant K374T receptor in the endoplasmic reticulum because of the cell's quality control detecting misfolding of the receptor, a fate common to many G-protein–coupled receptor (GPCR) mutants that cause decreased cell-surface expression (29–36).

Functional assays revealed that the ability of NPY to inhibit forskolin-induced cAMP production was not statistically different in HEK293 cells transfected with K374T only or cotransfected with wt and K374T as compared with cells transfected with only the wt receptor. Therefore, the functional properties of K374T appear normal.

We conclude that in transfected HEK293 cells the ~35% reduction in cell-surface expression of NPY-Y1R(K374T) does not result in a functional impairment of the cells to NPY response. These observations, coupled with the lack of functional linkage of the K374T mutation in the subject's immediate family, suggest that K374T is a rare, nonfunctional polymorphism of the *NPY-Y1R* gene. One cannot exclude the possibility, however, that the lack of a statistically significant impairment of NPY signaling in HEK293 cells transfected with the K374T variant may be because of the presence of an excess of spare NPY-Y1 receptors (wt and/or mutant, depending on which type has been transfected) in the HEK293 cells relative to Gi. As such, a reduction in cell-surface expression of receptor would not be expected to reduce the maximal response to NPY.

It is not known, however, whether cell-surface NPY-Y1R expression and intracellular signaling are more tightly coupled in neuronal cells in vivo than in the heterologous expression system used for our in vitro studies. If neuronal cells do not possess spare endogenous NPY-Y1R, then a reduction in cell-surface expression would more likely give rise to a reduction in NPY signaling. Therefore, if neuronal cells did not express spare cell-surface NPY-Y1R, it is possible that the decreased expression of the K374T variant might result in a more marked decrease in NPY responsiveness than that observed in HEK293 cells. Certainly, even if this were the case, one could not conclude that the K374T variant was solely causative of our subject's GDPP because of the lack of segregation between the K374T variant and GDPP in the subject's family. However, it would remain possible that the K374T variant of the NPY-Y1R, in combination with other potentially genetic factors, could have contributed to the subject's GDPP.

In summary, we have identified a heterozygous K374T variant in the human *NPY-Y1R* gene in a girl with GDPP. Linkage of this variant with GDPP in the subject's immediate family was not evident. Further experiments showed that although the cell-surface expression of K374T is partially impaired in the heterozygous state, HEK293 cells expressing the variant respond normally to NPY. We cannot formally rule out the possibility that neuronal cells expressing K374T may suffer reduced functional activity toward NPY. Taken altogether, however, our data suggest that the K374T variant is a rare, nonfunctional polymorphism of the *NPY-Y1R* gene and that mutations of the highly conserved *NPY-Y1R* gene do not represent a frequent mechanism underlying human idiopathic central pubertal disorders.

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