TAC3/*TACR3* Mutations Reveal Preferential Activation of Gonadotropin-Releasing Hormone Release by Neurokinin B in Neonatal Life Followed by Reversal in Adulthood

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Context: Mutations in *TAC3* and *TACR3* (encoding neurokinin B and its receptor) have been identified in Turkish patients with idiopathic hypogonadotropic hypogonadism (IHH), but broader populations have not yet been tested and genotype-phenotype correlations have not been established.

Objective: A broad cohort of normosmic IHH probands was screened for mutations in *TAC3*/*TACR3* to evaluate the prevalence of such mutations and define the genotype/phenotype relationships.

Design and Setting: The study consisted of sequencing of *TAC3*/*TACR3*, *in vitro* functional assays, and neuroendocrine phenotyping conducted in tertiary care centers worldwide.

Patients or Other Participants: 345 probands, 18 family members, and 292 controls were studied.

Intervention: Reproductive phenotypes throughout reproductive life and before and after therapy were examined.

Main Outcome Measure: Rare sequence variants in *TAC3*/*TACR3* were detected.

Results: In *TACR3*, 19 probands harbored 13 distinct coding sequence rare nucleotide variants [three nonsense mutations, six nonsynonymous, four synonymous (one predicted to affect splicing)]. In *TAC3*, one homozygous single base pair deletion was identified, resulting in complete loss of the neurokinin B decapeptide. Phenotypic information was available on 16 males and seven females with coding sequence variants in *TACR3*/*TAC3*. Of the 16 males, 15 had microphallus; none of the females had spontaneous thelarche. Seven of the 16 males and five of the seven females were assessed after discontinuation of therapy; six of the seven males and four of the five females demonstrated evidence for reversibility of their hypogonadotropism.

Conclusions: Mutations in the neurokinin B pathway are relatively common as causes of hypogonadism. Although the neurokinin B pathway appears essential during early sexual development, its importance in sustaining the integrity of the hypothalamic-pituitary-gonadal axis appears attenuated over time. **(*J Clin Endocrinol Metab* 95: 2857–2867, 2010)**

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Abbreviations: EV, Empty vector; hpg, hypothalamic-pituitary-gonadal (axis); IHH, idiopathic hypogonadotropic hypogonadism; IP, inositol phosphate; nIHH, normosmic IHH; NKB, neurokinin B; NK3-R, tachykinin receptor 3; T, testosterone.

Humans with defects in GnRH secretion and/or action with resulting idiopathic hypogonadotropic hypogonadism (IHH) have been instrumental in defining the genetic control of this hormone. Human, animal, cellular, and bioinformatic models have been integrated to identify and understand the roles of genes affecting GnRH neuronal migration [*KAL1* (1), *FGFR1* (2), *FGF8* (3), *NELF* (4), *PROK2* (5–8), *PROKR2* (6, 8)], GnRH secretory activity [*KISS1-R* (9, 10), *GNRH1* (11–13)], pituitary GnRH responsiveness [*GNRHR* (14)], and, in some cases, functions yet to be clearly understood [*CHD7* (15)]. The newest proteins on this list are neurokinin B (NKB, encoded by *TAC3*) and its cognate G protein-coupled receptor, NK3-R (encoded by *TACR3*) (16). NKB is a member of the tachykinin superfamily of neuropeptides that includes substance P and neurokinin A (17). Mutations in the genes encoding this ligand-receptor pair were recently identified in a Turkish population of normosmic IHH (nIHH) patients (16).

The mechanism(s) by which mutations in the NKB pathway cause GnRH deficiency and IHH are not yet clear. However, NKB is expressed in the same neurons that express kisspeptin, a member of the RF amide family of proteins (sharing the common C-terminal sequence Arg-Phe-NH2) (18, 19). Although mutations in the gene encoding kisspeptin (*KISS1*) have yet to be reported in humans with GnRH deficiency, mutations in the gene encoding the kisspeptin receptor (*KISS1-R* or *GPR54*) have been identified in nIHH probands as well as an individuals with central precocious puberty (9, 10, 20–23). Kisspeptin is a powerful stimulus for GnRH-induced LH secretion, whereas paradoxically, NKB agonists appear to have an excitatory or inhibitory effect on GnRH-induced gonadotropin secretion in rodent models depending on gender and sex steroid milieu (24–27). This variability in response to NKB seems at odds with the discovery of loss of function mutations in the NKB signaling pathway in hypogonadotropic patients (16).

Because the original mutations in *TAC3* and *TACR3* were described only in Turkish patients, the goal of this study was to examine the prevalence of TAC3 and TACR3 mutations in a much larger, international cohort of patients. Once putative mutations were identified, their functional consequences were studied *in vitro*. The phenotypic consequences of mutation-carrying patients were investigated at several developmental windows including neonatal life, adolescence/early adulthood, and late adulthood (after sex steroid therapy) as evaluated by physical examination, gonadotropin pulse pattern, and fertility outcome.

The data from these studies demonstrate that functionally validated rare sequence variants within the tachykinin pathway profoundly impact the functioning of the hypothalamic-pituitary-gonadal (hpg) axis in late gestation. However, the effect of these same mutations appears to attenuate over time because a significant proportion of patients carrying mutations exhibited partial or complete reversal of their hypogonadotropism in adult life. These data thus suggest that patients with genetic mutations in this system may not require lifelong sex steroid replacement and may be capable of spontaneous fertility. Finally, these findings have importance for assembling the hierarchy of the various neuroendocrine and genetic determinants of GnRH secretion in the human during both adolescent puberty and the "mini-puberty" of neonatal life.

Patients and Methods

All study activities fell under research protocols approved by the Massachusetts General Hospital Institutional Review Board.

Patient cohorts

The *TAC3* and *TACR3* genes were screened in two groups: patients affected by nIHH, and prospectively recruited volunteers determined to have normal reproductive function by history and physical examination (192 Caucasians screened for all coding exons, and 100 Brazilians screened only for five nucleotide variants: G18D, I249V, Y256H, W275X, and R295S). Patients were referred directly to the Reproductive Endocrine Associates of Massachusetts General Hospital or the Developmental Endocrinology Unit of the Clinical Hospital in Sao Paulo, Brazil, for clinical evaluation, or they were referred by their physicians to participate in genetic studies. Whenever possible, patients were interviewed by both a physician and a genetics counselor using an Institutional Review Board-approved questionnaire to obtain a thorough medical and family history.

The diagnosis of nIHH was based on the failure to undergo normal sexual maturation by age 18 yr, low serum sex steroid levels in the setting of inappropriately low or normal gonadotropin levels, and no other abnormality detected on cranial imaging (28).

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Olfactory testing employed the University of Pennsylvania identification test whenever possible (29). A score of at least the fifth centile based on sex and age was deemed normal. Of the 345 IHH patients who were screened, 107 were normosmic by olfactory testing, four were hyposmic, and the remaining 234 were normosmic by self-report.

The 345 IHH probands represented a diverse racial mix: 213 were Caucasian (including 37 from Turkey), 25 were Asian, one was American Indian/Alaskan native, 12 were African American, one was mixed native American/Caucasian, and 93 were unknown [of these, 60 of 93 were Brazilian (30)] (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Of the nIHH probands, 248 were male, 95 were female, and two were unknown. These ratios are consistent with previously published reports demonstrating a male predominance in nIHH (31).

Of the nIHH pedigrees, 89 were familial and 80 were sporadic, with the remaining unknown as to their mode of inheritance. Of the familial cases, 43 could be classified as autosomal dominant, 27 as autosomal recessive, and two as X-linked.

Clinical studies

Of the 345 IHH patients screened, 97 were admitted to the General Clinical Research Center of Massachusetts General Hospital for blood sampling every 10 min for 12–24 h to assess endogenous GnRH-induced LH secretion as previously described (32).

Clinical assays

Blood-sampling studies were performed over a 29-yr period; therefore, two different immunoassay systems were used for the measurement of LH. Originally, LH measurements were made via RIA with a limit of detection of 0.8 IU/liter (33). Later, this assay was switched to an automated microparticle enzyme immunoassay (AxSYM System; Abbott Laboratories, Abbott Park, IL) with a limit of detection of 1.6 IU/liter. The microparticle enzyme immunoassay was calibrated using the same reference preparations as the RIA to make results comparable across data sets. The data regarding the presence or absence of LH pulses are based on the respective limits of detection of each system and integrated by virtue of a common standard in both assays that permitted interconversion.

Mutation analysis

Genomic DNA was extracted from peripheral blood leukocytes. Probands were screened by PCR amplification of exon segments and direct sequencing. The coding region of the *TAC3* and *TACR3* genes were amplified using PCR. The *TAC3* gene (chromosome 12; GenBank accession no. NM 013251) consists of 10 exons. The *TACR3* gene (chromosome 4; GenBank accession no. NM 001059) consists of five exons. Primers are outlined in Supplemental Data 1. All amplified products were sequenced using the AmpliTaq Dye Terminator Cycle Sequencing Kit and an ABI PRISM 377 DNA sequencer (Perkin-Elmer Corp., Foster City, CA). All sequence variations were found on both strands and confirmed in a separate PCR. All nucleotide changes were assessed for their presence in the National Center for Biotechnology Information database of single nucleotide polymorphisms, the expressed sequence tags database, and among control alleles.

Of the 345 probands in the cohort, 284 were also screened for mutations in genes known to be involved in IHH, including *FGFR1* (n = 332), *KISS1-R* (n = 336), *NELF* (n = 274), *GNRHR* (n = 343), *FGF8* (n = 332), *GNRH1* (n = 200), *KAL1* (n = 334), *PROK2* (n = 330), and *PROKR2* (n = 330).

Functional studies

African green monkey kidney fibroblast cells (COS-7 cells) were transiently transfected with 0.5 μg of either WT, G18D, I249V, Y256H, R295S, or Y315C NK3-R or empty vector (EV) (pcDNA3.1+) using GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA). Generation of NK3-R mutations and inositol phosphate (IP) assays are described in Supplemental Data 2.

Results

IHH and *TACR3*

Ethnic and gender distribution

Rare nucleotide variants in *TACR3* were identified in 19 of 345 nIHH probands for a total prevalence of 5.5%. When the frequency of variants was examined in different racial subgroups, nucleotide changes were identified in 10 of 213 Caucasian probands (4.7%; four of the 10 probands were Turkish, for a 10.8% frequency in that ethnic subgroup), two of 25 were Asian probands (8%), and one of 12 was an African-American proband (8.3%) (Table S1). In probands where the racial designation was uncertain, one of 11 Hispanic probands (9%), four of 60 Brazilian probands (6.7%) (30), and one additional individual also harbored variants (Supplemental Table 1). Rare nucleotide variants were identified in 15 of 247 males (6.1%) and four of 95 females (4.2%) and thus occurred at approximately equal frequency in both genders.

Variants

Of the 345 nIHH probands, 19 were found to harbor 13 distinct coding sequence nucleotide variants in *TACR3* that were not observed in 292 controls (Table 1; also see Fig. 2). With the exception of P353S, which had previously been reported in the Turkish population (16), all other variants were novel. Three of the 13 variants were nonsense mutations (S27X, W280X, W275X); six of 13 variants were nonsynonymous (*i.e.* changing the amino acid: G18D, I249V, Y256H, R295S, Y315C, P353S); and the remaining four variants were synonymous (*i.e.* not changing the amino acid: L58L, V98V, T246T, S448S). However, the homozygous G>A transition underlying T246T occurred in the last base pair of exon 2 and was predicted to affect splicing (NNSPLICE 0.9). Of the nonsense, nonsynonymous, and putative splice site changes (n = 10), six were homozygous [S27X, W275X (present in three probands), Y256H, Y315C, P353S, T246T], and five variants

TABLE 1. Probands bearing rare variants in *TACR3* and *TAC3*

Proband	Change (nucleotide)	Change (amino acid)	Functional consequences	Inheritance	Race	Coding mutation in other genes
TACR3						
Nonsense mutations						
1	[c. 80 C>A]+[c. 80 C>A]	S27X/S27X	PTC		C	None[1]
2	[c. 623 G>A]+[=]	W208X/N	PTC	F	C	None[1]
3	[c. 824 G>A]+[c. 824 G>A]	W275X/W275X	PTC	S	C	None[2]
4	[c. 824 G>A]+[c. 824 G>A]	W275X/W275X	PTC	F	C	None[1]
5	[c. 824 G>A]+[c. 824 G>A]	W275X/W275X	PTC	F		None[4]
6	[c. 824 G>A]+[=]	W275X/N	PTC	S	C	None[1]
7	[c. 824 G>A]+[=]	W275X/N	PTC	F	C	None[1]
8	[c. 824 G>A]+[=]	W275X/N	PTC	S	C	None[2]
9	[c. 172 C>T]+[=] and [c. 824 G>A]+[=]	L58 L/N and W275X/N	N/A and PTC	F		None[4]
Nonsynonymous changes						
10	[c. 53 G>A]+[=]	G18D/N	=	S		None[4]
11	[c. 745 A>G]+[=]	I249V/N	=	F	H	None[3]
12	[c. 766 T>C]+[c. 766 T>C]	Y256H/Y256H	↓↓↓ IP	S	C	None[1]
13	[c. 885 A>C]+[=]	R295S/N	↓↓↓ IP	F		None[4]
14	[c. 944 A>G]+[c. 944 A>G]	Y315C/Y315C	↓↓↓ IP	F		None[2]
15	[c. 1057 C>T]+[c. 1057 C>T]	P353S/P353S	↓↓↓ Calcium[a]		C	None[1]
Synonymous changes						
16	[c. 294 G>C]+[=]	V98V/N	N/A	S	AA	Not screened
17	[c. 738 G>A]+[c.738 G>A]	T246T/T246T	Loss of splice site	S	A	GnRH1: het T58S 2
18	[c. 1344 C>T]+[=]	S448S/N	N/A	(Adopted)	A	None[2]
19	[c. 1344 C>T]+[=]	S448S/N	N/A		C	None[1]
TAC3						
20	[c. 60 Gdel]+[c. 60 Gdel]	G20fsX39/G20fsX39	PTC	F	A	None[2]

[1] Screened for GnRHR, KISS1-R, FGFR1, FGF8, PROK2, PROKR2, NELF, GnRH1; [2] screened for GnRHR, KISS1-R, FGFR1, FGF8, PROK2, PROKR2, NELF; [3] screened for GnRHR, KISS1-R, FGF8; [4] screened for GnRHR, KISS1-R, FGFR1, FGF8, PROK2, PROKR2, GnRH1. N/A, Not applicable; N, normal allele; PTC, premature termination codon; F, familial; S, sporadic; C, Caucasian; A, Asian; AA, African-American; H, Hispanic; =, IP accumulation not different from WT; ↓↓↓, IP accumulation reduced compared to WT.

[a] Previously published (16).

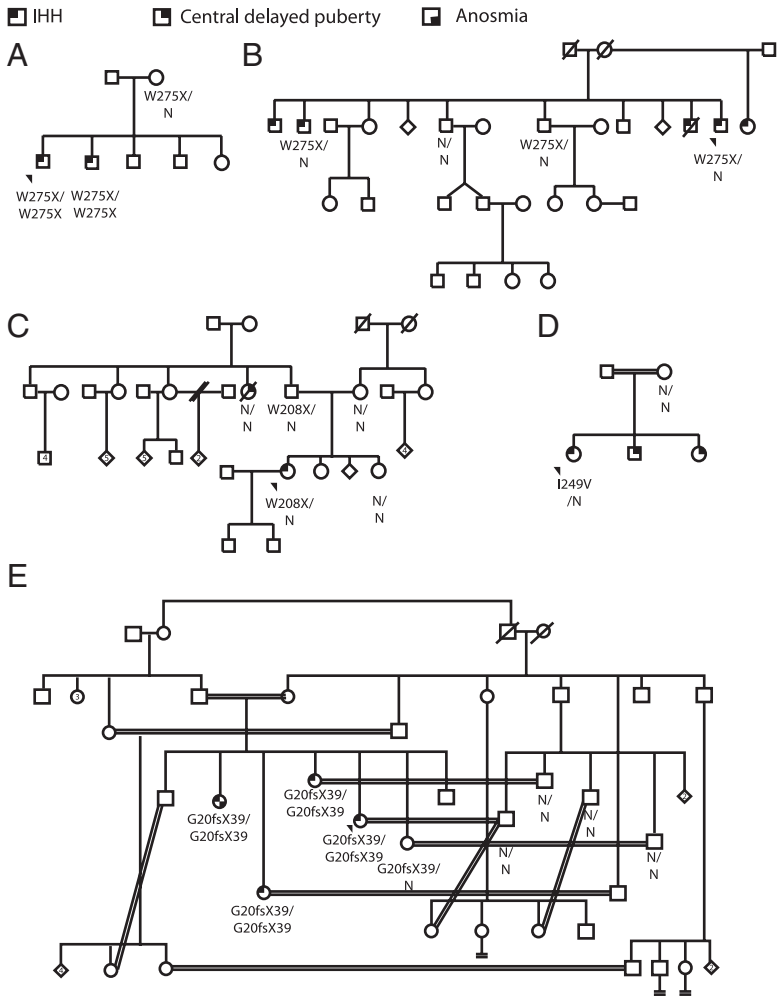

FIG. 1. Pedigrees of familial cases of *TACR3* and *TAC3* mutations. With only one exception, all the affected family members had the same change as their respective probands. A, Proband 4; B, proband 7; C, proband 2; D, proband 11; E, proband 20.

were heterozygous [G18D, W208X, I249V, W275X (present in four probands), R295S].

Of the eight probands bearing homozygous *TACR3* variants, three were familial (only one of the three appeared to have an autosomal recessive mode of inheritance), three were sporadic, and two were unknown. Of the 11 probands bearing heterozygous (n = 10) or possible compound heterozygous (n = 1) variants, five were familial (one dominant, two recessive, and two unknown), four were sporadic, and two were unknown.

In familial cases, the identified nucleotide variants did not arise *de novo* but rather were present in at least one parent and all family members who were affected by IHH and whose DNA was available (Fig. 1). This observation suggests that *TACR3* does not undergo frequent spontaneous mutagenesis.

Functional studies

In WT NK3-R transfected cells, NKB caused a 16.7 ± 3.9-fold induction of IP production; in contrast, cells transfected with EV failed to show any IP induction. Y256H, R295S, and Y315C NK-3R variants showed complete loss of function, with IP induction of 1.2 ± 0.2-fold, 3.2 ± 0.6-fold, and 1.7 ± 0.5-fold, respectively, over unstimulated levels. In contrast, cells transfected with the G18D or I249V NK3-R variant had a 13.5 ± 3.5-fold and 16.1 ± 4.4-fold IP induction, respectively, in response to 10^{-7} M NKB, not significantly different from cells transfected with WT NK3-R (Fig. 2).

Clinical phenotypes

Probands carrying rare variants in *TACR3* exhibited a broad range of phenotypes. Of males carrying coding sequence variants in *TACR3* and in whom information was available (n = 15 probands and one family member), 15 of 16 or 94% had microphallus [stretched penile length of less than 10.5 cm (34)] as assessed by questionnaire or physical examination at first presentation (in most cases) (Table 2). Surprisingly, despite this high prevalence of microphallus, only two probands (no. 7 and no. 13) had cryptorchidism. Testes size was generally small but did range from 1–12 ml, suggesting that some probands had undergone partial sexual maturation (35) (Table 2). Of females carrying sequence variants in *TACR3* and in whom information was available (n = 3), none had spontaneous thelarche or menarche (Table 3).

Probands were also evaluated after treatment was discontinued (most commonly sex steroids). In striking contrast to the signs of severe hypogonadotropism that accompanied their initial presentation (pre treatment), several probands with *TACR3* mutations showed evidence for spontaneous activity of their hpg axis post treatment in adulthood. Three males [no. 6 with W275X/N, no. 8 with W275X/N, no. 12 with Y256H/Y256H (functionally validated)] exhibited significant increases in testicular volume while on androgens, suggestive of endogenous gonadotropin stimulation of Sertoli cell complement (Table 2). Notably, no. 6 and no. 8 also achieved fertility in the absence of gonadotropin/GnRH therapy.

Four male probands underwent blood sampling every 10 min to assess endogenous GnRH-induced LH pulsatility (Table 2 and Fig. 3). Within this group, no. 12 [Y256H/Y256H (functionally validated)] had 5-ml testes and a testosterone (T) level of 65 ng/dl pre treatment. After androgen treatment, he had 12-ml testes, T level of 222

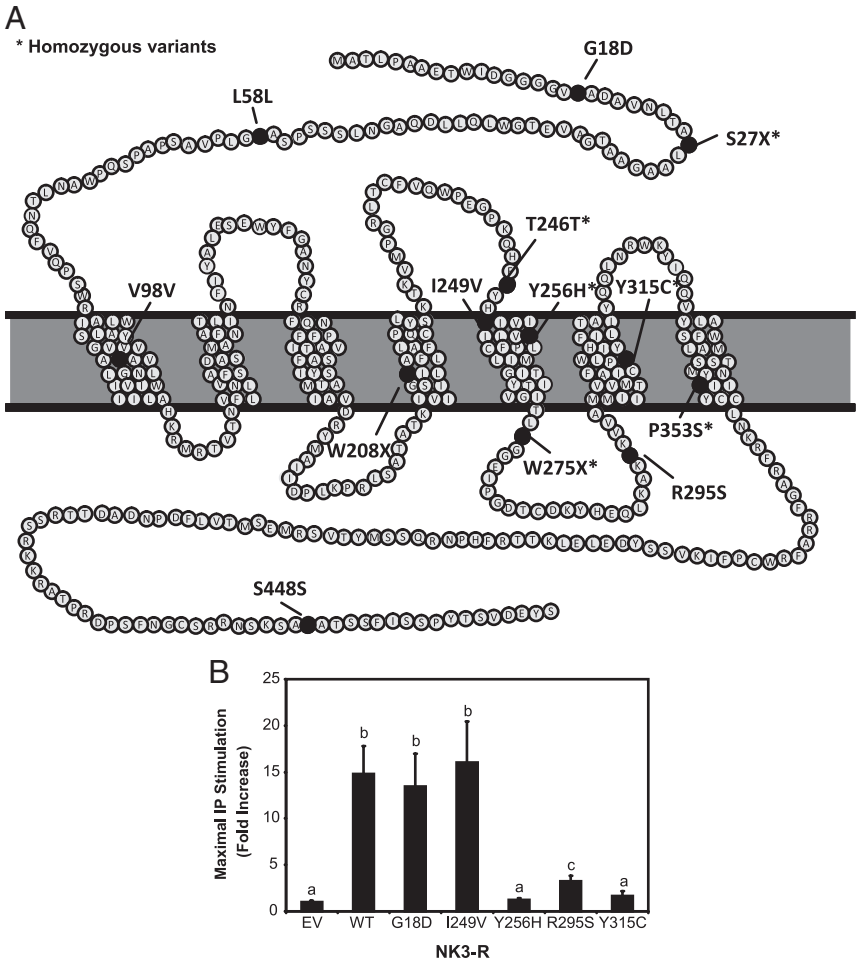

FIG. 2. A, Schematic of mutations in NK3-R. B, Effects of mutations in *TACR3* on NKB-mediated activation of signal transduction. COS-7 cells transfected with wild-type (WT), G18D, I249V, Y256H, R295S, and Y315C NK3-R or EV were treated with NKB (10^{-7} M) for 1 h. A significant increase in IP accumulation occurred in cells transfected with WT, G18D, or I249V NK3-R. In contrast, there was a marked reduction in NKB-stimulated IP production in cells transfected with Y256H, R295S, or Y315C NK3-R, or with EV. *a*, *b*, and *c* denote significantly different fold increases in IP accumulation.

ng/dl, and four LH pulses during 12 h of blood sampling every 10 min. Similarly, patient 16 (V98V/N) had a T level of 16 ng/dl before treatment but 246 ng/dl after treatment, accompanied by five LH pulses in 12 h. Proband 6 (W275X/N) had a complete absence of GnRH-induced LH pulses before starting sex steroid therapy; after treatment, two LH pulses were detectable. Despite this modest change in his neuroendocrine profile, years later, this patient conceived a child in the absence of GnRH or gonadotropin therapy (genetic paternity not confirmed). Proband 7 (W275X/N) also had no LH pulses before androgen therapy but after treatment demonstrated robust LH pulses (although his T levels remained subnormal) (Table 2 and Fig. 3). Notably, one of the brothers of proband 7 (7b) who carried the same W275X mutation demonstrated an active LH pattern on his post treatment sampling study but with normal T levels (Figs. 1B and 3 and Table 2). Another brother with IHH also showed a "normalized" hpg axis after treatment based on hormone levels, but no DNA is available to confirm his *TACR3* genotype (Fig. 1B).

IHH and *TAC3*

For *TAC3*, one female proband from a consanguineous family (no. 20) was found to harbor a homozygous frameshift mutation (c.G60del) leading to a premature termination codon in the precursor upstream of the NKB decapeptide (Supplemental Fig. 1). After treatment with sex steroids, this individual conceived a child without fertility medications, but she subsequently suffered an early pregnancy loss. One of her sisters carrying the same homozygous mutation (no. 20b) also conceived spontaneously, and she carried the pregnancy to term (Table 3 and Fig. 1). Sister 20c has not conceived but has regular menstrual cycles (Table 3 and Fig. 1). Sister 20d has had a positive withdrawal bleed to a progesterone challenge but does not cycle spontaneously (Table 3 and Fig. 1).

Discussion

This report focuses on reproductive phenotypes before and after sex steroid therapy in hypogonadotropic patients with rare nucleotide variants in the NKB pathway. Although information regarding the probands and family members comes from several different sources with variable depth of phenotyping, therapies, and intensity of follow-up, two striking biological features emerge. Most males carrying *TACR3* variants for whom physical examination evidence was available (94%) had microphallus identified either neonatally or later in life. This frequency stands in sharp contrast to an 8% prevalence of microphallus in an nIHH cohort (n = 42) selected without consideration of genetic etiology (35). Because phallic growth is strongly influenced by the integrity of the *in utero* activity of the hpg axis in late gestation (36), this consistent presence of microphallus suggests that NKB-stimulated signaling plays an important role as a driver of GnRH secretory activity during this critical window of development. Second, the majority of patients who were assessed longitudinally after discontinuation of sex steroid

TABLE 2. Phenotypic characterization of male probands and family members harboring rare variants in *TACR3* before and after treatment

	Before treatment				After treatment				
Proband	Testis size; phallus size	T levels (ng/dl)	No. of pulses/mean LH (q 10 min sampling × 12 h for LH)	Treatment	Testis size; phallus size	T levels (ng/dl)	No. of pulses/mean LH (q 10 min sampling × 12 h for LH)	Fertility	Evidence for neuroendocrine recovery
1	–	–	–	T	0.5 ml; 6 cm	–	–	No	–
3	"Small phallus"[a]	14	–	T	4–5 ml; 9.5 cm	–	–	No	–
4	1 ml; 7 cm	–	–	T	–	–	–	No	–
5	2 ml; 7 cm	35	–	T	3 ml; 10 cm	–	–	No	–
6	2 ml; 5 cm	16	0/1.2	T	8 ml; "increased phallus"	38	2/3.2	S	Spontaneous fertility
7	2 ml; "small phallus"	57	0/0.8	T, GnRH pump, hCG	10 ml; "NL phallus"	68	8/7.9	No	↑ LH pulses
8	6 ml; 4 cm	<28	–	T, hCG+FSH	25 ml	285	–	S	Spontaneous fertility
9	6 ml; 12.5 cm	34	–	T	8 ml; 12.5 cm	–	–	No	–
10	2 ml	<14	–	–	–	–	–	–	–
12	5 ml; "small phallus"	65	–	T	12 ml; "NL phallus"	222	4/7.5	Not tried	↑ T
13	2 ml; 6 cm	30		T	9 cm	–	–	No	–
15	–	–	–	T, hCG	6 ml; 8 cm	"Low"	–	No	No
16	12 ml; "small phallus"[a]	16	–	T	13 ml; 10 cm	246	5/7.4	Not tried	↑ T
17	"Small phallus"[a]	–	–	T	3 ml	–	–	No	–
19	1–2 ml; "small phallus"	–	–	T	1–2 ml; 7 cm	–	–	Yes	–
Family members									
4b	–	–	–	–	–	–	–	–	–
7b	4 ml; "small phallus"	35	0/1.7	T, GnRH	11 ml; "NL phallus"	505	6/9.1	No	↑ LH pulses

NL, Normal; –, unknown; S, spontaneous; hCG, human chorionic gonadotropin; ↑, increased.

[a] These assessments were made at birth; all others were made at the first presentation for abnormal pubertal development.

TABLE 3. Phenotypic characterization of female probands and family members harboring rare variants in *TACR3* and *TAC3* before and after treatment

	Before treatment				After treatment				
Proband	Breast	Menarche	No. of pulses/mean LH (q 10 min sampling × 12 h for LH)	Treatment	Breast	Menarche	No. of pulses/mean LH (q 10 min sampling × 12 h for LH)	Fertility	Evidence for neuroendocrine recovery
TACR3									
2	No	No	0/0.8	E/P	III	Yes	0/0.9	No	No
11	No	No	0/1.6	E/P	IV	Yes	–	–	–
14	–	–	–	E/P	–	Yes	–	–	–
18	No	No	–	E/P[a]	–	Yes	–	–	–
TAC3									
20	No	No	–	E/P	Yes	Yes	–	S	Spontaneous fertility, spontaneous vaginal bleeding
Family members									
20b	No	No	–	E/P	Yes	Yes	–	S	Spontaneous fertility
20c	No	No	–	E/P	Yes	Yes	–	No	Spontaneous regular vaginal bleeding
20d	No	No	–	E/P	Yes	Yes	–	No	Vaginal bleeding after progesterone monotherapy

E/P, Estrogens/progesterone; S, spontaneous; –, unknown.

[a] Also clomiphene, pulsatile GnRH, and gonadotropins.

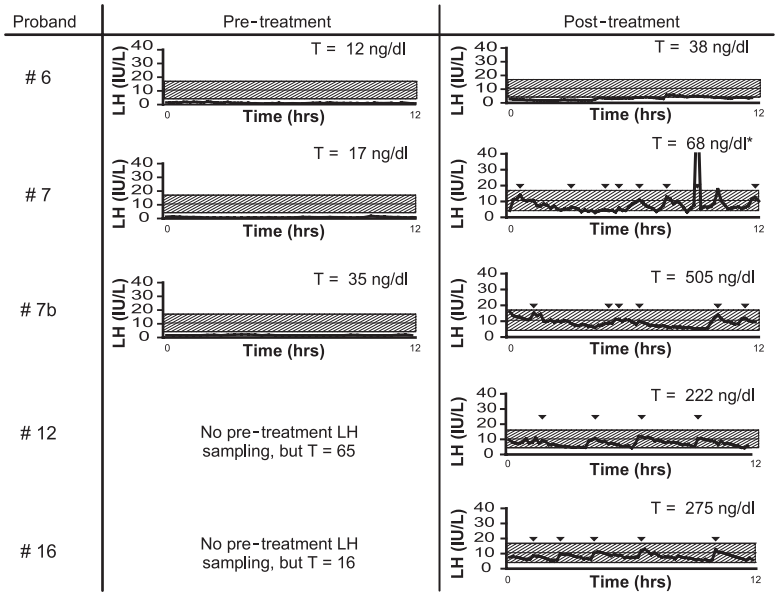

FIG. 3. Blood sampling every 10 min regarding reversal probands. *Left,* Studies before treatment. *Right,* Studies after discontinuation of treatment. *, T value represents a single blood sample taken within 5 months of the sampling study.

therapy exhibited evidence of spontaneous partial or complete recovery of their reproductive axis (10 of 12 or 83%), as determined by increases in LH pulsatility, T, testicular volume, the presence of withdrawal bleeding, spontaneous menstrual cycles, and/or spontaneous pregnancy (10 individuals with rare sequence variants: 9 of 10 with termination, frameshift, or functionally validated mutations; and one of 10 with heterozygous synonymous change). Although determining the true incidence of amelioration of IHH is limited by the difficulty of assessing patients off therapy, the high prevalence of reversal reported here stands in sharp distinction to the much lower reported incidence of recovery from GnRH deficiency in a patient cohort bearing a variety of genetic mutations (~10%) (28). Thus, *TAC3/TACR3* genetic defects may portend future prognosis in IHH and foreshadow the possibility of discontinuing sex steroid or gonadotropin therapy later in life. More importantly, perhaps, this striking phenotype strongly suggests that the role of the NKB system in GnRH secretion may be less critical in adult life than during late gestation and the early neonatal period. This finding is in contrast to patients with *DAX1* mutations causing adrenal hypoplasia congenita (IHH and adrenal insufficiency), who can have active hpg axes in neonatal life followed by adolescent hypogonadotropism (37). Moreover, the *TAC3/TACR3* phenotype appears to be distinct from the phenotypes associated with another secretory product of the same neurons, kisspeptin, and its cognate receptor, KISS1-R, where reversal of IHH has not yet been described. Of course, this latter point must be interpreted with caution because the number of well-studied mutations in the KISS1/KISS1-R system is much smaller at this time (9, 10, 20, 21).

Nonsense mutations in the ligand (*TAC3*) and receptor (*TACR3*) provide an important opportunity to examine the most severe dosing of gene impairment, thereby allowing a contrast of phenotypic severity as well as a contrast between the two sexes. For example, two females with a homozygous frameshift mutation in *TAC3* conceived spontaneously. Another continued having spontaneous regular menstrual cycles after discontinuation of sex steroid treatment. Because the homozygous *TAC3* mutation led to a complete absence of peptide, these patients demonstrate that the ligand, NKB, is dispensable for GnRH synthesis and secretion during the adult period. Because the tachykinin pathway is already well known to be promiscuous, other tachykinins, or even completely different ligands might compensate for their absence of NKB (17).

In parallel to this ligand mutation, four men had homozygous nonsense mutations in *TACR3*. Although patient 3 (homozygous W275X) was born with a microphallus, no follow-up is available to determine whether his hypogonadal state persisted or showed evidence for recovery later in life. Similarly, little follow-up information is available on the other male homozygous patients: no. 1 (S27X/S27X), no. 4 and no. 5 (W275X/W275X), as well as no. 17 (with the presumed homozygous splice site mutation T246T/T246T). However, proband 12 (Y256H/Y256H; validated loss-of-function *in vitro*) presented at age 21 with a history of microphallus and a hypogonadal T level (65 ng/dl) but subsequently demonstrated robust LH pulses off therapy with T levels of 222 ng/dl later in adulthood. This observation suggests that patients with markedly abnormal signaling through the NKB receptor can still achieve robust GnRH synthesis and secretion during adulthood.

Using traditional Mendelian principles, patients bearing heterozygous mutations in autosomal recessively inherited genes, such as those encoding NKB and its receptor, would not be predicted to manifest a disease phenotype. However, heterozygous mutations in the gene encoding another G protein-coupled receptor [prokineticin 2 receptor (*PROKR2*)] have now been reported as contributors to the IHH disease phenotype, either alone or in the presence of heterozygous mutations in other as-yet-to-be-identified genes/pathways (8). Thus, patients with homozygous nonsense mutations in *TACR3* might be hypothesized to have more severe phenotypes than those

bearing heterozygous mutations. Three probands with homozygous W275X presented with microphallus; however, three of four men bearing heterozygous nonsense mutations also had microphallus, suggesting that despite a smaller number of intact *TACR3* alleles, the severity of early androgen deficiency was comparable. Importantly, whereas the vast majority of the patients with mutations in this series were screened for mutations in the other genes currently known to cause IHH, strikingly only one rare heterozygous variant (13) was identified in another gene (proband 17, bearing heterozygous T58S in GnRH1) (Table 1).

Despite the number of mutations identified in the NKB pathway, our knowledge of how this ligand/receptor pair influences GnRH secretion remains incomplete. In rodents and sheep, NKB and its receptor are coexpressed in neurons that express kisspeptin and dynorphin (18, 19), raising the possibility of neurokinins acting indirectly to modulate GnRH secretion. Like many other genes involved in the pathogenesis of GnRH deficiency, NKB and its receptor are also expressed in various other organs (uterus and ovary) (38, 39), opening the possibility that mutations in this pathway can act at multiple levels of the hpg axis to cause dysfunction. In contrast to patients with nIHH, mice with targeted deletions of *Tacr3*, at least in the strains tested to date, do not have gross reproductive defects (40), and mice in which the *TAC3*-equivalent gene *Tac2* has been selectively deleted have yet to be reported.

Mutations in the NKB system are relatively common as causes of hypogonadism, occurring in more than 5% of a nIHH population that spans several ethnic groups. Although additional studies will help to illuminate the biology and interactions of the neurokinin pathway, the severe hypogonadotropism observed during early neonatal life in patients with mutations in the ligand/receptor pair juxtaposed against later markers of recovery suggests that NKB and its receptor play disparate roles in different windows of reproductive development.

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