# Variations in *PROKR2*, But Not *PROK2*, Are Associated With Hypopituitarism and Septo-optic Dysplasia

Mark J. McCabe,* Carles Gaston-Massuet,* Louise C. Gregory, Kyriaki S. Alatzoglou, Vaitsa Tziaferi, Oualid Sbai, Philippe Rondard, Koh-hei Masumoto, Mamoru Nagano, Yasufumi Shigeyoshi, Marija Pfeifer, Tony Hulse, Charles R. Buchanan, Nelly Pitteloud, Juan-Pedro Martinez-Barbera, and Mehul T. Dattani

Developmental Endocrinology Research Group, Clinical and Molecular Genetics Unit (M.J.M., L.C.G., K.S.A., V.T., M.T.D.), and Neural Development Unit (C.G.-M., J.-P.M.-B.), University College London (UCL)-Institute of Child Health, London WC1N 1EH, United Kingdom; Centre National de la Recherche Scientifique Unité Mixte de Recherche 5203 (O.S., P.R.), Institut de Génomique Fonctionelle, Institut National de la Santé et de la Recherche Médicale Unité 661, Université Montpellier 1 et 2, 34090 Montpellier, France; Department of Anatomy and Neurobiology (K.M., M.N., Y.S.), School of Medicine, Kinki University, Osaka 589-8511, Japan; Department of Endocrinology (M.P.), University Medical Centre, SI-1525 Ljubljana, Slovenia; Guy's and St Thomas' Hospital (T.H.), London SE1 9RT, United Kingdom; King's College Hospital (C.R.B.), London SE5 9RS, United Kingdom; and Endocrinology, Diabetes and Metabolism (N.P.), University of Lausanne, 1015 Lausanne, Switzerland

**Context:** Loss-of-function mutations in *PROK2* and *PROKR2* have been implicated in Kallmann syndrome (KS), characterized by hypogonadotropic hypogonadism and anosmia. Recent data suggest overlapping phenotypes/genotypes between KS and congenital hypopituitarism (CH), including septo-optic dysplasia (SOD).

**Objective:** We screened a cohort of patients with complex forms of CH (n = 422) for mutations in *PROK2* and *PROKR2.*

**Results:** We detected 5 *PROKR2* variants in 11 patients with SOD/CH: novel p.G371R and previously reported p.A51T, p.R85L, p.L173R, and p.R268C—the latter 3 being known functionally deleterious variants. Surprisingly, 1 patient with SOD was heterozygous for the p.L173R variant, whereas his phenotypically unaffected mother was homozygous for the variant. We sought to clarify the role of PROKR2 in hypothalamopituitary development through analysis of *Prokr2*$^{-/-}$ mice. Interestingly, these revealed predominantly normal hypothalamopituitary development and terminal cell differentiation, with the exception of reduced LH; this was inconsistent with patient phenotypes and more analogous to the healthy mother, although she did not have KS, unlike the *Prokr2*$^{-/-}$ mice.

**Conclusions:** The role of PROKR2 in the etiology of CH, SOD, and KS is uncertain, as demonstrated by no clear phenotype-genotype correlation; loss-of-function variants in heterozygosity or homozygosity can be associated with these disorders. However, we report a phenotypically normal parent, homozygous for p.L173R. Our data suggest that the variants identified herein are unlikely to be implicated in isolation in these disorders; other genetic or environmental modifiers may also impact on the etiology. Given the phenotypic variability, genetic counseling may presently be inappropriate. **(*J Clin Endocrinol Metab* 98: E547–E557, 2013)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2012-3067 Received August 13, 2012. Accepted December 17, 2012.
First Published Online February 5, 2013

* M.J.M. and C.G.-M. contributed equally to this work.

Abbreviations: ACTHD, ACTH deficiency; AVP, arginine vasopressin; CPHD, combined pituitary hormone deficiency; E, embryonic day; FGF8, fibroblast growth factor 8; GHD, GH deficiency; GI, gastrointestinal; HA, hemagglutinin; HH, hypogonadotrophic hypogonadism; HPE, holoprosencephaly; $IP_1$, D-myo-inositol monophosphate; KS, Kallmann syndrome; MRI, magnetic resonance imaging; OT, oxytocin; PROK2, prokineticin-2; PROKR2, PROK2 receptor; SOD, septo-optic dysplasia; TSHD, TSH deficiency.

Congenital hypopituitarism is a rare condition that may be associated with complex midline defects of the forebrain; these encompass a wide spectrum of phenotypes ranging from cleft palate to septo-optic dysplasia (SOD), holoprosencephaly (HPE), and incompatibility with life. SOD is defined as the combination of 2 of the following: 1) midline forebrain defects (eg, absent septum pellucidum, agenesis of the corpus callosum); 2) optic nerve hypoplasia; or 3) anterior pituitary hypoplasia and/or pituitary hormone deficiencies (1).

Both HPE and SOD are highly heterogeneous conditions, usually sporadic with a multifactorial etiology. However, an increasing number of early developmental transcription factors and associated pathway genes have been implicated in the etiology of HPE (eg, *SHH*, *ZIC2*, *SIX3*, *TGIF*) and SOD (eg, *HESX1*, *SOX2*, *SOX3*, *OTX2*) (2–7). These genes are expressed in regions that determine the formation of forebrain and related midline structures such as the hypothalamus and pituitary, and mutations in these genes are therefore associated with marked phenotypic heterogeneity (8).

Recently, we identified autosomal recessive and dominant mutations in fibroblast growth factor 8 (*FGF8*) in HPE and SOD (9). Although *Fgf8* had previously been shown to maintain anterior pituitary cellular proliferation in mice through the regulation of *Lhx3* (10), loss of function mutations in *FGF8* in humans had so far only been associated with Kallmann syndrome (KS) and hypogonadotrophic hypogonadism (HH) (11). Our study was the first to provide a genetic link between these midline disorders and KS/HH, thus suggesting that other genes implicated in the pathogenesis of KS/HH, such as *FGFR1* (receptor of *FGF8*), *KAL1*, prokineticin-2 (*PROK2*), or its receptor *PROKR2*, could also play a role in disorders such as HPE and SOD.

Heterozygous and homozygous loss of function mutations/variations in *PROK2* and *PROKR2* are implicated in KS/HH (Figure 1) (12–15). These proteins appear to be important for murine olfactory bulb development and subsequent GnRH neuronal migration from this region to the ventral forebrain (16, 17). Expression of *Prok2* has been reported in the ependymal and subependymal layers of the olfactory bulbs, preoptic area, and median eminence in mice (18, 19).

In a recent collaborative study of 103 patients, including 68 patients with SOD, we showed that 4 patients with hypopituitarism harbored functionally significant mutations/variations in *PROKR2* (20). Another recent report described 2 other patients with hypopituitarism who bore functionally significant *PROKR2* variations (21). Therefore, to further investigate the role of *PROK2/PROKR2* in hypothalamo-pituitary disorders, we expanded the screen to 422 patients (male:female ratio, 1.1:1) with hypopitu-

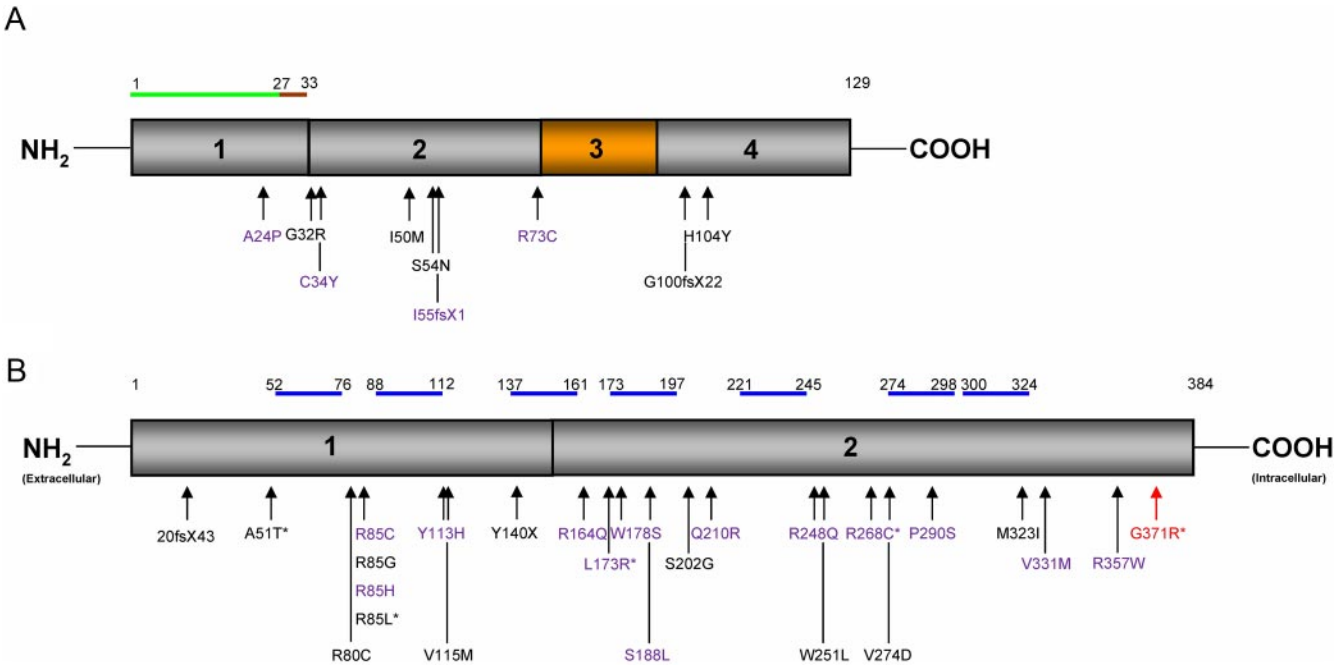


**Figure 1.** Structure of PROK2 and PROKR2. A, Ligand PROK2 consists of 4 exons with the alternative splicing event of exon 3 (orange) resulting in PROK1. The first 27 amino acids consist of the signaling peptide (green) followed by the AVITGA motif (brown), which is essential for PROK2 bioactivity. B, PROKR2 is a G protein-coupled receptor, thus spanning the membrane 7 times (blue; transmembrane domains) and encoded by 2 exons. Multiple variations have been detected in both proteins (indicated under figures by arrows). Asterisks indicate variants that have been detected herein, with the p.G371R variant of PROKR2 being the only novel variant (red). Purple type indicates variants that are known to be functionally deleterious in vitro and have been detected in KS (13, 15). Amino acid numbers are indicated above the figures.

itarism and SOD or HPE, which to our knowledge, is the largest cohort screened to date.

## Patients and Methods

### Patients

A total of 422 patients were recruited between 1998 and 2010; 168 were recruited at the London Centre of Pediatric Endocrinology, based at Great Ormond Street Hospital for Children and University College London (UCL) Hospitals in London; the remainder were referred from national (n = 157) and international (n = 97) centers. Ethical committee approval was obtained from the UCL Institute of Child Health/Great Ormond Street Hospital for Children Joint Research Ethics Committee, and informed written consent was obtained from patients and/or parents. Of the 422 patients screened, 375 (89%) had SOD and its variants, whereas 47 (11%) had HPE or midline clefts.

### Mutation analysis

Primers for the PCR amplification (35 cycles) of the coding region of human *PROK2* (NM_001126128) and *PROKR2* (NM_144773) were designed using the online Primer3 program (http://frodo.wi.mit.edu/primer3). PCR parameters are available on request. Amplified DNA was then analyzed for mutations by direct sequencing, using BigDye version 1.1 sequencing chemistry (Applied Biosystems, Foster City, California) and analysis on a 3730X1 DNA Analyzer (Applied Biosystems/Hitachi, Tokyo, Japan). For any novel mutations/sequence variations detected in either gene, 480 ethnically matched controls (if available) were then screened at the corresponding residue. Changes were checked with reference to the dbSNP database (www.ncbi.nlm.nih.gov/SNP) and 1000 Genomes Project (www.1000genomes.org).

### Functional analysis of novel variants

For cell surface quantification by ELISA, HEK-293 cells were cultivated in DMEM supplemented with 10% fetal calf serum and transfected by electroporation with a Gene Pulser Xcell eukaryotic system (Bio-Rad, Hercules, California) as described previously (13). Then, $10^7$ cells were transfected with 2 $\mu$g of recombinant pRK5 plasmid vectors coding for the N-terminal hemagglutinin (HA)-tagged wild-type or mutant PROKR2 and made up to a total amount of 10 $\mu$g plasmid DNA with empty vector. Twenty hours after transfection, HEK-293 cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 5 min. The cells were then either permeabilized, using .05% Triton X-100 for 5 min, or not permeabilized as previously described (13). HA-tagged proteins were detected using monoclonal
anti-HA peroxidase antibody 12CA5 (Roche Diagnostics, Mannheim, Germany) at 0.5 $\mu$g/ml. Because PROKR2 is a Gq-coupled receptor, we examined the signaling properties of novel variants by measuring intracellular calcium release and the accumulation of D-myo-inositol monophosphate ($IP_1$) in HEK-293 cells in response to PROK2 ligand using IP-One HTRF assay kit (Cisbio Bioassays, Condolet, France), as previously described (13, 22). PROKR2 can also couple to Gs protein to generate cAMP, so we additionally evaluated this pathway to better characterize the functionality of novel PROKR2 variants using cAMP HTRF assay kit as previously described (13, 22).

### Immunohistochemistry and in situ hybridization of *Prokr2* knockout mice

*Prokr2* null and wild-type embryos were collected at embryonic day (E) 18.5 and fixed with 4% paraformaldehyde (Sigma, St Louis, Missouri) and dehydrated to 100% ethanol to be embedded in paraffin. Paraffin sections (7 $\mu$m) were used for both immunohistochemistry and in situ hybridization. In short, immunohistochemistry was performed by dewaxing sections with histoclear, followed by hydration and antigen retrieval using microwave with citric acid buffer (10 mM citric acid, .05% Tween 20 [pH 6.0]). Antibodies were obtained from hybridoma bank [Developmental studies hybridoma bank (University of Iowa) and National Hormone and Peptide Program (Harbor-University of California, Los Angeles Medical Center)] and used at 1:1000 concentration in 5% inactivated sheep serum. For immunofluorescence, secondary goat antirabbit biotinylated antibody 1:300 (Dako, Carpinteria, California) was used, followed by 1:500 streptavidin (Sigma). 3,3-Diaminobenzidine (DAB; Vector Laboratories, Burlingame, California) staining was used in accordance with the manufacturer's protocol. Slide in situ hybridization on paraffin sections was performed as described in Gaston-Massuet et al (23). To quantify the number of LH cells, 3 different sections at different axial levels were selected from 3 embryos per genotype. Quantification of arginine vasopressin (AVP), oxytocin (OT), and Ghrh neurons was performed using 3 sections of equivalent axial level between mutant and wild-type from the supraoptic to the tubular area of the hypothalamus. Data are presented as mean number of cells ± SEM, with Student's *t* test used for statistical analysis and a $P < .05$ value considered statistically significant.

## Results

### Mutation analysis

No mutations/variations were found in *PROK2*, whereas 11 unrelated patients exhibited mutations/variations within the coding region of *PROKR2*, 9 of whom have variations that have been previously described in KS and shown to be functionally significant (p.L173R [n = 4], p.R268C [n = 4], and p.R85L [n = 1]) (Supplemental Figure 1). p.L173R is known to disrupt cell-surface targeting of the receptor, whereas the latter 2 variants affect G protein coupling (13). The remaining 2 patients harbored the p.A51T and the novel p.G371R variations. None of the 11 patients with *PROKR2* variations had changes in *FGF8*, *FGFR1*, *KAL1*, *NELF*, *CHD7*, *WDR11*, *HESX1*, *SOX3*, or *SHH*.

### Patient with *PROKR2* (p.R85L) variant

The c.254G>T, p.R85L variant was detected in heterozygosity in a male Caucasian patient (II) who presented at 6 years of age with combined pituitary hormone deficiency (CPHD; GH deficiency [GHD], TSH deficiency

**Table 1.** Phenotypes in Patients with *PROKR2* Variations

| Patient No. | Mutation | Status | Sex | Ethnicity | Age, y | Endocrinopathy | MRI | Eyes | Other Features |
|---|---|---|---|---|---|---|---|---|---|
| I | p. A51T | HT | F | Chinese | 0.9 | GHD | APH | SOD, BL ONH | Pigmentary changes of right optic nerve |
| II | p. R85L | HT | M | Caucasian | 6.0 | GHD, TSHD, ACTHD, GnD | APA, EPP, hypoplastic infundibulum | Normal | Normal smell |
| III | p.L173R | HT | F | Caucasian | 0.1 | GHD, ACTHD | APH, partially descended PP | Normal | Hyperinsulinism treated with diazoxide and resolved; GI dysmotility |
| IV[a] | p.L173R | HT | M | Caucasian | 0.6 | GHD, ACTHD, TSHD, DI | Absent septum pellucidum, heterotopic gray matter, schizencephaly | SOD, BL ONH | Neonatal jaundice; spastic quadriplegia, epilepsy; sleep apnea and uncoordinated swallow diagnosed at age 18 yr |
| V | p.L173R | HT | M | Caucasian | 0.7 | GHD, ACTHD TSHD | APH, EPP, hypoplastic stalk | Normal | Hypopituitarism diagnosed in infancy; unilateral undescended testis, puberty induced age 12.5 yr; normal gonadotropins on retesting |
| VI | p.L173R | HT | M | Caucasian | 1.0 | GHD, ACTHD, TSHD | APH, EPP | SOD, BL ONH, retinal detachment, PHPV (L) | Autistic spectrum disorder and sleep disturbance |
| VII[b] | p.R268C | HT | M | Pakistani | 1.3 | Normal | APH, dysgenesis of CC, Dandy-Walker malformation, cerebellar hypoplasia | SOD, BL ONH, atypical visual evoked potential | Epilepsy, developmental delay, sleep disorder |
| VIII | p.R268C | HM | M | African[c] | 1.2[d] | GHD, TSHD, ACTHD, GnD | APH, EPP, hypoplastic stalk | SOD, Rt ONH | Neonatal hypoglycemia, BL undescended testes |
| IX[b] | p.R268C | HT | M | Caucasian | 0.8 | GHD, TSHD, ACTHD, GnD | EPP, absent infundibulum | SOD, BL ONH | Micropenis, BL undescended testes, developmental delay |
| X | p.R268C | HT | M | Caucasian | 1.4 | GHD, TSHD | Agenesis of CC, focal malformation of Rt mesial frontal cortex | SOD, BL small ODs | Neonatal hypoglycemia, congenital heart disease, developmental delay, GORD |
| XI | p. G371R | HT | M | Caucasian | 4.5 | GHD | APH | SOD, BL ONH, astigmatism (L) | Mild aggression |

Abbreviations: HT, heterozygous; HM, homozygous; M, male; F, female; Ht, height; Wt, weight; SDS, SD score; GnD, gonadotrophin deficiency; DI, diabetes insipidus; APA, anterior pituitary aplasia; APH, anterior pituitary hypoplasia; EPP, ectopic posterior pituitary; PP, posterior pituitary; CC, corpus callosum; ODs, optic discs; ONH, optic nerve hypoplasia; BL, bilateral; L, left; Rt, right; PHPV, persistent hyperplastic vitreous; GORD, gastroesophageal reflux disease. Age denotes age at presentation. The table shows endocrine deficits, ocular phenotypes, and results of MRI in patients with *PROKR2* variations.

[a] Patient deceased at age 24 years; cause of death unknown.

[b] Patient phenotype has been recently reported (22).

[c] Patient of mixed Black African and Caribbean origin.

[d] Initial presentation with neonatal hypoglycemia; commenced hydrocortisone and $T_4$ treatment on day 7 of life.

[TSHD], and ACTH deficiency [ACTHD]). He had normal visual acuity and normosmia, and magnetic resonance imaging (MRI) of the brain showed an absent anterior pituitary and an ectopic/undescended posterior pituitary. Puberty was induced with gonadotropins at the age of 23 years, and the patient has since remained on testosterone (Table 1 and Supplemental Table 1).

### Patients with *PROKR2* (p.L173R) variant

The 4 patients (III, IV, V, VI) carrying the heterozygous c.518T>G, p.L173R variant were all Caucasian and presented with variable phenotypes. Two patients (IV, VI) had SOD, and all four patients had multiple anterior pituitary hormone deficiencies, with the additional diagnosis of diabetes insipidus in patient IV (Table 1 and Supplemental Table 1). Patient III presented in the neonatal period with profound hypoglycemia and was diagnosed with cortisol deficiency (cortisol <30 nmol/L). Despite hydrocortisone treatment, she had increasing glucose requirements to maintain normoglycemia (up to 15 mg/kg/min) and had an inappropriately increased insulin concentration (11.4 mU/L) when hypoglycemic (blood glucose <3 mmol/L). A diagnosis of congenital hyperinsulinism was made, and diazoxide was commenced. Hyperinsulinism resolved by the age of 1 year, and the diagnosis of GHD was subsequently confirmed by glucagon provocation with commencement of recombinant human GH. She has since been diagnosed as having gastrointestinal (GI) dysmotility. In 3 of the 4 patients, the heterozy-

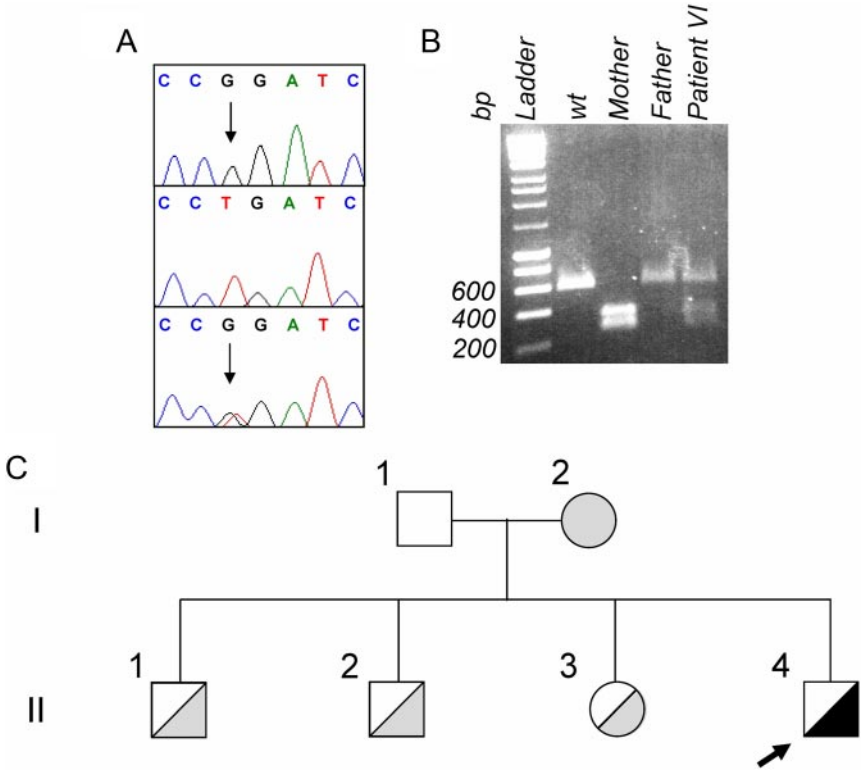


**Figure 2.** Parental screening of patient VI and pedigree mapping. A, Direct sequencing of *PROKR2* in patient VI and his parents shows that the patient is heterozygous for the p.L173R variation (bottom panel, arrow), whereas the mother is homozygous (top panel) and the father has the wild-type sequence (middle panel). B, The genotypes were confirmed by restriction digest of PCR products using the BsaWI enzyme that does not cut in the *WT* sequence, thus leaving a band of 596 bp. Incubation with the patient's DNA results in 3 fragments, including the full-length product plus the cleaved 342- and 254-bp products, whereas the parent homozygous for the variant only shows the 342- and 254-bp products. C, Pedigree map of the patient's family presenting 2 generations. Black shading represents affected members, whereas unaffected carriers are marked by gray. The proband (II.4) was heterozygous for the p.L173R variation, whereas his mother (I.2) was the unaffected, homozygous carrier. She had 3 other children (II.1–3), all of whom were heterozygous for the variation but lacked a hypopituitary phenotype.

gous variation was inherited from 1 of the parents. Only 1 of the parents manifested a phenotype; the heterozygous mother of patient V exhibited mild anosmia and delayed menarche with reported normal fertility. Surprisingly, the mother of patient VI was homozygous for the p.L173R variation and yet asymptomatic (Figure 2), with no evidence of abnormal gonadotropin secretion (peak LH, 30.3 IU/L; peak FSH, 8.6 IU/L, in response to GnRH; estradiol, 593 pmol/L). She had 3 other children (without fertility treatment) who were also heterozygous for the variation (data not shown). One male sibling of patient VI had a sleep disorder with behavioral problems, whereas another male sibling had epilepsy; their older sister was phenotypically normal.

### Patients with *PROKR2* (p.R268C) variant

The *PROKR2* c.802C>T, p.R268C variant was detected both in heterozygosity (VII, IX, X) and in homozygosity (VIII). All 4 patients were male and had SOD. Three patients had optic nerve hypoplasia and a hypoplastic anterior pituitary on MRI, whereas patient X had SOD with agenesis of the corpus callosum and small optic discs, but no anterior pituitary hypoplasia at the time of presentation (Table 1 and Supplemental Table 1). Patients VII and X had additional brain abnormalities (cerebellar hypoplasia, Dandy-Walker cyst, focal abnormality of mesial frontal cortex) with/without epilepsy and developmental delay. Three of the 4 patients had multiple pituitary hormone deficiencies. Although patient VII has not yet developed any pituitary hormone deficiencies, he is under regular clinical follow-up. Both parental DNA samples were only available for patient IX; the unaffected father was the heterozygous carrier. Only the maternal DNA sample for patient X was available, and she was not a carrier; parental DNA could not be obtained for patients VII or VIII.

### Patients with other *PROKR2* variations

Patient I is a female of Chinese origin with SOD who first presented at the age of 11 months with bilateral optic nerve hypoplasia and pigmentary changes of the right optic nerve. Pituitary MRI showed a hypoplastic anterior pituitary, with a eutopic posterior pituitary. She developed GHD (peak GH to stimulation, 3.3 $\mu$g/L) by the age of 6 years and commenced treatment with recombinant human GH. She entered puberty (breast stage 2) at the age of 10.3 years, and she is followed up regularly. To date, no other pituitary hormone deficiencies have been identified (Table 1 and Supplemental Table 1). Sequencing analysis revealed a heterozygous missense variation in *PROKR2* (c.151G>A, p.A51T). Her unaffected mother was also heterozygous for this change. Although p.A51T occurs at a highly conserved residue, it has also been detected in 1 of our 480 controls and has recently been determined as functionally benign (21). Therefore, no further functional work was conducted.

The novel *PROKR2* sequence variant (c.1111C>G, p.G371R) was detected in heterozygosity in a male patient (XI). He presented with SOD including unilateral optic nerve hypoplasia, anterior pituitary hypoplasia, and GHD

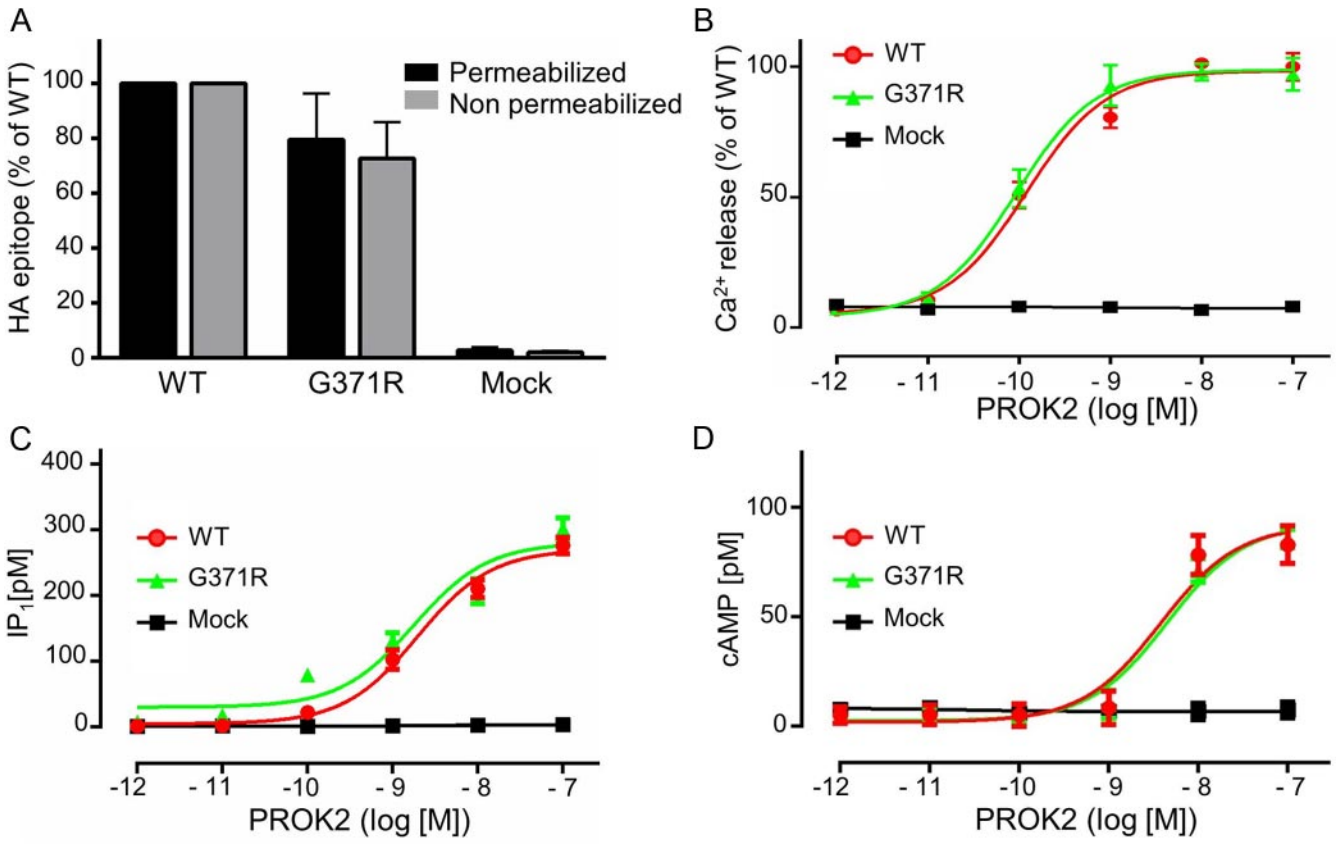


**Figure 3.** Functional analysis of the HA-tagged novel variant G371R. A, Surface and total cell levels of the PROKR2 mutants. Amounts of HA-tagged receptors at the cell surface (nonpermeabilized cells, gray histograms) and in permeabilized cells (black histograms) were quantified by ELISA. B–D, Functional assays comparing wt PROKR2 and the PROKR2 p.G371R variant against negative control (mock). Induction of protein signaling through either the novel mutant or wt PROKR2 receptor by ligand PROK2 treatment resulted in similar levels ($P > .05$) of intracellular $Ca^{2+}$ turnover (B) and $IP_1$ (C) and cAMP (D) accumulation, indicating that p.G371R is not a pathogenic variant. Data are presented as mean ± SEM of 3 independent experiments.

(Table 1 and Supplemental Table 1). The sequence variant occurred at a highly conserved residue located within the intracellular C-terminal region of PROKR2 and was not detected in any of our 480 controls. The total amount and the amount at the cell surface of the variant are similar (Figure 3A), indicating that the trafficking properties of the p.G371R variant are not impaired compared to the wild-type receptor. Further functional analysis showed that the signaling activity of the variant was similar to that of the wild-type receptor for both $Ca^{2+}$ release and $IP_1$ accumulation (Figure 3, B and C). As shown in Figure 3D, the accumulation of cAMP from the variant after PROK2 stimulation was also comparable to that of the wild-type PROKR2. These results showed that the p.G371R variant does not alter either Gq or Gs signaling pathways, although we cannot exclude the possibility that this variation may cause defects in other aspects of PROKR2 signaling, such as Gi-protein coupling, which were not investigated in this study.

### Analysis of the hypothalamic-pituitary axis in the *Prokr2*-null embryos

Using immunohistochemistry against a variety of pituitary terminal differentiation markers (GH, ACTH, TSH, and α-glycoprotein subunit [gonadotropes and thyrotropes]), we aimed to investigate whether the phenotype in *Prokr2* knockout mice at E18.5 would be comparable to the phenotypes we observed in our patients (Figure 4). Extensive *Prokr2* expression has been reported in the developing preoptic region of the brain that contains the hypothalamic nuclei (24), whereas in humans, expression of *PROKR2* has been shown by RT-PCR in the pituitary gland and central nervous system postnatally (25, 26). Expression of each of the markers was consistent between wild-type and mutant mice with a reduction in pituitary size, consistent with a reported reduction in global embryo size in the mutants (17). LH-immunoreactive cell number was significantly reduced in *Prokr2*$^{-/-}$ embryos (Figure 4, K, L, K′, L′, and M). In situ hybridization using specific probes to the endocrine hypothalamic neurons (median eminence and paraventricular/supraoptic nuclei) expressing *Avp*, *OT*, and *Ghrh* showed no overt differences between wild-type and *Prokr2*$^{-/-}$ embryos at E18.5 either morphologically (Figure 5, A–F) or quantitatively (Figure 5H). Our results indicate that mProkr2 is dispensable for proper formation of the hypothalamic-pituitary

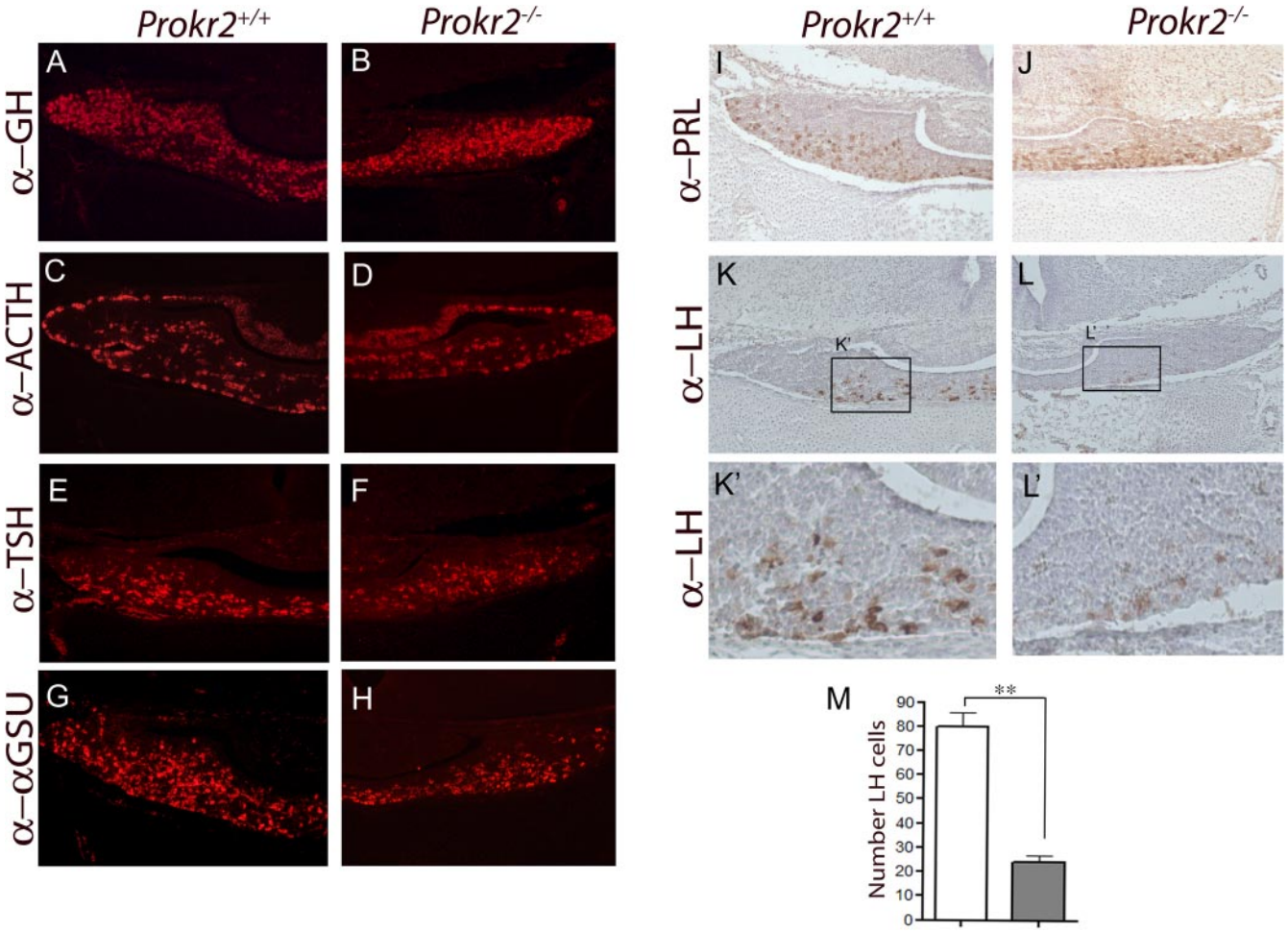


**Figure 4.** mProkr2-deficient embryos exhibit normal terminal differentiation of hormone-producing cells. Coronal sections through the pituitary gland of the wild-type, *Prokr2*$^{+/+}$ (A, C, E, G, I, K) and *Prokr2*$^{-/-}$ (B, D, F, H, J, L) embryos immunostained against GH (A, B), ACTH (C, D), TSH (E, F), $\alpha$-glycoprotein subunit hormone (G, H), prolactin (I, J), and LH (K, L). Immunoreactivity of GH, ACTH, TSH, $\alpha$-GSU, and PRL show no difference between wild-type and *Prokr2*-deficient embryos. Although the pituitary glands of *Prokr2*$^{-/-}$ embryos appear smaller, this is due to an overall reduction in head size of these mutant embryos. The number of LH-expressing cells appears reduced in *Prokr2*$^{-/-}$ embryos (K, L, and K′, L′, enlarged boxed areas in K and L, respectively), and quantification of LH cells demonstrates a statistically significant reduction in *Prokr2*$^{-/-}$ $P < .05$ (M). $\alpha$-GSU, $\alpha$-glycoprotein subunit hormone; PRL, prolactin. Scale bar in A represents 150 $\mu$m.

axis. The down-regulation in LH in the *Prokr2*-null embryos agrees with the role of mProkr2 in regulating GnRH neuronal migration previously reported by Matsumoto et al (16).

## Discussion

In this study, we have identified 11 patients with variable congenital hypopituitarism/SOD, who presented with sequence variations in *PROKR2* (Table 1). Because the parental carriers included both maternal and paternal carriers, there is no suggestion of a parent of origin effect. Despite its established role in KS (14), we could not implicate the corresponding ligand, *PROK2*, in hypopituitarism. Our results of the largest cohort of patients with congenital hypopituitarism, including both CPHD and SOD, screened to date are consistent with our recently published data (20). Here, we report the identification of *PROKR2* variants in patients with craniofacial/midline disorders and hypopituitarism, thus suggesting an overlap in genotypes/phenotypes between these conditions and KS (20). In our cohort, 9 of 11 patients were found to harbor previously described *PROKR2* variations that had been shown to be functionally deleterious in vitro; the lack of dominant-negative effects of these variants suggests that their functional significance in vivo remains debatable (13). These variations are present in approximately 2% of our cohort with SOD; thus *PROKR2* variations occur more frequently than any other genetic abnormalities identified in association with SOD to date (27). However, the extent to which these variations contribute to the phenotype is yet to be established. Of the 9 patients above, *PROKR2* variations were detected both in homozygos-

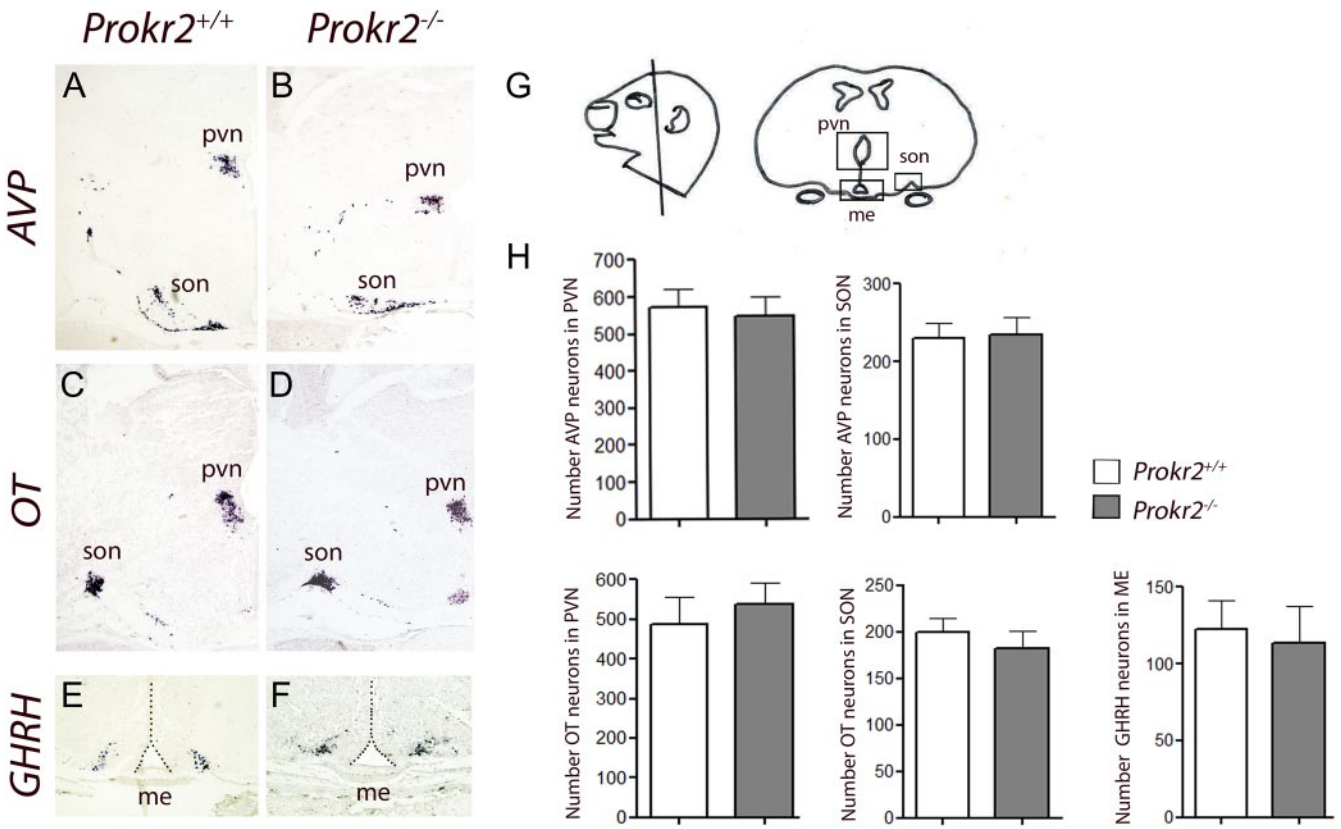


**Figure 5.** *Prokr2* null embryos exhibit normal development of the neuroendocrine hypothalamus. Coronal sections through the brain of mouse embryos (represented in G) at E18.5, wild-type *Prokr2*$^{+/+}$ (A, C, E), and mutant *Prokr2*$^{-/-}$ (B, D, F), hybridized with *Avp* (A, B), *OT* (C, D), and *Ghrh* (E, F). *Avp*- and *OT*-expressing neurons in the paraventricular and supraoptic nuclei appear similar between *Prokr2*$^{+/+}$ and *Prokr2*$^{-/-}$ embryos, suggesting that *Prokr2* is not required for the development of these structures. Expression of *Ghrhr* in the arcuate nucleus at the level of the median eminence appears unaltered between genotypes (E, F). H, Plotted graphs representing numbers of *AVP*, *OT*, and *Ghrh* neurons indicate no difference in the number of *AVP*, *OT*, and *Ghrh* neurons between wild-type (white columns) and *Prokr2*$^{-/-}$ embryos. arc, Arcuate nucleus; pvn, paraventricular nuclei; son, supraoptic nuclei; me, median eminence. Scale bar in A represents 300 $\mu$m.

ity (n = 1) and in heterozygosity (n = 8). Interestingly, 1 patient (VI) with SOD/CPHD and structural pituitary abnormalities was heterozygous for the *PROKR2* p.L173R variation, whereas his phenotypically normal mother was homozygous for the same variant; this variant has previously been identified in several patients with KS and was shown to disrupt cell-surface targeting of the receptor in vitro (12, 13, 28). We sought to better understand the phenotypes observed in our patients and the lack of a phenotype in the healthy homozygous mother by investigating a possible role for mProkr2 in the development and integrity of the hypothalamic-pituitary axis, using *Prokr2* null embryos as a model. Our study of the homozygous knockout *Prokr2*$^{-/-}$ mice revealed a morphologically normal pituitary and hypothalamus with normal hormone-secreting cells except for LH$^{+ve}$ gonadotrophs, which were significantly reduced in the mutants and consistent with the previously reported KS-like phenotypes that these mice exhibit (16, 17). These observations suggest that in the absence of *Prokr2*, the murine hypothalamic-pituitary axis develops normally. Although this may not be a human model, the parallels between the normal hypothalamic-pituitary axis between our knockout mice and the healthy mother of patient VI are compelling and suggest that the variants identified in this report may not be sufficient to cause a hypothalamic-pituitary phenotype in isolation. Any contributory mechanisms of *Prokr2/PROKR2* in the pathogenicity of hypopituitarism remain to be proven. In this cohort, the mother who was homozygous for the *PROKR2* p.L173R variation clearly did not have KS (normosmic and fertile with 4 children that were conceived without assistance, and with normal gonadotropins and estrogen). In addition, 2 of the 4 patients who had reached pubertal age (II, VIII) required the induction of puberty and remained on testosterone treatment into adulthood, whereas the other 2 (IV, V) progressed through puberty spontaneously and remained off sex steroid treatment. Therefore, the possibility that *Prokr2/PROKR2* is not, in isolation, causative of KS either must be considered; it may, however, contribute by modifying a phenotype caused by a defect in another gene(s) or environmental factor(s), as recently postulated by Raivio et al (20).

Comparison of our results with those already in the literature supports the above notion. First, we detected functionally significant variants at the p.R85 and p.R268 alleles in heterozygosity or homozygosity in patients with phenotypes ranging from CPHD to SOD. They had previously only been detected in KS and healthy controls (as had the p.L173R variant), albeit only in heterozygosity in the latter (12). Our data therefore strongly suggest that another gene or environmental factor is causing the more severe CPHD-SOD phenotypes. Indeed, although not proven in any of our hypopituitary patients, digenic cases of KS involving *PROKR2* have been reported previously (12, 15, 28–30). In a recent study by Sarfati et al (28), male KS patients presenting with homozygous variations in *PROKR2* were significantly more likely to exhibit cryptorchidism, microphallus, lower mean testicular volumes, and circulating gonadotropins than their heterozygous counterparts (28). This difference in the gene-dosage of *PROKR2* supports a contributory role to the pathogenesis of KS. No differences were observed between homozygous or heterozygous females, although this was attributed to the very low number of cases of the former (n = 4). Thus, the extent to which *PROKR2* variants contribute to either hypopituitarism or KS-associated phenotypes remains to be established. Care must be taken with the interpretation of results when patients presenting with such disorders also exhibit variations in the *PROKR2* gene, particularly with respect to genetic counseling. Other genes known to be associated with these disorders should be screened and, should these be negative for mutations, then one cannot rule out the presence of an as yet unidentified mutated gene or possible environmental factors.

In addition to presenting with CPHD/SOD, some of our patients with *PROKR2* variants had additional manifestations including epilepsy, sleep abnormalities, GI dysmotility, and diabetes insipidus. Although such variants have been detected for the first time in patients with the latter 2 disorders herein, a possible association is not altogether surprising given that prokineticin ligands and receptors are involved in various systems and processes including circadian rhythms in the brain, angiogenesis, neurogenesis, pain perception, immune responses, hematopoiesis, reproduction, and GI smooth muscle contraction (31–37). Epilepsy, sleep disorders, and diabetes insipidus are suggestive of a forebrain/hypothalamic phenotype (35, 36, 38). *Avp* and *Prok2* mRNA both colocalize to a significant number of hypothalamic neurons in rats, and there is evidence of interaction between these pathways through AVP receptor-null mice (39, 40). However, *Prokr2* knockout mice exhibited a morphologically normal hypothalamus with quantitatively normal expression of *Avp*, *OT*, and *Ghrh*. These data do not, however, exclude a role for *PROKR2* in postnatal development of these hypothalamic disorders in our patients. However, considering that our patient with diabetes insipidus and epilepsy (IV) was heterozygous for the same variant that the unaffected mother of patient VI had in homozygosity, it appears unlikely that mutated *PROKR2* is causative of these phenotypes. Additionally, we cannot definitively state that the GI dysmotility phenotype in patient III was caused by her heterozygous *PROKR2* variation, this again being the same as the mother of patient VI. Any contributions of *PROKR2* variants to these phenotypes would necessitate further studies, particularly with the aid of a postnatal murine model.

We have identified variations in *PROKR2* at a higher frequency in SOD than any other previously described genetic factor; we also describe other clinical features in association with these variations, including GI and hypothalamic disorders (eg, diabetes insipidus). However, the role of PROKR2 is controversial; heterozygous and homozygous variants occurring across the protein induce comparable phenotypes or, as we have shown, more severe phenotypes in cases of the former than the latter. This is compounded by the incidence of homozygosity in the healthy mother of a heterozygous child with a severe form of hypopituitarism in the form of SOD. Our analyses of the pituitary and hypothalamus in *Prokr2* knockout mice are largely inconsistent with the patient phenotypes, yet strongly support the normal presentation of the mother and her 3 unaffected (with respect to hypopituitarism) heterozygous children, although one needs to note the reduced LH in the murine null mutants. The number of genetically assigned causes of both KS and hypopituitarism is low, accounting for 30% of KS and 5–10% of SOD and related midline disorders, and given that none of our patients harbored mutations in any of the known genes in these disorders, there are clearly other genetic/environmental factors yet to be discovered. Subjects with sequence variants in *PROKR2* represent a unique cohort; further careful genetic investigation is likely to aid in the identification of the missing genetic or epigenetic modifiers that interact with this pathway in humans to account for the phenotypic heterogeneity. Further research into uncovering these additional factors would help define the role, if any, of *PROKR2* in these and other disorders discussed herein.

## Acknowledgments

Address all correspondence and requests for reprints to: Professor Mehul Dattani, Institute of Child Health and Great Ormond Street Children's Hospital, Pediatric Endocrinology, 30 Guilford Street, London WC1N 1EH, United Kingdom. E-mail: m.dattani@ich.ucl.ac.uk.

This work was supported by Birth Defect Foundation-Newlife Project Grant 08-09/29 (to M.J.M. and M.T.D.); in part by a grant from the British Society for Pediatric Endocrinology and Diabetes (to V.T. and M.T.D.) (M.T.D. is also funded by Great Ormond Street Children's Charity); Wellcome Trust Grants 084361 and 086545 (to J.-P.M.-B., M.T.D., and L.C.G.); and by the National Institute for Health Research, Great Ormond Street Hospital for Children/UCL Institute of Child Health Specialist Biomedical Research Centre, Grant 11ND01 (to C.G.-M.).

Disclosure Summary: The authors have nothing to disclose.

## References

1. **Webb EA, Dattani MT.** Septo-optic dysplasia. *Eur J Hum Genet*. 2010;18:393–397.
2. **Kelberman D, Rizzoti K, Avilion A, et al.** Mutations within Sox2/SOX2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans. *J Clin Invest*. 2006;116:2442–2455.
3. **Dattani MT, Martinez-Barbera JP, Thomas PQ, et al.** Mutations in the homeobox gene HESX1/Hesx1 associated with septo-optic dysplasia in human and mouse. *Nat Genet*. 1998;19:125–133.
4. **Ragge NK, Brown AG, Poloschek CM, et al.** Heterozygous mutations of OTX2 cause severe ocular malformations. *Am J Hum Genet*. 2005;76:1008–1022.
5. **Abe Y, Oka A, Mizuguchi M, et al.** EYA4, deleted in a case with middle interhemispheric variant of holoprosencephaly, interacts with SIX3 both physically and functionally. *Hum Mutat*. 2009;30:E946–E955.
6. **Paulussen AD, Schrander-Stumpel CT, Tserpelis DC, et al.** The unfolding clinical spectrum of holoprosencephaly due to mutations in SHH, ZIC2, SIX3, and TGIF genes. *Eur J Hum Genet*. 2010;18:999–1005.
7. **Ribeiro LA, Quiezi RG, Nascimento A, Bertolacini CP, Richieri-Costa A.** Holoprosencephaly and holoprosencephaly-like phenotype and GAS1 DNA sequence changes: report of four Brazilian patients. *Am J Med Genet A*. 2010;152A:1688–1694.
8. **Alatzoglou KS, Dattani MT.** Genetic forms of hypopituitarism and their manifestation in the neonatal period. *Early Hum Dev*. 2009;85:705–712.
9. **McCabe MJ, Gaston-Massuet C, Tziaferi V, et al.** Novel FGF8 mutations associated with recessive holoprosencephaly, craniofacial defects, and hypothalamo-pituitary dysfunction. *J Clin Endocrinol Metab*. 2011;96:E1709–E1718.
10. **Ericson J, Norlin S, Jessell TM, Edlund T.** Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. *Development*. 1998;125:1005–1015.
11. **Falardeau J, Chung WC, Beenken A, et al.** Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. *J Clin Invest*. 2008;118:2822–2831.
12. **Dode C, Teixeira L, Levilliers J, et al.** Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *PLoS Genet*. 2006;2:1648–1652.
13. **Monnier C, Dode C, Fabre L, et al.** PROKR2 missense mutations associated with Kallmann syndrome impair receptor signalling activity. *Hum Mol Genet*. 2009;18:75–81.
14. **Pitteloud N, Zhang C, Pignatelli D, et al.** Loss-of-function mutation in the prokineticin 2 gene causes Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci USA*. 2007;104:17447–17452.
15. **Cole LW, Sidis Y, Zhang C, et al.** Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. *J Clin Endocrinol Metab*. 2008;93:3551–3559.
16. **Matsumoto S, Yamazaki C, Masumoto KH, et al.** Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2. *Proc Natl Acad Sci USA*. 2006;103:4140–4145.
17. **Prosser HM, Bradley A, Caldwell MA.** Olfactory bulb hypoplasia in Prokr2 null mice stems from defective neuronal progenitor migration and differentiation. *Eur J Neurosci*. 2007;26:3339–3344.
18. **Zhang C, Truong KK, Zhou QY.** Efferent projections of prokineticin 2 expressing neurons in the mouse suprachiasmatic nucleus. *PLoS One*. 2009;4:1–12.
19. **Ng KL, Li JD, Cheng MY, Leslie FM, Lee AG, Zhou QY.** Dependence of olfactory bulb neurogenesis on prokineticin 2 signaling. *Science*. 2005;308:1923–1927.
20. **Raivio T, Avbelj M, McCabe MJ, et al.** Genetic overlap in Kallmann syndrome, combined pituitary hormone deficiency, and septo-optic dysplasia. *J Clin Endocrinol Metab*. 2012;97:E694–E699.
21. **Reynaud R, Jayakody SA, Monnier C, et al.** PROKR2 variants in multiple hypopituitarism with pituitary stalk interruption. *J Clin Endocrinol Metab*. 2012;97:E1068–E1073.
22. **Trinquet E, Fink M, Bazin H, et al.** D-myo-inositol 1-phosphate as a surrogate of D-myo-inositol 1,4,5-tris phosphate to monitor G protein-coupled receptor activation. *Anal Biochem*. 2006;358:126–135.
23. **Gaston-Massuet C, Andoniadou CL, Signore M, et al.** Genetic interaction between the homeobox transcription factors HESX1 and SIX3 is required for normal pituitary development. *Dev Biol*. 2008;324:322–333.
24. **Martin C, Balasubramanian R, Dwyer AA, et al.** The role of the prokineticin 2 pathway in human reproduction: evidence from the study of human and murine gene mutations. *Endocr Rev*. 2011;32:225–246.
25. **Soga T, Matsumoto S, Oda T, et al.** Molecular cloning and characterization of prokineticin receptors. *Biochim Biophys Acta*. 2002;1579:173–179.
26. **Lin DC, Bullock CM, Ehlert FJ, Chen JL, Tian H, Zhou QY.** Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. *J Biol Chem*. 2002;277:19276–19280.
27. **McNay DE, Turton JP, Kelberman D, et al.** HESX1 mutations are an uncommon cause of septooptic dysplasia and hypopituitarism. *J Clin Endocrinol Metab*. 2007;92:691–697.
28. **Sarfati J, Guiochon-Mantel A, Rondard P, et al.** A comparative phenotypic study of Kallmann syndrome patients carrying monoallelic and biallelic mutations in the prokineticin 2 or prokineticin receptor 2 genes. *J Clin Endocrinol Metab*. 2010;95:659–669.
29. **Canto P, Munguia P, Soderlund D, Castro JJ, Mendez JP.** Genetic analysis in patients with Kallmann syndrome: coexistence of mutations in prokineticin receptor 2 and KAL1. *J Androl*. 2009;30:41–45.
30. **Abreu AP, Kaiser UB, Latronico AC.** The role of prokineticins in the pathogenesis of hypogonadotropic hypogonadism. *Neuroendocrinology*. 2010;91:283–290.
31. **Li M, Bullock CM, Knauer DJ, Ehlert FJ, Zhou QY.** Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle. *Mol Pharmacol*. 2001;59:692–698.

32. **Lecouter J, Lin R, Tejada M, et al.** The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: localization of Bv8 receptors to endothelial cells. *Proc Natl Acad Sci USA*. 2003;100:2685–2690.
33. **Battersby S, Critchley HO, Morgan K, Millar RP, Jabbour HN.** Expression and regulation of the prokineticins (endocrine gland-derived vascular endothelial growth factor and Bv8) and their receptors in the human endometrium across the menstrual cycle. *J Clin Endocrinol Metab*. 2004;89:2463–2469.
34. **Zhou QY, Cheng MY.** Prokineticin 2 and circadian clock output. *FEBS J*. 2005;272:5703–5709.
35. **Prosser HM, Bradley A, Chesham JE, Ebling FJ, Hastings MH, Maywood ES.** Prokineticin receptor 2 (Prokr2) is essential for the regulation of circadian behavior by the suprachiasmatic nuclei. *Proc Natl Acad Sci USA*. 2007;104:648–653.
36. **Kishi T, Kitajima T, Tsunoka T, et al.** Possible association of prokineticin 2 receptor gene (PROKR2) with mood disorders in the Japanese population. *Neuromolecular Med*. 2009;11:114–122.
37. **Caronia LM, Martin C, Welt CK, et al.** A genetic basis for functional hypothalamic amenorrhea. *N Engl J Med*. 2011;364:215–225.
38. **Sanabria ER, Scorza FA, Bortolotto ZA, Calderazzo-Filho LS, Cavalheiro EA.** Disruption of light-induced c-Fos immunoreactivity in the suprachiasmatic nuclei of chronic epileptic rats. *Neurosci Lett*. 1996;216:105–108.
39. **Masumoto KH, Nagano M, Takashima N, et al.** Distinct localization of prokineticin 2 and prokineticin receptor 2 mRNAs in the rat suprachiasmatic nucleus. *Eur J Neurosci*. 2006;23:2959–2970.
40. **Li JD, Burton KJ, Zhang C, Hu SB, Zhou QY.** Vasopressin receptor V1a regulates circadian rhythms of locomotor activity and expression of clock-controlled genes in the suprachiasmatic nuclei. *Am J Physiol Regul Integr Comp Physiol*. 2009;296:R824–R830.