

ORIGINAL ARTICLE

The GPR54 Gene as a Regulator of Puberty

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ABSTRACT

BACKGROUND

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Puberty, a complex biologic process involving sexual development, accelerated linear growth, and adrenal maturation, is initiated when gonadotropin-releasing hormone begins to be secreted by the hypothalamus. We conducted studies in humans and mice to identify the genetic factors that determine the onset of puberty.

METHODS

We used complementary genetic approaches in humans and in mice. A consanguineous family with members who lacked pubertal development (idiopathic hypogonadotropic hypogonadism) was examined for mutations in a candidate gene, GPR54, which encodes a G protein-coupled receptor. Functional differences between wild-type and mutant GPR54 were examined in vitro. In parallel, a *Gpr54*-deficient mouse model was created and phenotyped. Responsiveness to exogenous gonadotropin-releasing hormone was assessed in both the humans and the mice.

RESULTS

Affected patients in the index pedigree were homozygous for an L148S mutation in GPR54, and an unrelated proband with idiopathic hypogonadotropic hypogonadism was determined to have two separate mutations, R331X and X399R. The in vitro transfection of COS-7 cells with mutant constructs demonstrated a significantly decreased accumulation of inositol phosphate. The patient carrying the compound heterozygous mutations (R331X and X399R) had attenuated secretion of endogenous gonadotropin-releasing hormone and a left-shifted dose-response curve for gonadotropin-releasing hormone as compared with six patients who had idiopathic hypogonadotropic hypogonadism without GPR54 mutations. The *Gpr54*-deficient mice had isolated hypogonadotropic hypogonadism (small testes in male mice and a delay in vaginal opening and an absence of follicular maturation in female mice), but they showed responsiveness to both exogenous gonadotropins and gonadotropin-releasing hormone and had normal levels of gonadotropin-releasing hormone in the hypothalamus.

CONCLUSIONS

Mutations in GPR54, a G protein-coupled receptor gene, cause autosomal recessive idiopathic hypogonadotropic hypogonadism in humans and mice, suggesting that this receptor is essential for normal gonadotropin-releasing hormone physiology and for puberty.

THE NEUROENDOCRINE AND GENETIC control of sexual maturation at puberty remains one of the great mysteries of human biology. The secretion of gonadotropin-releasing hormone by the hypothalamus represents the first known step in the reproductive cascade — initiating pulsatile release of gonadotropins, gonadal secretion of sex steroids, pubertal development, and gametogenesis. Although the central role of gonadotropin-releasing hormone in the reproductive hierarchy of all mammals is undisputed, little is understood of the genetic factors that modulate its secretion. The identification of such factors is critical for advancing the understanding of normal reproduction and for providing insight into disorders of the pubertal process.

We used complementary genetic approaches in humans and mice to study a gene involved in the onset of puberty. Idiopathic hypogonadotropic hypogonadism in humans is characterized by the absence of spontaneous sexual maturation in the face of concentrations of gonadotropins in the low-normal range. Affected patients have a complete or partial absence of luteinizing hormone pulsations induced by endogenous gonadotropin-releasing hormone¹ and have normal responsiveness to physiological replacement with exogenous gonadotropin-releasing hormone. These observations localize the defect to an abnormality of gonadotropin-releasing hormone synthesis, secretion, or activity.²⁻⁴

Using linkage analysis and candidate-gene screening, we identified mutations in a G protein-coupled receptor gene, *GPR54*, in a large, consanguineous Saudi Arabian family with idiopathic hypogonadotropic hypogonadism and in one unrelated black male proband in the United States. Through a complementary approach, we generated *Gpr54*-deficient mice with a phenotype that demonstrated a lack of adult sexual development and low circulating gonadotropin concentrations — features that closely resemble their human counterparts.

METHODS

FAMILY HISTORY

A large Saudi Arabian family in which there had been three marriages between first cousins sought medical attention for infertility (Fig. 1). Six of the 19 offspring (4 men and 2 women) met the standard diagnostic criteria for idiopathic hypogonadotropic hypogonadism (inappropriately low gonadotropin concentrations in the presence of prepubertal

concentrations of sex steroids, normal anterior pituitary function, and normal findings on imaging of the brain) and had responsiveness to exogenous, pulsatile gonadotropin-releasing hormone, as previously reported.⁵ The collection of blood samples for genetic studies and the clinical protocols were approved by the Subcommittee on Human Studies of the Massachusetts General Hospital, and all participants provided written informed consent.

MUTATION ANALYSIS

Linkage to a 1.06-Mb interval on chromosome 19p13.3 was previously demonstrated.⁶ Mutation analysis of candidate genes, beginning with *GPR54*, was initiated with the use of DNA extracted from whole blood. The sequence of *GPR54* complementary DNA (cDNA) (GenBank accession number AY253981) was aligned with the published genomic sequence⁷ to identify the genomic structure. Details of polymerase-chain-reaction (PCR) amplifications, sequencing, control populations, transient transfections, the targeting construct, and biochemical assays are provided in Supplementary Appendix 1 (available with the full text of this article at <http://www.nejm.org>).

ADDITIONAL POPULATIONS

To determine whether the observed base-pair changes in *GPR54* were normal variants, control populations of 80 North American persons (primarily anonymous blood donors), 50 Middle Eastern persons, and 50 black persons from North America were also screened. An additional 63 patients with normosmic idiopathic hypogonadotropic hypogonadism and 20 patients with anosmic idiopathic hypogonadotropic hypogonadism (Kallmann's syndrome) were also screened for coding-sequence mutations in *GPR54*. Six patients with normosmic idiopathic hypogonadotropic hypogonadism who had participated in dose-response studies of exogenous, pulsatile gonadotropin-releasing hormone and who were negative for *GPR54* mutations on genomic screening were selected for comparisons of genotypes and phenotypes.

ALLELE-SPECIFIC CLONING

To demonstrate that there were base-pair changes on separate alleles, specific PCR products were cloned into a pCRII-TOPO plasmid vector (Invitrogen). Colonies were grown, and their DNA sequenced.

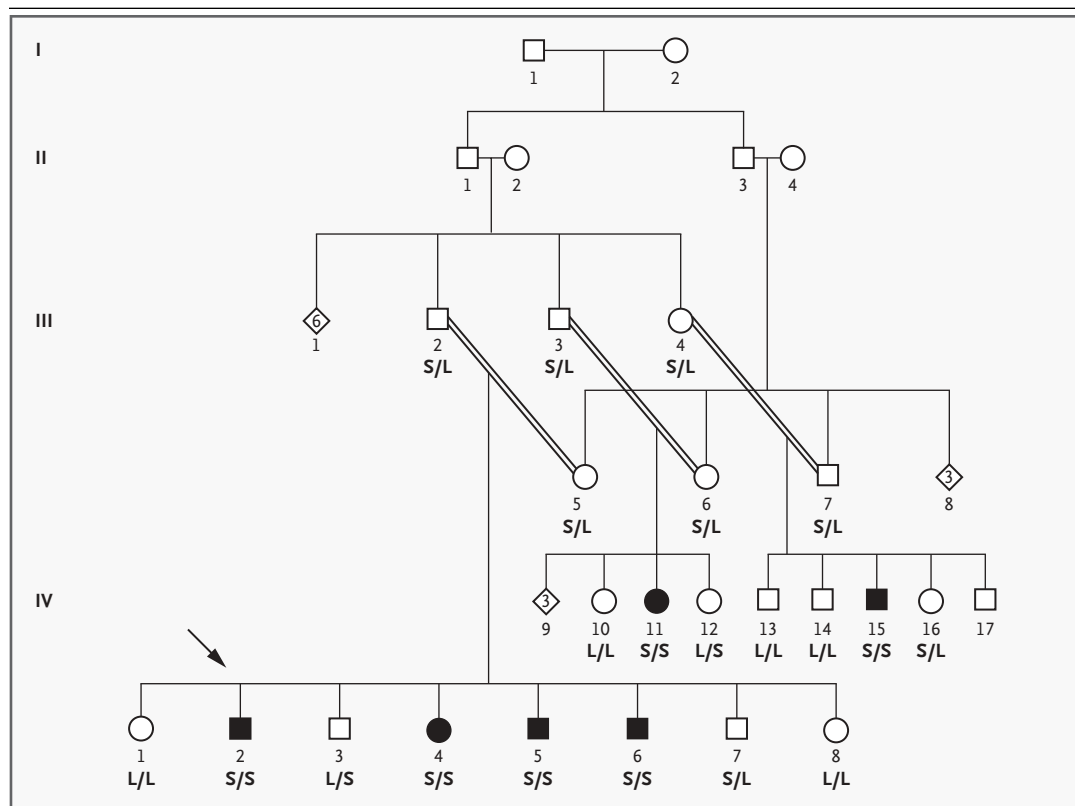


Figure 1. Pedigree of a Family with Idiopathic Hypogonadotropic Hypogonadism.

Squares represent male family members, circles female family members, diamonds multiple family members with the number given within, and solid symbols affected family members; the proband is indicated with an arrow. L denotes leucine, and S serine at position 148 of the *GPR54* gene. Adapted with permission from Bo-Abbas et al.⁵ and Acierno et al.⁶

REVERSE-TRANSCRIPTASE PCR

Subjects in whom coding sequence changes were identified in *GPR54* were further screened by means of reverse-transcriptase (RT)-PCR to rule out cryptic splicing events. Total RNA was extracted from lymphoblastoid cell lines, and *GPR54* cDNA was amplified and sequenced.

GENERATION OF MUTANT CONSTRUCTS

The sequence of the mammalian expression vector pCMVSPORT 6 containing full-length wild-type *GPR54* (clone CS0DE005YC17, Invitrogen) was confirmed by direct sequencing and found to contain a polyA tail. Site-directed mutagenesis was performed to introduce the three mutations (L148S, R331X, and X399R) into this vector. In addition, a stop codon was introduced immediately after the polyA tail (the construct was called “X399R polyA stop”).

STUDIES OF GPR54 SIGNALING

A natural ligand for *GPR54*, kisspeptin-1 (encoded by the gene *KISS1*), has been identified by three separate groups.⁸⁻¹⁰ Its C-terminal decapeptide kisspeptin-1 112-121⁹ was demonstrated to be the minimal-length peptide required for the full stimulation of *GPR54* (the Gq class of G proteins, coupled to phospholipase C). Stimulation of *GPR54* by kisspeptin-1 112-121 has been shown to increase phosphatidylinositol turnover.¹⁰

Kidney (COS-7) cells from African green monkeys were transiently transfected with 1.5 μ g of each *GPR54* construct or empty vector (pCMVSPORT6) per well, were stimulated with varying doses of kisspeptin-1 112-121 for 45 minutes, and were subsequently extracted with 20 mM of formic acid. Supernatants were loaded onto anion-exchange columns, and inositol phosphates were extracted.¹¹ Assays were performed in triplicate.

QUANTITATIVE RT-PCR

Quantitative RT-PCR was performed on RNA isolated from immortalized lymphoblasts obtained from patients (TaqMan One-Step RT-PCR Master Mix, Applied Biosystems). Different primers and probes capable of specifically amplifying the R331X and X399R alleles were used. Samples were run in quadruplicate in a minimum of two independent experiments. The β -actin gene was used as an endogenous control to standardize the assays in terms of expression levels.

GENOTYPE-PHENOTYPE CORRELATIONS

The patient carrying mutations R331X and X399R was admitted to the General Clinical Research Center of the Massachusetts General Hospital. Blood sampling was performed every 10 minutes for 12 hours. The patient then received gonadotropin-releasing hormone subcutaneously every two hours, and his dose was titrated while he was an outpatient until his pituitary-gonadal axis had normalized. After 11 months of treatment, the patient underwent a dose-response study in which four doses of gonadotropin-releasing hormone spanning 1.5 logarithmic orders (7.5 to 250 ng per kilogram of body weight per bolus) were administered intravenously in random order, and luteinizing hormone was sampled frequently.¹² Pulsatile hormone secretion was assessed with the use of the modified version of the method of Santen and Bardin.^{13,14}

STUDIES IN MICE

The transgenic mice (*Gpr54^{tm1PPL}*) were maintained as an inbred stock on a 129S6/SvEv genetic background. The gene-targeting strategy engineered a germ-line deletion of transmembrane loops 1 and 2 and the encompassing domains (Fig. 2A). Correct targeting was verified for the 3' and 5' arms by Southern blotting and PCR (Fig. 2B). The generation of a null *Gpr54* allele was confirmed by RT-PCR (Fig. 2C). Genotyping was performed by PCR (see Supplementary Appendix 2, available with the full text of this article at <http://www.nejm.org>). All experiments were performed under the authority of a U.K. Home Office Project License and were approved by a local ethics panel.

Gonadotropin-Releasing Hormone Injection

Wild-type female mice were staged with the use of vaginal smears. Wild-type female mice at diestrus and *Gpr54* $-/-$ female mice received four intraperi-

toneal injections of 25 ng of gonadotropin-releasing hormone (Sigma) at 30-minute intervals.¹⁵ The mice were killed 30 minutes after the last injection. Blood samples and pituitary specimens were treated as previously described,¹⁵ except that the pituitary specimens were homogenized in 0.3 ml of phosphate-buffered saline.

Hormone Assays

The sensitivity of the immunoradiometric assay for luteinizing hormone was 0.07 ng per milliliter (intraassay variation, 6.0 percent; interassay variation, 12.5 percent), and the sensitivity of the radioimmunoassay for follicle-stimulating hormone was 2 ng per milliliter (intraassay variation, 10 percent; interassay variation, 18 percent). Gonadotropin-releasing hormone was measured by radioimmunoassay,¹⁶ with a detection limit of 0.2 pg per tube (0.83 pg per milliliter) and an intraassay variation of 13 percent. Testosterone was measured by radioimmunoassay, with a sensitivity of 0.2 nmol per liter (intraassay variation, 6.0 percent; interassay variation, 18 percent). 17β -estradiol was measured by enzyme-linked immunosorbent assay (ELISA), with a sensitivity of 10 pg per milliliter (intraassay variation, 3.9 percent; interassay variation, 10 percent).

Histologic Studies

Mouse tissues were dissected and fixed for four hours in 4 percent formaldehyde and were then washed three times in 0.01 percent phosphate-buffered saline. Ovaries, testes, and adrenal glands were wax-embedded and sectioned at 3 to 4 μ m. Tissue sections were stained with hematoxylin and eosin. Mammary glands were dissected and fixed for 2 to 4 hours at 23°C in fixative (six parts absolute ethanol to three parts chloroform to one part glacial acetic acid), washed in 70 percent ethanol for 15 minutes, rehydrated, and stained overnight in carmine alum stain that has been boiled for 20 minutes in 500 ml of distilled water. Slides were washed in increasing concentrations of ethanol (70 percent, 95 percent, and 100 percent) for 15 minutes each, cleared in xylene for 30 minutes, and mounted.

RESULTS**MUTATION ANALYSIS**

Linkage in a consanguineous Saudi Arabian family was previously demonstrated on chromosome 19p13.3 with a maximal two-point lod score of

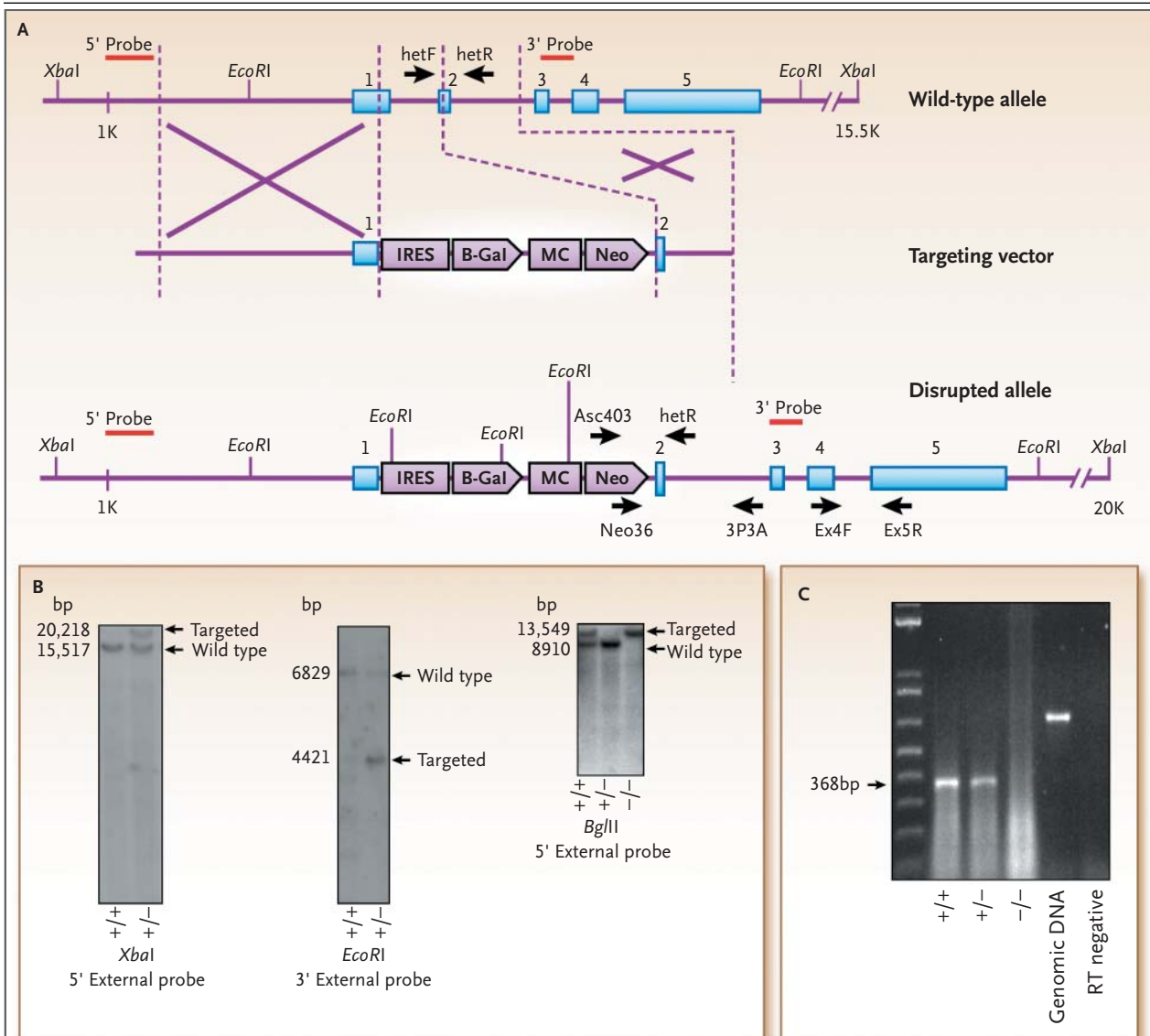


Figure 2. Targeted Deletion in the *Gpr54* Locus in Mice.

Panel A shows a schematic representation of the allele of *Gpr54* that was targeted for deletion (Panel A). Blue boxes represent exons, and purple boxes resistance cassettes and markers. Key restriction sites, primers, and probes are shown above the loci. Panel B shows the correct targeting of the 5' and 3' arms as demonstrated by Southern blotting. The arrows indicate the mutant and wild-type bands detected by diagnostic restriction digestion and probing. The sizes are given in numbers of base pairs. Southern blots show the expected pattern in heterozygous, wild-type, and mutant mice. In mice that are homozygous for the *Gpr54* deletion (Panel C), transcription 3' of the locus has been ablated. RT-PCR analysis of the segment spanning exons 4 and 5 shows that a detectable transcript is absent. IRES denotes internal ribosome entry site, B-Gal beta-galactosidase gene, MC MC promoter, Neo neomycin resistance gene, and Ex exon.

5.17.⁶ The candidate region on chromosome 19 contained 23 known genes,⁷ including GPR54, which is expressed in the human brain, pituitary gland, and placenta, as assessed with the use of RT-PCR.^{9,10} GPR54 has five exons and contains an open reading frame of 1197 bp that encodes a 398-amino-acid protein.

A homozygous single-nucleotide variant (443T>C) in exon 3, which substitutes a serine for the normal leucine at position 148 (L148S) in the second intracellular loop, was found in all six affected persons in the Saudi pedigree and did not occur in a homozygous state in any unaffected family members (all references to base-pair positions are

reported according to standard numbering and nomenclature¹⁷) (see Supplementary Appendix 3, available with the full text of this article at <http://www.nejm.org>). This variant does not appear to be a polymorphism, since it occurs only in affected family members; is absent in 160 chromosomes from unrelated, unaffected controls from the United States and 100 chromosomes from controls from the Middle East; is present in an amino acid residue that is conserved among species including mouse, rat, amphioxus, and pufferfish (GenBank accession numbers AF343726, BAB55447, and AAM18884, and Fugu Genome Server¹⁸ accession number SINFRUP00000071513,¹⁹ respectively); and changes the polarity of the encoded amino acid from hydrophobic to neutral.

Of the 63 unrelated patients with normosmic idiopathic hypogonadotropic hypogonadism and the 20 patients with Kallmann's syndrome, one black man with idiopathic hypogonadotropic hypogonadism was discovered to have a heterozygous C-to-T transition at nucleotide 991 in exon 5, in which an arginine at residue 331 was replaced with a premature stop codon (991C>T [R331X]). In addition, a heterozygous T-to-A transversion was identified at nucleotide 1195 in exon 5, which replaced the stop codon at residue 399 with an arginine (1195T>A [X399R]) (Supplementary Appendix 3). This nonstop mutation results in the continuation of the open reading frame to the polyA signal, with no intervening stop codon. Neither change was identified in the 160 chromosomes from North American controls or the 100 chromosomes from black controls.

To confirm that the nonsense and nonstop mutations are found on separate chromosomes, allele-specific cloning was performed. Seventeen clones contained either R331X or X399R, and no clones contained both, confirming that the two variants occur on separate alleles (making the patient a compound heterozygote) (data not shown).

RT-PCR

RT-PCR products generated from the Saudi Arabian proband and the compound heterozygote were of the expected size for all segments. Sequence analysis of these products revealed that the newly identified mutations did not result in cryptic splicing (data not shown).

FUNCTIONAL ASSAYS

To determine whether the identified changes in GPR54 affect the function of the receptor, inositol

phosphate production was measured in COS-7 cells in response to kisspeptin-1 112–121. The maximal inositol phosphate response of the cells that were transfected with the mutant L148S and R331X constructs was decreased by 65 percent and 67 percent, respectively, as compared with the cells that were transfected with the wild-type gene (Fig. 3A and 3B). RT-PCR of COS-7 cells transfected with the X399R construct revealed a transcript that contained 3' untranslated region, polyA tail, and expression-vector sequence (data not shown). In the absence of the physiologic stop codon at position 399 (but with a stop codon in the vector sequence), the *in vitro* transcript resulted in an elongated receptor protein. Because this *in vitro* construct did not accurately mimic *in vivo* physiology, it was not used in the functional studies. The X399R polyA stop construct, which makes a protein identical to that encoded by the nonstop transcript, stimulates inositol phosphate production that is 61 percent of that of wild-type GPR54 (Fig. 3C). No inositol phosphate stimulation was observed with pCMVSPORT 6.

QUANTITATIVE RT-PCR

Expression analysis of the GPR54 alleles was performed by means of real-time PCR, with the use of lymphoblastic messenger RNA (mRNA) as a template. The mutant alleles were expressed at concentrations correlated with concentrations of control lymphoblasts. When compared with the standardized control mRNA, the mean (\pm SE) total concentration of GPR54 mRNA in the compound-heterozygous patient was 17.6 ± 1.6 percent of the normal concentration ($P < 0.001$ by Student's *t*-test); the expression concentration of the R331X allele was 17.9 ± 1.9 percent of the normal concentration, and the expression concentration of the X399R allele was 2.5 ± 0.3 percent of the normal concentration (Fig. 3D).

ENDOCRINOLOGIC PHENOTYPING

A base-line profile of luteinizing hormone in the proband carrying the heterozygous mutations R331X and X399R is shown in Figure 4A. The patient had low concentrations of luteinizing hormone and low testosterone concentrations, particularly as compared with the mean (± 2 SD) luteinizing hormone level measured in 20 normal men.^{20,21} Nonetheless, nine low-amplitude pulses of luteinizing hormone are present, as determined by formal pulse analysis. The responses to four doses of intravenous gonadotropin-releasing hormone are compared with the mean amplitude of luteinizing hormone

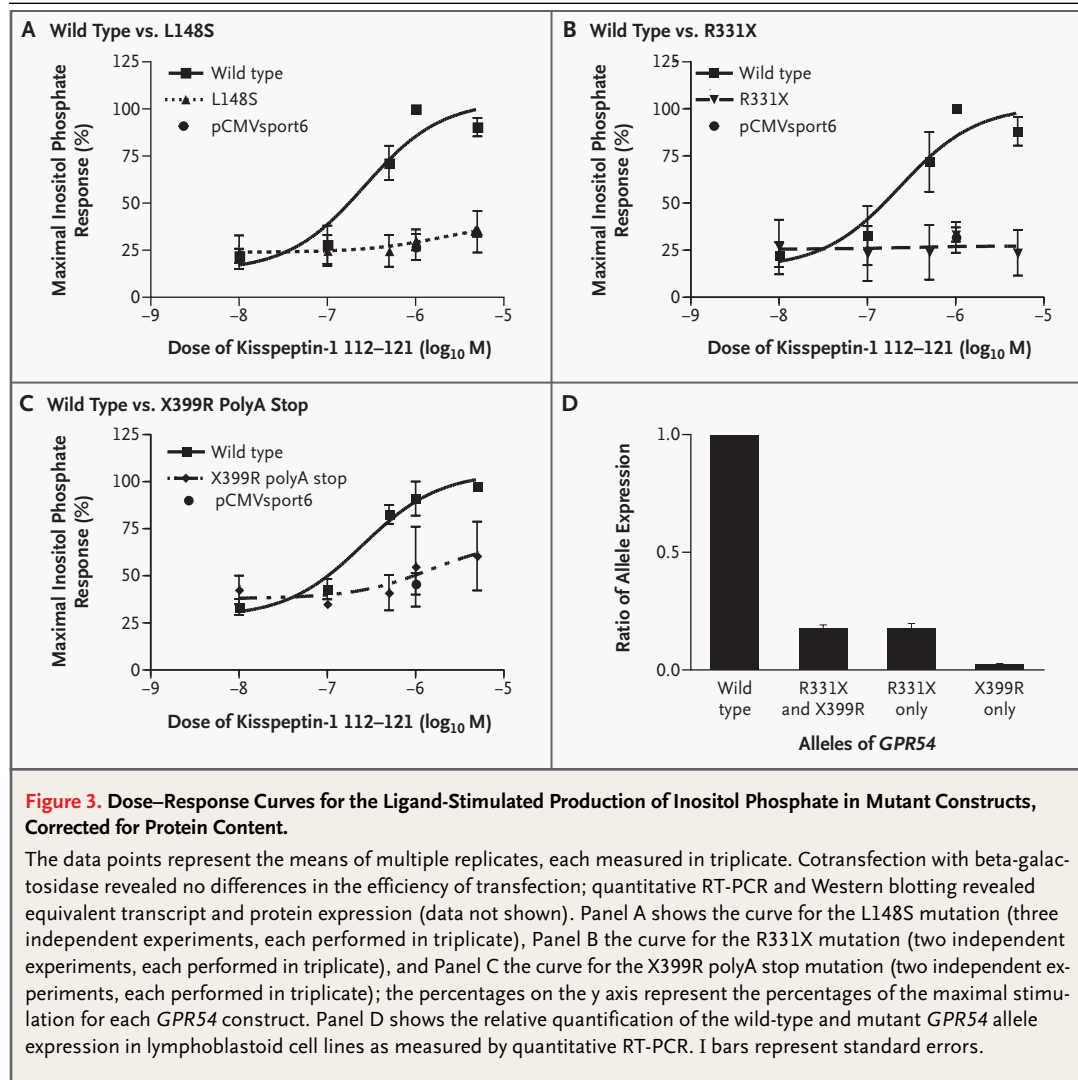


Figure 3. Dose–Response Curves for the Ligand-Stimulated Production of Inositol Phosphate in Mutant Constructs, Corrected for Protein Content.

The data points represent the means of multiple replicates, each measured in triplicate. Cotransfection with beta-galactosidase revealed no differences in the efficiency of transfection; quantitative RT-PCR and Western blotting revealed equivalent transcript and protein expression (data not shown). Panel A shows the curve for the L148S mutation (three independent experiments, each performed in triplicate), Panel B the curve for the R331X mutation (two independent experiments, each performed in triplicate), and Panel C the curve for the X399R polyA stop mutation (two independent experiments, each performed in triplicate); the percentages on the y axis represent the percentages of the maximal stimulation for each *GPR54* construct. Panel D shows the relative quantification of the wild-type and mutant *GPR54* allele expression in lymphoblastoid cell lines as measured by quantitative RT-PCR. I bars represent standard errors.

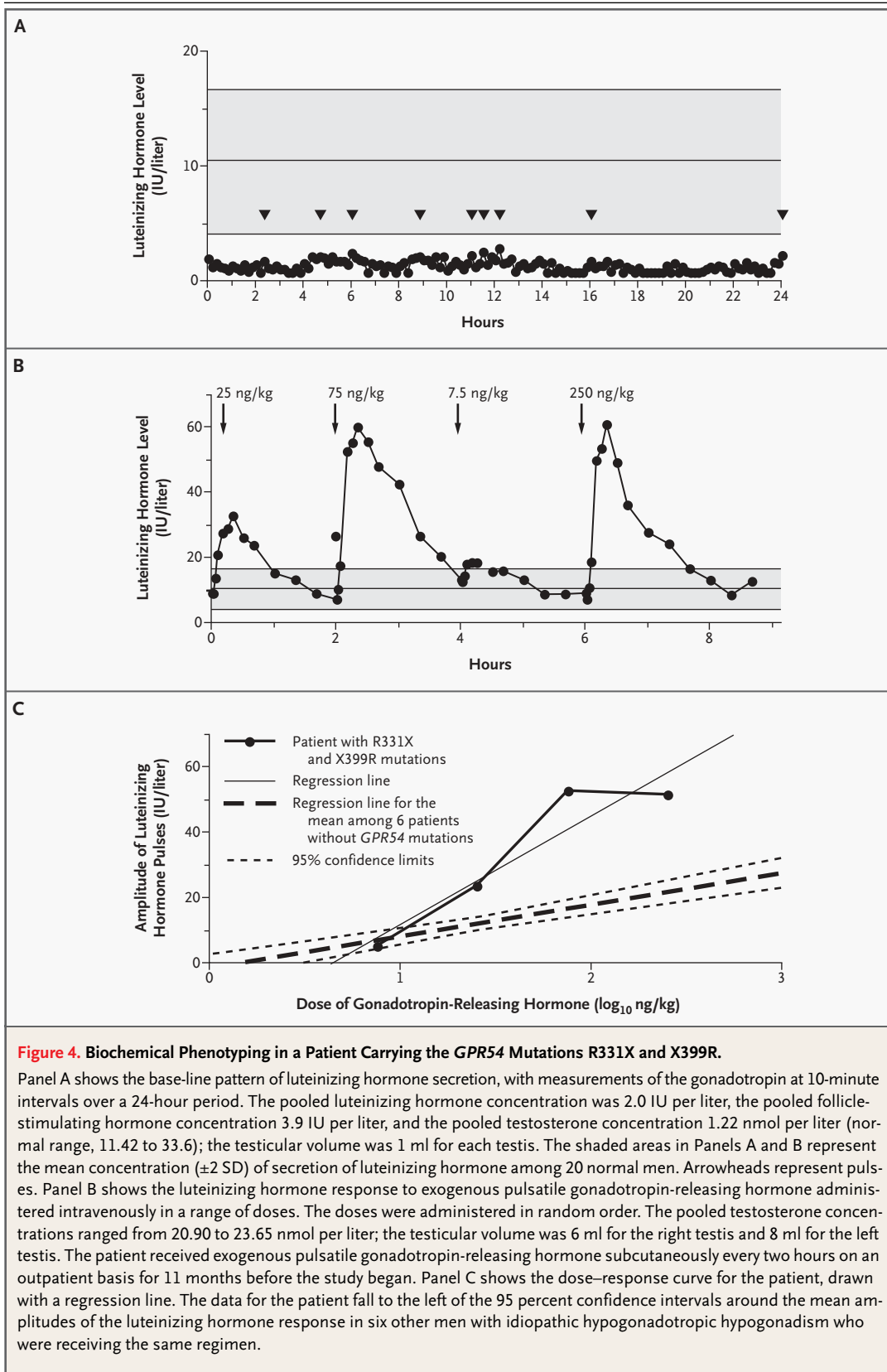
pulses in six other patients with idiopathic hypogonadotropic hypogonadism who were treated with the same regimen (Fig. 4B and 4C). The dose–response curve for the proband is shifted leftward as compared with the 95 percent confidence intervals around the mean responses in men with idiopathic hypogonadotropic hypogonadism who did not have any *GPR54* mutations.

PATHOPHYSIOLOGY, ANATOMY, AND BEHAVIOR OF HOMOZYGOUS *GPR54*-DEFICIENT MICE

Homozygous mutant mice (*Gpr54* $-/-$) (Fig. 2A and 2B) were viable and obtained at the expected mendelian frequency from heterozygous breeding pairs (Fig. 2B and Supplementary Appendix 2). RT-PCR analysis of transcripts showed no detectable transcription in the 3' end of the homozygous

Gpr54^{tm1PTL} allele (Fig. 2C). *Gpr54* $+/-$ mice were phenotypically normal and were fertile. *Gpr54* $-/-$ mice did not display any of the physiologic changes associated with sexual maturation. The testes of male *Gpr54* $-/-$ mice were significantly smaller than those of age-matched controls (Fig. 5A) (mean weight in nine *Gpr54* $-/-$ mice, 0.05 ± 0.00 g; mean weight in eight age-matched controls, 0.18 ± 0.01 g; $P < 0.001$ by the unpaired Mann–Whitney U test) and did not contain spermatozoa in the lumen of the seminiferous tubules or the epididymis (Fig. 5C, 5D, 5E, and 5F). Primary spermatocytes were present, but there were very few haploid spermatids, which suggests that spermatogenesis had been initiated but had stopped before the meiotic-division stage.

Male mice also lacked development of second-



ary sex glands, including the preputial gland (Fig. 5G and 5H), the seminal vesicles, and the prostate (not shown). In the adrenal glands of the mutant animals, the innermost region of the cortex, which normally regresses at puberty, was still present (Fig. 5I and 5J). Sexual mounting behavior was also not observed among the male mice. No gross morphologic abnormalities were found in the central nervous system of *Gpr54*^{-/-} mice, and the mutant mice thrived, apart from the reproductive defect.

Female mutant mice also had defective sexual development; they had small vaginal openings and did not become pregnant after appropriate mating exposure. Vaginal smears consisted of nonkeratinized epithelia and mucus strands similar to those observed in immature female mice, indicating the lack of an estrus cycle. The uterine horns in female *Gpr54*^{-/-} mice were threadlike, and the ovaries were significantly smaller than normal (mean weight in nine wild-type mice, 5.7±0.7 mg; mean weight in eight mutant mice, 1.0±0.1 mg; $P < 0.001$ by the unpaired Mann-Whitney U test) (Fig. 5B). Mammary tissue showed no postpubertal maturation of branched epithelial ducts (Fig. 5K and 5L). The ovaries contained primary and secondary follicles and occasionally an early antral follicle but no large graafian follicles or corpora lutea (Fig. 5M and 5N).

ENDOCRINOLOGIC PHENOTYPES IN *GPR54*-DEFICIENT MICE

Male *Gpr54*^{-/-} mice had significantly lower blood testosterone concentrations than age-matched *+/+* controls (mean among 12 mutant mice, 0.1±0.02 pg per milliliter; mean among 11 wild-type mice, 4.6±1.6 pg per milliliter; $P < 0.001$ by the unpaired Mann-Whitney U test). The testosterone concentrations in male *Gpr54*^{-/-} mice were similar to those observed in female *Gpr54*^{+/+} mice (mean among eight female mice, 0.2±0.02 pg per milliliter) (Fig. 6A). The 17 β -estradiol concentrations in female *Gpr54*^{-/-} mice were similar to those in *Gpr54*^{+/+} females at nonestrus stages of the reproductive cycle (Fig. 6B) and to the base-line serum estradiol concentrations in male *Gpr54*^{+/+} mice (data not shown). No *Gpr54*^{-/-} females were identified that had a 17 β -estradiol concentration similar to that found at estrus (mean concentration among five wild-type females, 96.5±16.3 pg per milliliter) (Fig. 6B).

The lack of an estrus cycle in female mice was not caused by an inability of gonadal tissue to respond to gonadotropins. Female *Gpr54*^{-/-} mice could be

induced to ovulate after sequential injection of the gonadotropins pregnant mares serum and human chorionic gonadotropin (data not shown). The lack of an estrus cycle and the failure to produce sperm in *Gpr54*^{-/-} mice were caused by a significant reduction in the serum follicle-stimulating hormone concentration ($P = 0.009$) and a more moderate decrease in the luteinizing hormone concentration (Fig. 6C and 6D). Possible explanations of the reduced concentrations of circulating gonadotropins include an absence of pituitary gonadotropes, an inability of existing gonadotropes to respond to stimulation by gonadotropin-releasing hormone, and a lack of gonadotropin-releasing hormone production. This last possibility was ruled out because there was no significant difference between normal and mutant mice in the concentration of gonadotropin-releasing hormone in hypothalamic extracts (Fig. 6E).

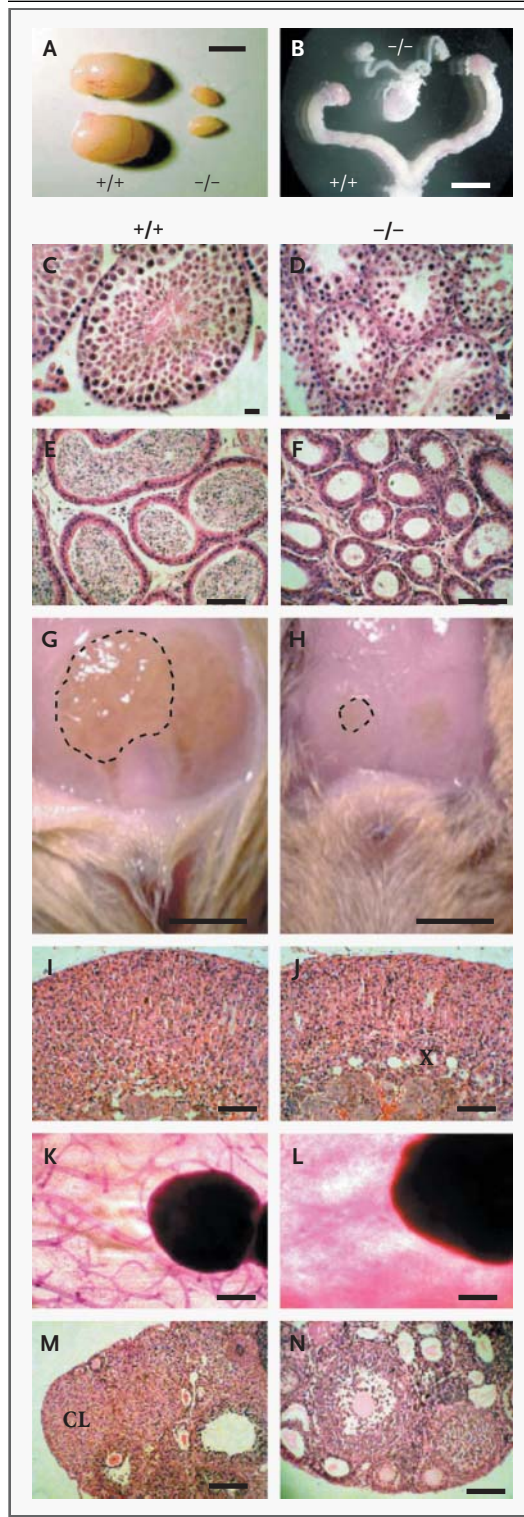
In addition, measurements of pituitary luteinizing hormone and follicle-stimulating hormone showed that although the total amount of each hormone was lower in *Gpr54*^{-/-} mice than in wild-type mice, significant quantities of each hormone were found, indicating that the pituitary gonadotropes are present in *Gpr54*^{-/-} mice and are capable of synthesizing luteinizing hormone and follicle-stimulating hormone. Furthermore, in adult female *Gpr54*^{-/-} mice, luteinizing hormone was secreted into the bloodstream in response to the injection of gonadotropin-releasing hormone (Fig. 6F), and there was a corresponding depletion in pituitary luteinizing hormone (Fig. 6G). Studies of the secretion of follicle-stimulating hormone in response to the injection of gonadotropin-releasing hormone had similar results (data not shown). Although the absolute concentration of serum luteinizing hormone after the injection of gonadotropin-releasing hormone was lower in *Gpr54*^{-/-} mice than in *+/+* mice, the proportional increase in the luteinizing hormone concentration was similar (an increase by a factor of five from base line). These responses are consistent with a first exposure to gonadotropin-releasing hormone.^{22,23}

DISCUSSION

In primates, the hypothalamic-pituitary-gonadal axis is fully active during neonatal life, followed by a mysterious period of dormancy during childhood. The triggers of the onset of gonadotropin-releasing hormone secretion at puberty are as unclear as

Figure 5. Gonadal Anatomy and Secondary Sexual Characteristics of *Gpr54* $-/-$ Mice.

Panel A shows the reduction in the size of the testes (wild-type as compared with mutant male mice), and Panel B shows the small ovaries and uteri found in female *Gpr54* $-/-$ mice; the scale bars represent 0.5 cm. In Panels C through N, the wild-type mouse is represented by the left-hand column and the mutant mouse is represented by the right-hand column. Panel D shows the reduction in the number of spermatozoa in the seminiferous tubules, as compared with Panel C; the scale bars represent 50 μ m. Panels E and F show the presence and absence, respectively, of sperm in the epididymis; the scale bars represent 100 μ m. Panel H shows reduced development of the preputial gland, as compared with Panel G; the scale bars represent 1 cm. Panels I and J show the absence and presence, respectively, of the prepubescent zone X in the adrenal gland; the scale bars represent 20 μ m. Panel L shows reduced mammary-duct formation, as compared with Panel K (the dark mass is lymph node); the scale bars represent 0.5 cm. Panels M and N show the presence and absence, respectively, of graafian follicles and corpora lutea; CL denotes corpus luteum; the scale bars represent 300 μ m.



those that halt its secretion at the end of the neonatal period. Insight into this process has been gained through the study of various diseases in humans and animal models in which genetic defects cause abnormalities of sexual maturation. Mutations in both *GPR54* in humans and *Gpr54* in mice cause hypogonadotropic hypogonadism, pubertal delay, and sexual infantilism that can be corrected by the administration of exogenous gonadotropin-releasing hormone. Taken together, these observations establish that the effect of *GPR54* on gonadotropin-releasing hormone secretion is conserved in multiple mammalian species and is a genetic determinant of sexual maturation.

GPR54 is a member of the rhodopsin family of G protein-coupled receptors whose sequences are most similar to those of members of the galanin-receptor family (35 to 40 percent identity).⁹ Although galanin and galanin-like peptide appear not to bind to *GPR54*,^{9,24} endogenous peptides derived from a precursor protein, kisspeptin-1, have recently been identified that do display agonist activity.⁸⁻¹⁰ The longest of these peptides is kisspeptin-1 68-121, or metastin, so called because of its ability to suppress metastatic potential in melanoma and breast-cancer cell lines.²⁵⁻²⁷ Metastin is secreted into the circulation by the placenta in relatively large quantities throughout gestation, although its physiologic role in pregnancy remains unknown.²⁸

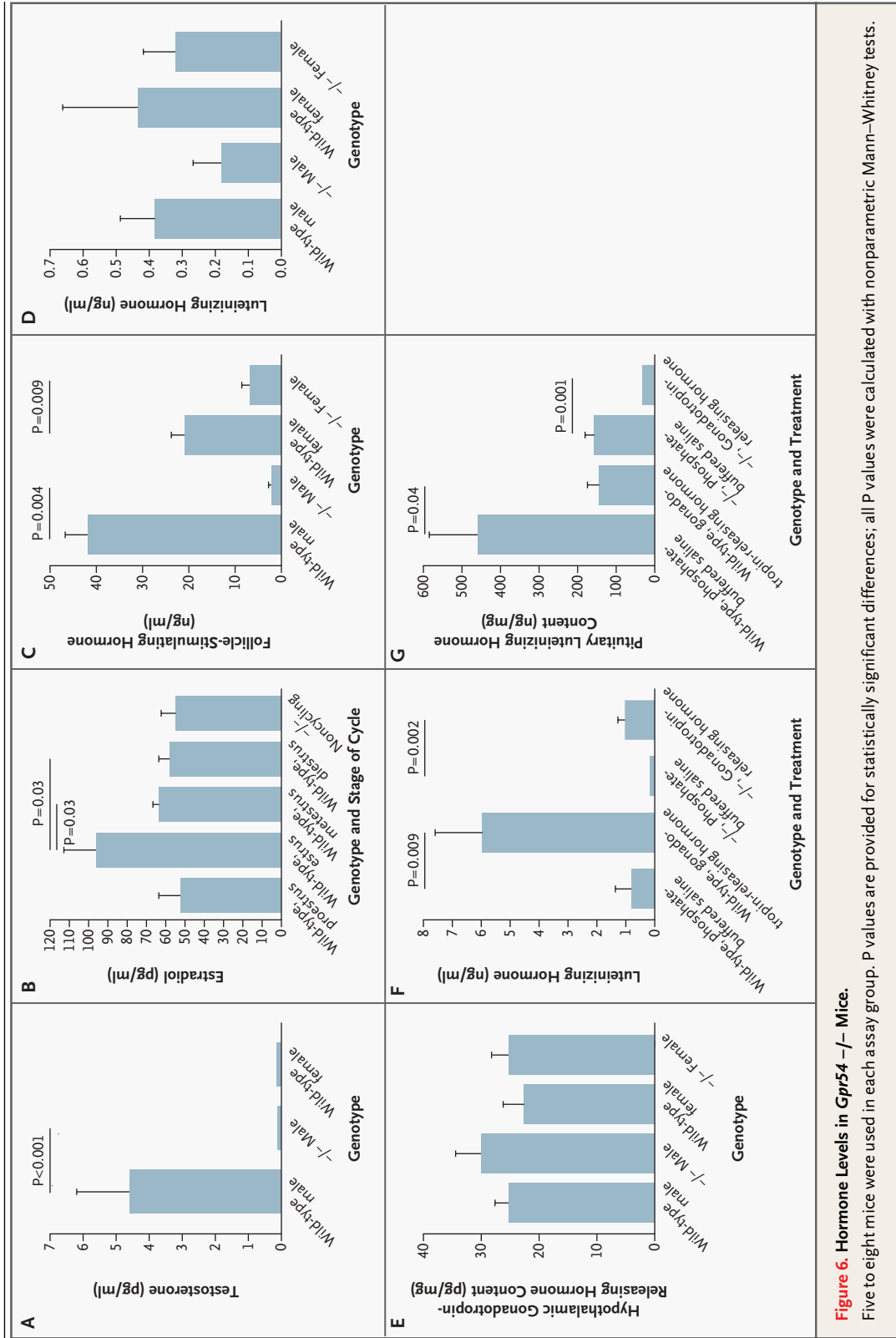


Figure 6. Hormone Levels in *Gpr54* $-/-$ Mice. Five to eight mice were used in each assay group. P values are provided for statistically significant differences; all P values were calculated with nonparametric Mann-Whitney tests.

In this study, a variety of mutations in GPR54 were identified in patients with idiopathic hypogonadotropic hypogonadism. The index family was found to carry a homozygous L148S substitution. When expressed in cell lines, the L148S mutant construct markedly reduced inositol phosphate production as compared with the wild-type construct. A male patient with sporadic idiopathic hypogonadotropic hypogonadism was also found to be a compound heterozygote for the mutations R331X and X399R, which gave rise to nonsense and nonstop transcripts. It has been shown that mRNAs with premature termination codons can be targeted to nonsense-mediated decay,²⁹ whereas mRNAs without an in-frame termination codon can be subject to nonstop decay, a recently identified degradation pathway that is initiated when the ribosome reaches the 3' terminal of the mRNA.^{30,31}

We hypothesized that this combination of nonstop and nonsense mutations in GPR54 in the patient with sporadic idiopathic hypogonadotropic hypogonadism would result in the absence of a functional receptor. Quantitative RT-PCR confirmed the presence of dramatically reduced concentrations of GPR54 mRNA in the patient with the R331X and X399R alleles. Moreover, this total concentration appeared to be composed almost exclusively of the nonsense transcript. These results indicate that the contribution of the nonstop transcript was almost negligible, supporting the hypothesis of nonstop decay. In the unlikely event that a protein were produced by the X399R transcript, our findings regarding the X399R polyA stop construct suggest that it would function poorly.

The clinical phenotype of the patient carrying the R331X and X399R mutations was associated with a neuroendocrine profile involving low-amplitude pulses of luteinizing hormone, suggesting reduced secretion of gonadotropin-releasing hormone. This notion is supported by the leftward-shifted dose-response curve as compared with those for other patients with idiopathic hypogonadotropic hypogonadism who were undergoing the same therapy, suggesting that this patient was more sensitive to exogenous gonadotropin-releasing hormone. Since these studies were performed, deletions in GPR54 have been described in a separate family with idiopathic hypogonadotropic hypogonadism, although the phenotypic features of this family were not detailed.³² Although the investigators in that case agree with our conclusion that the frequency of GPR54 mutations as a cause of idiopathic hypogo-

nadotropic hypogonadism is low (4 of 113 total cases), GPR54 reveals a new direction for the exploration of other genes that are essential for the secretion of gonadotropin-releasing hormone.

The *Gpr54*-deficient mice had striking physiological similarities to the patients with idiopathic hypogonadotropic hypogonadism, including a lack of sexual maturation associated with low concentrations of gonadotropins. In addition, their gonads remained sensitive to exogenous gonadotropins, and their pituitary gonadotropes remained responsive to stimulation by gonadotropin-releasing hormone. This strong similarity between the findings in the patients and those in the mouse model establishes a central role for GPR54 in gonadotropin-releasing hormone secretion and the onset of sexual maturation among mammalian species. Moreover, the use of *Gpr54*-deficient mice permitted the quantitation of their hypothalamic gonadotropin-releasing hormone concentrations, which were normal in the face of their hypogonadotropism. The presence of normal concentrations of gonadotropin-releasing hormone in the hypothalamus of *Gpr54*-deficient, sexually immature mice is reminiscent of prepubertal rats and monkeys who have normal numbers of gonadotropin-releasing hormone-containing neurons, normal mRNA concentrations, and normal concentrations of gonadotropin-releasing hormone in the hypothalamus.^{33,34} Extrapolation from the *Gpr54*-deficient mice to nonhuman primates and humans suggests that GPR54 may have a substantial effect on the processing or secretion of gonadotropin-releasing hormone.

There are three possible mechanisms that may allow abnormalities in GPR54 to cause pubertal delay. The first possibility is that defects in the metastin-GPR54 system perturb gonadotropin-releasing hormone neuronal migration that is analogous to the abnormal axonal targeting that occurs in the X-linked form of Kallmann's syndrome (idiopathic hypogonadotropic hypogonadism with anosmia).³⁵⁻³⁷ In vitro, the metastin-GPR54 system induces an "adhesive phenotype" with inhibition of chemotaxis, focal adhesions and stress fibers, and phosphorylation of focal adhesion kinase and paxillin.⁸ However, the normal content of gonadotropin-releasing hormone in the hypothalamus of *Gpr54*-deficient mice argues that there has been an appropriate migration of the neurons containing gonadotropin-releasing hormone from their origin in the olfactory placode to their destination in the hypothalamus. The possibility remains, how-

ever, that there is a subtle defect in the terminal migration or differentiation of these neurons within the hypothalamus.

The second possibility is that GPR54 modulates the activity of gonadotropin-releasing hormone at the level of the pituitary. The presence of small but detectable pulses of luteinizing hormone induced by gonadotropin-releasing hormone in the patient with the R331X and X399R mutations and his leftward-shifted dose-response curve suggest that pituitary responsiveness in the GPR54-deficient patient is, if anything, enhanced, suggesting that loss-of-function mutations in GPR54 do not diminish the sensitivity of gonadotropes to gonadotropin-releasing hormone.

The third possibility is that GPR54 regulates the release of gonadotropin-releasing hormone at the level of the hypothalamus. This hypothesis is supported by three observations: the low-amplitude pulses of luteinizing hormone in the patient carrying the R331X and X399R mutations, his leftward-shifted dose-response curve, and the normal content of gonadotropin-releasing hormone in the hypothalamus of Gpr54-deficient mice. Further studies will be required to determine the precise mechanisms of action within the hypothalamus as well as the physiological functions of the peptide ligands for GPR54.

Currently, it appears that the frequency of GPR54 mutations as a cause of idiopathic hypogonadot-

ropic hypogonadism is not high. However, the elucidation of the role of GPR54 as a regulator of gonadotropin-releasing hormone-related physiology and puberty may well provide a seminal clue, offering a new perspective on other candidate genes that may control sexual maturation and puberty. These include the genes affecting the biosynthesis, processing, and secretion of the putative ligands, kisspeptin-metastin; the transcriptional regulation of GPR54 itself; and the signaling pathways downstream of the gene. Our data from patients with idiopathic hypogonadotropic hypogonadism and a mouse model provide strong evidence that GPR54 is a key regulator of the biology of puberty.

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