



DLG2 variants in patients with pubertal disorders

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Purpose: Impaired function of gonadotropin-releasing hormone (GnRH) neurons can cause a phenotypic spectrum ranging from delayed puberty to isolated hypogonadotropic hypogonadism (IHH). We sought to identify a new genetic etiology for these conditions.

Methods: Exome sequencing was performed in an extended family with autosomal dominant, markedly delayed puberty. The effects of the variant were studied in a GnRH neuronal cell line. Variants in the same gene were sought in a large cohort of individuals with IHH.

Results: We identified a rare missense variant (F900V) in *DLG2* (which encodes PSD-93) that cosegregated with the delayed puberty. The variant decreased *GnRH* expression in vitro. PSD-93 is an anchoring protein of NMDA receptors, a type of glutamate receptor that has been implicated in the control of puberty in laboratory animals. The F900V variant impaired the interaction

between PSD-93 and a known binding partner, Fyn, which phosphorylates NMDA receptors. Variants in *DLG2* that also decreased GnRH expression were identified in three unrelated families with IHH.

Conclusion: The findings indicate that variants in *DLG2*/PSD-93 cause autosomal dominant delayed puberty and may also contribute to IHH. The findings also suggest that the pathogenesis involves impaired NMDA receptor signaling and consequently decreased GnRH secretion.

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INTRODUCTION

Puberty results from reactivation of gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus.¹ In patients with isolated hypogonadotropic hypogonadism (IHH), puberty either fails to occur completely, is incomplete, or occurs during adulthood, rather than during adolescence.² The disorder can be inherited in an X-linked, autosomal dominant, autosomal recessive, or oligogenic fashion.³ IHH is caused by variants in genes that regulate the embryonic migration of GnRH neurons into the hypothalamus and/or the function of GnRH neurons. Variants in more than 40 genes have been implicated in the etiology.^{3,4}

Isolated, self-limited delayed puberty is a milder condition in which puberty occurs at an abnormally late age in adolescence.² It is often inherited in an autosomal dominant fashion, although other modes of inheritance have also been described.^{4,5} IHH and self-limited delayed puberty can occur within the same family, suggesting that the same genetic

defects can underlie both conditions.^{2,5} Consistent with this concept, individuals with self-limited delayed puberty have an increased frequency of potentially pathogenic variants in IHH genes.^{2,5} Recent studies have implicated variants in several genes involved in GnRH migration and function in the pathogenesis of delayed puberty, including *EAP1*,⁶ *HS6ST1*,⁷ and *IGSF10*.⁸ However, in the great majority of families with delayed puberty, the genetic etiology remains unknown.

Here we studied an extended family with markedly delayed puberty and found a rare missense variant (F900V) in *DLG2* that cosegregated with the phenotype. We found evidence that this variant impairs GnRH expression in vitro. We also identified rare missense variants in *DLG2* that impaired GnRH expression in vitro in three nuclear families with IHH. Interestingly, PSD-93, the protein encoded by *DLG2*, serves as an anchoring protein for NMDA receptors, and NMDA signaling has been strongly implicated in the regulation of puberty in laboratory animals.¹ We found that the variants

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identified in the families with delayed puberty and IHH interfered with the binding of PSD-93 to Fyn, a nonreceptor type protein kinase that phosphorylates NMDA receptors to promote signaling. Taken together, the evidence strongly suggests that *DLG2/PSD-93* participates in regulating the onset of human puberty and reproduction and, consequently, that variants in this gene cause pubertal disorders.

MATERIALS AND METHODS

Subjects

An extended family with extremely delayed puberty

We evaluated an extended family in which multiple members showed a marked delay in the onset of puberty. The proband was evaluated at the National Institutes of Health (NIH) Clinical Center and the extended family members were evaluated either at the Clinical Center or through phone interviews.

Subjects with isolated hypogonadotropic hypogonadism

Subjects were enrolled in a study at the Massachusetts General Hospital to investigate the genetic causes of hypogonadotropic hypogonadism, which included exome sequencing of probands and parents for the genetic causes of IHH (ClinicalTrials.gov identifier: NCT00494169).

This study was approved by the institutional review boards (IRBs) at participating institutions. All adult subjects and parents of minors provided written informed consent and children provided written assent.

This study was approved by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institute of Neurological Disorders and Stroke (NINDS), and Massachusetts General Hospital.

Exome sequencing

Exome sequencing was performed at three different sequencing centers: NIH Intramural Sequencing Center (NISC), Broad Institute, or NICHD Molecular Genomics Core (MGC), depending on where the subjects were recruited. For the three subjects with IHH, the BAM files that contained the sequence alignment data were carefully examined on Integrative Genomics Viewer (IGV) to seek any significant variant in 40 genes associated with IHH (gene list is available in Supplemental Method 1).^{9,10}

Sanger sequencing

The identified variants were confirmed by Sanger sequencing. The primer sets used for polymerase chain reaction (PCR) amplification and sequencing are listed in Supplemental Method 2.

Expression vectors and mutagenesis

Dlg2 complementary DNA (cDNA) was cloned from 7-day-old mouse brain messenger RNA (mRNA) and subcloned into expression vector, pcDNA3.1(-) (Invitrogen, USA). The identified variants were introduced using a site-directed mutagenesis kit (Invitrogen, USA), and sequences of the

entire insert were confirmed with Sanger sequencing. The position in the murine ortholog that corresponds to the human F900V variant is chr7: 92438043T>G (ENSMUST00000107196.9, GRCm38/mm10), and the evolutionary conservation is shown in Supplemental Fig. 1.

Pull-down experiment, mass spectrometry, coimmunoprecipitation, and western blot

For pull-down experiments, lysates of HEK293T cells expressing GFP alone or PSD-93-GFP (protein encoded by wild-type (WT) *Dlg2* and tagged with GFP) or F900V-PSD-93-GFP (protein encoded by F900V *Dlg2* and tagged with GFP) were mixed sequentially with rat hypothalamus lysates and GFP antibody that were then bound to protein A-Sepharose beads. The eluted proteins were analyzed using mass spectrometry at the NINDS mass spectrometry core facility. An identified protein, Fyn, was coexpressed with WT *Dlg2* or F900V *Dlg2* in HEK293T cells, and the interaction was analyzed by coimmunoprecipitation and western blot. The detailed methods are described in Supplemental Method 3.

Assessment of GluN2B phosphorylation

HEK293T cells were transfected with GluN1, GluN2B, Fyn, and WT PSD-93 or the F900V variant. After 4 hours, APV (50 μ M) and MgCl₂ (20 mM) were added to the medium to block cell death caused by excitotoxicity. After 24 hours following transfection, cells were lysed with 1% SDS lysis buffer and analyzed by western blot with antibodies to phosphorylated-GluN2B (rabbit anti-pY1472-GluN2B, Millipore, catalog number AB5403) and GluN2B (mouse anti-GluN2B, NeuroMab).

Culture and transfection of GnRH-expressing cell lines (GT1-7 and HEK293)

GT1-7 was a generous gift from Pamela Mellon.¹¹ GT1-7 cells (passage 5–15) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax (Gibco, USA) medium containing 10% fetal bovine serum (FBS) and 1% pen-strep. The medium was changed every 3 days. 0.3 million cells/well were plated in 12-well plates and after 24 hours the cells were transfected with empty vector (EV), mutant (Mut) *Dlg2/PSD-93*, or WT *Dlg2/PSD-93* expression vector using lipofectamine 3000 (Invitrogen, USA) in Opti-MEM (Invitrogen, USA) for 4 hours. Then, the medium was changed to regular culture medium. The cells were collected after 48 hours for RNA extraction or GnRH immunoassay. The above experiments were also performed in HEK293 cells, which express *GNRH1* (<http://amp.pharm.mssm.edu/Harmonizome/gen>), except that the cells were collected at 24 hours post-transfection for analysis.

GnRH competitive enzyme-linked immunosorbent assay

GT1-7 cells were transfected with empty vector, WT *Dlg2/PSD-93* vector, or mutant *Dlg2/PSD-93* expression vector as described above, and after 24 hours the culture medium was removed and cells were lysed by 2 freeze–thaw cycles. GnRH

immunoassay was performed as previously described¹² using a commercially available kit (Phoenix Pharmaceuticals, USA) following the manufacturer's instruction. The reported detection limit is 10 pg/mL and reported intra-assay variation and interassay coefficients of variation were 10% and 15%, respectively.

mRNA expression of *Dlg2* in rat preoptic area (POA)

RNA from rat preoptic area was extracted using an RNeasy kit (Qiagen) following the manufacturer's protocol and was reverse transcribed into cDNA. The expression of *Gnrh1/GNRH1*, membrane-associated guanylate kinases (MAGUKs: *Dlg1*, *Dlg2*, *Dlg3*, *Dlg4*) and NMDA receptors (*GluN1/NR1*, *GluN2A/NR2A*, *GluN2B/NR2B*) was measured by real-time reverse transcription PCR (RT-PCR) using Taqman probes (Invitrogen, USA). Expression values were normalized to 18S ribosomal RNA (rRNA). The detailed probes used and methods are described in Supplemental Methods 4 and 5.

Protein expression of PSD-93 in mouse hypothalamus

Hippocampus and hypothalamus were dissected from mice at different ages, then analyzed by western blot. The detailed probes used and methods are described in Supplemental Methods 4 and 5.

Statistical analysis

Western blot bands after coimmunoprecipitation were quantified using ImageJ¹³ and compared using Student's *t* test. The raw data for empty vector, WT *Dlg2*/PSD-93, and mutant *Dlg2*/PSD-93 from each experiment were log-transformed to better fit the normal distribution and compared using one-way analysis of variance (ANOVA) followed by pair-wise comparisons using the Holm–Sidak correction for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

RESULTS

Clinical presentation in an extended family with markedly delayed puberty

The proband (Fig. 1a, III.7) was a young man who presented at 16 years of age with delayed puberty. He had Tanner stage 2 pubic hair, testicular volumes of 4 mL bilaterally, testosterone of 40 ng/dL, and significantly delayed bone age of 13 years 2 months. The patient's mother (II.5) and maternal grandmother (I.2) had menarche at age 18 years, and the maternal aunt (II.2) reported a history of breast development beginning at age 14 years, menarche at age 16.5 years, and 3 inches of height gain after 18 years of age. They had no history of infertility. The proband's older brother (III.6) had late pubertal onset at age 16 years with prolonged linear growth until age 21 years. Unaffected females in the family had menarche at approximately 12–14 years of age, and unaffected males also had pubertal onset (determined by detailed questionnaire) at a midnormal age. Subjects I.2, II.2, and II.5 had no problems with fertility. Subjects' hormonal profiles and imaging studies are summarized in Supplemental Table 1.

Identification of a variant in *DLG2*/PSD-93

Exome sequencing of affected family members (I.2, II.2, II.5, III.6, and III.7) and unaffected family members (II.3, III.1, III.2, III.3, and III.5) revealed a single rare, nonsynonymous variant that cosegregated precisely with the delayed puberty phenotype. This missense variant (c.2698T>G:p.F900V, NM_001142699.1) occurred in *DLG2*, which encodes PSD-93. The variant was confirmed by Sanger sequencing (Supplemental Fig. 2). It was found in only one individual in gnomAD and was predicted to be deleterious to protein function by all four applied in silico analyses (CADD, SIFT, MutationTaster, PolyPhen-2).^{14–17} An extensive search for any rare variants in 40 genes known to cause hypogonadotropic hypogonadism or delayed puberty revealed only a single, heterozygous, rare, missense variant

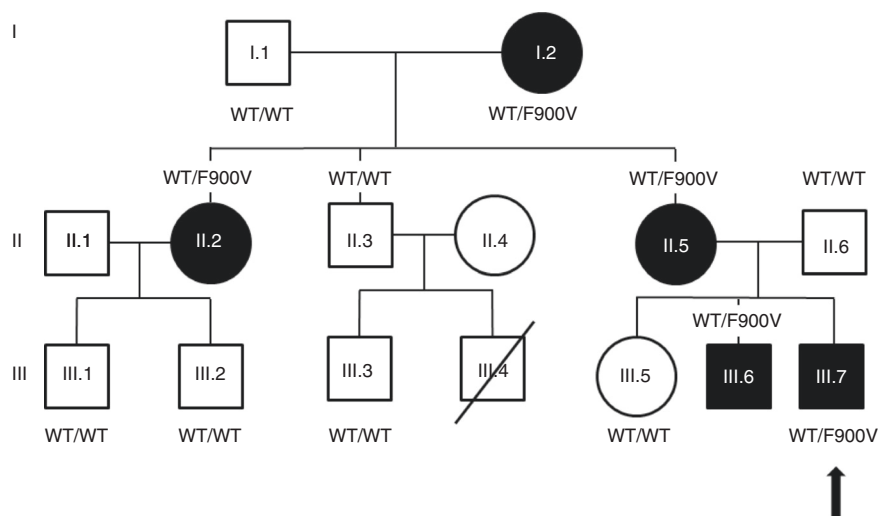


Fig. 1 Family with markedly delayed puberty. Pedigree. Arrow indicates proband. Closed symbol, delayed puberty; open symbol, normal timing of puberty. F900V denotes a variant in *DLG2*/PSD-93. *WT*, wild-type *DLG2*/PSD-93.

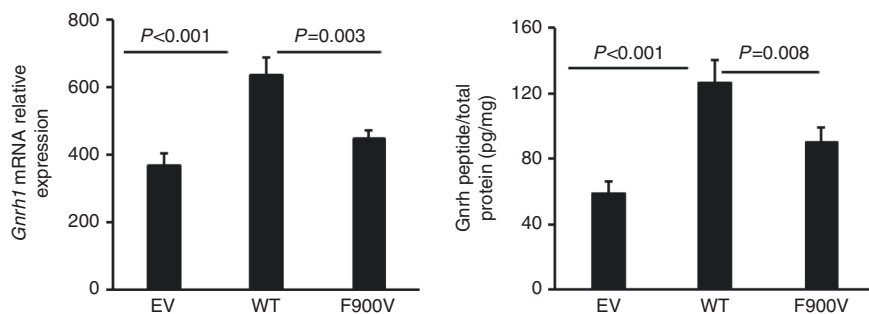


Fig. 2 Effect of F900V *DLG2/PSD-93* variant on *Gnrh1* expression in GT1-7 cells. In GT1-7 cells (between 13 and 15 passages in culture), wild-type (WT) PSD-93 expression stimulated *Gnrh1* expression compared with empty vector (EV) for both messenger RNA (mRNA) (normalized to 18S ribosomal RNA [rRNA], left panel, $P < 0.001$, $n = 10$) and GnRH peptide expression (normalized to total protein, right panel, $P < 0.001$, $n = 14$). The F900V PSD-93 mutant impaired this stimulation both in mRNA levels ($P = 0.003$, left) and intracellular peptide levels ($P = 0.008$, right).

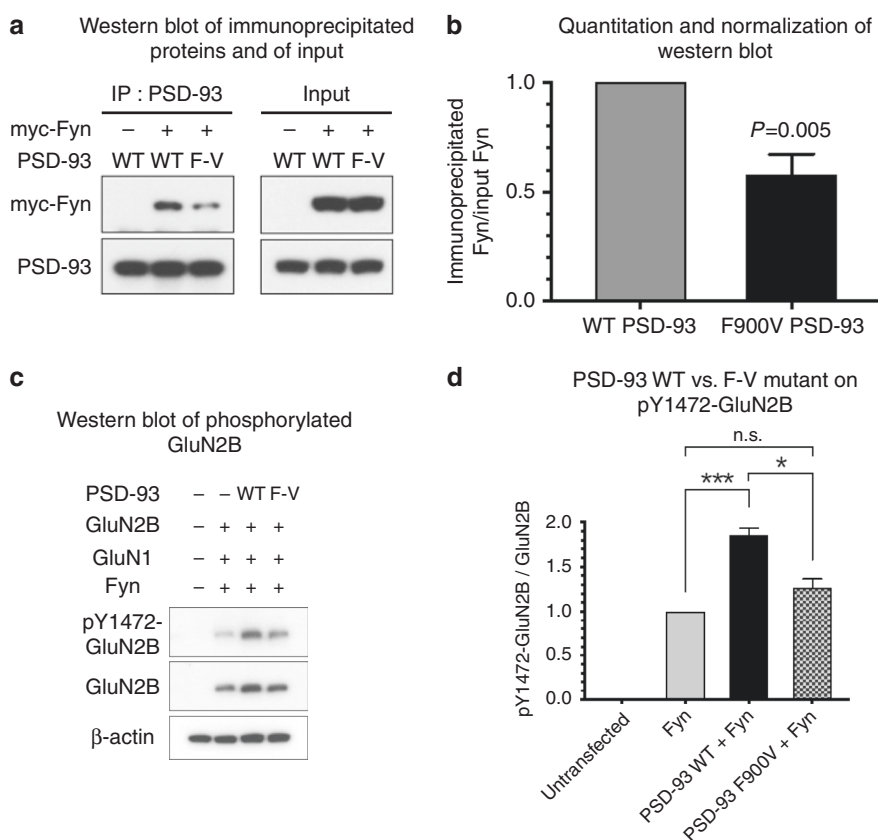


Fig. 3 Co-immunoprecipitation of PSD-93 and myc-Fyn in HEK293T cells and GluN2B phosphorylation. (a) Fyn tagged with myc and either wild-type (WT) or F900V mutant PSD-93 were coexpressed in HEK293T cells and immunoprecipitated with antibody against PSD-93. The input protein (prior to immunoprecipitation) and immunoprecipitated protein were analyzed by western blot using antibody against the myc tag. *F-V* F900V variant, *IP* immunoprecipitation. (b) The immunoprecipitated myc-Fyn bands were quantified and normalized to input. The findings indicate that the variant in PSD-93 decreased binding to Fyn ($n = 3$, $P = 0.005$). (c, d) Phosphorylation of GluN2B. Fyn, GluN1, GluN2B, and either WT or F900V PSD-93 were coexpressed in HEK293T cells and the phosphorylation of GluN2B at Y1472 was assessed by western blot. The F900V variant decreased phosphorylation ($n = 3$). *** $P = 0.0004$, * $P = 0.01$.

(c.568C>G:p.P190A, predicted to be deleterious by multiple silico analyses) in *TACR3* (an autosomal recessive cause of IHH) in the proband (III.7) and some affected family members (I.2, II.5, III.6). However, the *TACR3* variant was present in unaffected members (II.3 and III.5) and absent in affected subject II.2; thus, it failed to cosegregate with the delayed puberty phenotype.

The F900V variant decreases *Gnrh1/GNRH1* expression in vitro

We studied the effect of the F900V variant on *Gnrh1* expression in the mouse hypothalamic GnRH cell line GT1-7 (between 13 and 15 passages in culture). In these cells, endogenous *Dlg2* expression was minimal compared with mouse brain tissue (by real-time RT-PCR, Supplemental

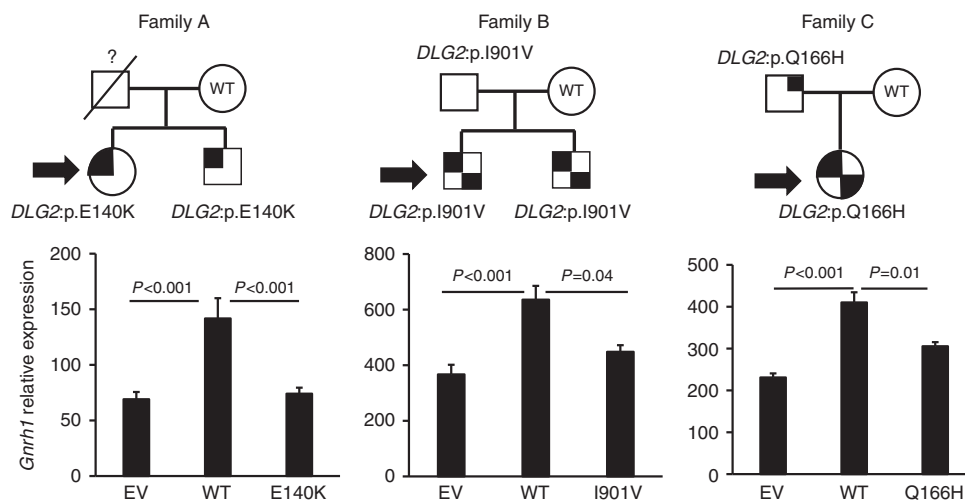


Fig. 4 Three unrelated families with variants in *DLG2/PSD-93*. Family A: two siblings with unaffected parents presented with normosmic hypogonadotropic hypogonadism. Each sibling was heterozygous for an E140K variant in *DLG2/PSD-93*. Neither DNA nor a pubertal history was available for the father of the affected individuals. Family B: two siblings with unaffected parents presented with Kallmann syndrome. Both siblings and the unaffected father were heterozygous for a I901V variant in *DLG2/PSD-93*. Family C: a patient presented with Kallmann syndrome. She and her father with delayed puberty were heterozygous for a Q166H variant in *DLG2*. Below each pedigree, the results of in vitro studies of the specific *Dlg2/PSD-93* variant found in that family are shown. Empty vector (EV), wild-type (WT) *Dlg2/PSD-93*, and mutant *Dlg2/PSD-93* were expressed in GT1-7 cells and *Gnrh1* messenger RNA (mRNA) expression was measured. The three *Dlg2/PSD-93* variants suppressed the stimulation of *Gnrh1* compared with WT *Dlg2/PSD-93* ($n = 8$). Arrow indicates probands. Left upper black quadrant, hypogonadotropic hypogonadism (IHH); right lower black quadrant, anosmia; right upper black quadrant, delayed puberty.

Fig. 3). Expression of WT PSD-93 stimulated both *Gnrh1* mRNA and peptide expression compared with empty vector ($P < 0.001$ for both mRNA and peptide, Fig. 2). F900V PSD-93 stimulated expression less than did WT ($P = 0.003$ for mRNA, $P = 0.008$ for peptide, Fig. 2). Similar mRNA results were obtained in GT1-7 cells studied between 5–7 passages and in HEK293 cells (Supplemental Fig. 4). These findings suggest that *DLG2/PSD-93* participates in the regulation of *Gnrh1* expression in GnRH neurons and that the identified variant, F900V, causes a partial loss of function in *DLG2/PSD-93*.

The F900V variant decreases binding of PSD-93 to Fyn and decreases GluN2B phosphorylation

DLG2 encodes the postsynaptic density protein, PSD-93. The identified variant is located in the guanylate kinase domain, which is not enzymatically active but instead serves as a binding site for other interacting proteins, such as MAP1A.¹⁸ Pull-down experiments using the WT and F900V mutant PSD-93 followed by mass spectrometry were used to identify differentially bound proteins and suggested that the F900V PSD-93 variant had decreased binding to Fyn. This effect on Fyn binding was confirmed by coimmunoprecipitation ($P = 0.005$, Fig. 3a, b). Fyn is a nonreceptor tyrosine protein kinase that phosphorylates GluN2B (NMDA receptor type 2B) to stabilize surface expression and to activate NMDA receptor signaling.^{19,20} This tyrosine phosphorylation of GluN2B is enhanced by interaction with PSD-93.^{19,20} We therefore coexpressed Fyn, GluN1, GluN2B, and PSD-93 in HEK293 cells and found that the F900V variant decreased the phosphorylation of GluN2B (Fig. 3c, d). Thus, our findings

support the following pathogenic mechanism: the F900V variant decreases PSD-93 binding to Fyn, which decreases Fyn phosphorylation of NMDA receptors, decreases NMDA receptor activity, and decreases GnRH expression, causing delayed puberty.

Identification of variants in *DLG2/PSD-93* in a large cohort of subjects with isolated hypogonadotropic hypogonadism (IHH)

We next hypothesized that *DLG2/PSD-93* variants might contribute not only to pubertal delay but also to more severe pubertal disorders, such as IHH. We therefore analyzed the exome sequence data from 1,367 IHH patients who had been studied at the Massachusetts General Hospital and identified 7 unrelated subjects who each carried a rare sequence variant in *DLG2/PSD-93*. Among these, there were three intronic variants and four missense variants. Vectors designed to express each of the four identified missense variants were transfected in GT1-7 cells. As noted in previous experiments, WT *Dlg2/PSD-93* stimulated *Gnrh1* mRNA expression (Fig. 4). Three of the four identified variants diminished this stimulation, indicating that the variants caused a loss of function (Fig. 4). Coimmunoprecipitation experiments showed that the three variants found in IHH patients that diminished *Gnrh1* expression also impaired the interaction of PSD-93 with Fyn (Supplemental Fig. 5). Of the three families with variants that affected protein function in vitro, family A had normosmic IHH and families B and C had Kallmann syndrome (IHH with olfactory dysfunction) (Table 1, Supplemental Table 1). In family A, two brothers had IHH. The detailed medical history of family A is not available, and the

Table 1 Characteristics of subjects with variants in *DLG2*.

ID	Index proband III-7	Family A	Family B	Family C
Phenotype	Delayed puberty	nIHH	KS	KS
First pubertal sign	Testicular enlargement at 16 years	None	None	Pubic hair at age 16 years
Cessation of growth	Still growing at 20 years	NA	NA	18–19 years
Sex of affected individual(s)	Male	Male/female	Male/male	Female
Variant in <i>DLG2/PSD-93</i>	c.2698T>G p.F900V	c.418G>A p.E140K	c.2701A>G p.I901V	c.498G>C p.Q166H
Affected siblings carrying the same variant	Yes	Yes	Yes	No sibling
Inherited from	Mother with delayed puberty	Presumably father with unknown timing of puberty	Father with history of small phallus	Father with delayed puberty
Domain in PSD-93	Guanylate kinase	Pre-PDZ	Guanylate kinase	Pre-PDZ
Allele frequency in gnomAD	1	11	Not found	1
Other variants in genes associated with IHH	<i>TACR3</i> (P190A)	None	<i>FGFR1</i> (T340A)	<i>TAC3</i> (R80C), <i>PCSK1</i> (W35R)
Other clinical findings	Found in unaffected family members	None	Found in father with small phallus	Found in affected father with delayed puberty
	None	NA	On hormone replacement	Hashimoto thyroiditis. On estradiol replacement.

IHH isolated hypogonadotropic hypogonadism, KS Kallmann syndrome, NA not available, nIHH normosmic isolated hypogonadotropic hypogonadism.

father was deceased. A missense variant (p.E140K) in *DLG2*, which diminished *Gnrh1* mRNA expression (Fig. 4), was identified in both brothers. Neither had any significant sequence variant in 40 genes previously associated with IHH. In family B, two male siblings had bilateral undescended testes, failure to undergo spontaneous puberty, and anosmia with olfactory nerve hypoplasia documented in the older brother. Their father had a history of small phallus but reported a normal timing of puberty. A missense variant in *DLG2* (p.I901V), which diminished *Gnrh1* mRNA expression (Fig. 4), was found in both brothers and the father. A rare sequence variant in a known gene for IHH, *FGFR1*, was found in the father and both brothers (Table 1). In family C, the proband failed to undergo spontaneous puberty. She failed a smell test, but her head magnetic resonance image (MRI) was reported as normal. Her father had a history of delayed puberty that required testosterone treatment. He had no problem conceiving. The proband and her father had a missense variant in *DLG2* (p.Q166H) that diminished *Gnrh1* mRNA expression (Fig. 4) and also rare sequence variants in two genes associated with IHH, *TAC3* and *PCSK1* (Table 1).

Temporal expression of *Dlg2/PSD-93* in the preoptic area

We observed mRNA expression of *Dlg2*, *Dlg4*, and NMDA receptors in the rat preoptic area and protein expression in mouse hypothalamus (Supplemental Figs. 6, 7, and 8), which varied with age. However, the temporal pattern of expression did not match the temporal pattern of reproductive maturation suggesting that, although *Dlg2/PSD-93* may be important for normal puberty, rising expression may not be a trigger for pubertal onset.

DISCUSSION

We report an extended family with extremely delayed puberty whose affected family members had a rare variant, F900V, in *DLG2/PSD-93*. Multiple lines of evidence indicate that the variant in *DLG2/PSD-93* is a cause of delayed puberty. First, the F900V variant cosegregated with delayed puberty in the extended family. Second, experimental estimates of exchangeability suggest that F to V substitutions, which are conservative, have less tendency to impair protein function than do radical amino acid substitutions but still frequently do have an effect.²¹ Three in silico prediction tools predicted that this specific variant alters protein function based on evolutionary conservation and protein structure considerations. Third, in GnRH-expressing cell lines, WT PSD-93 upregulated *Gnrh1* expression and the F900V variant impaired this stimulatory effect. Fourth, PSD-93 is an anchoring protein for NMDA receptors and has an important role in NMDA receptor signaling. NMDA receptors have been strongly implicated in regulating the timing of puberty in rodents and primates.¹ Fifth, the identified F900V variant impaired the ability of PSD-93 to bind Fyn and to stimulate NMDA receptor phosphorylation by Fyn. Finally, genome-wide association studies (GWAS) have recently implicated the

DLG2 gene locus in the normal timing of puberty in males²² and females (Supplemental Fig. 9).²³

Other variants in *DLG2/PSD-93*, which also impaired *Gnrh1* expression in vitro, were found in three families with IHH, suggesting that variants in this gene may also contribute to IHH, with and without anosmia. This shared genetic etiology between delayed puberty and IHH has also been observed for other genes that regulate GnRH neurons.² However, in the families that we studied with IHH, there appeared to be incomplete penetrance, suggesting that other genetic factors likely contribute to these more severe phenotypes. Indeed, in these families, we also identified variants in other genes that are associated with IHH (*FGFR1*, *TAC3*, and *PCSK1*). Although it is not known whether these other gene variants affect protein function, it is possible that they may have also contributed to the phenotype. This possibility is consistent with previous evidence that IHH may have an oligogenic cause.^{24,25} In contrast, in our extended family with self-limited delayed puberty, which is a milder phenotype, the inheritance appeared to be monogenic. The proband carried a rare deleterious variant in *TACR3*, a gene in which biallelic variants cause IHH.²⁶ However, the *TACR3* variant was present in unaffected members (II.3 and III.5) and absent in affected subject II.2. Thus, it failed to cosegregate with the delayed puberty phenotype, indicating that it is neither necessary nor sufficient to explain the phenotype. However, the data do not exclude the possibility that the *TACR3* variant could have contributed to the phenotype in those subjects carrying both variants.

PSD-93, the protein encoded by *DLG2*, serves as an anchoring protein for NMDA receptors, a specific class of ionotropic glutamate receptors, and there is strong evidence that NMDA receptors promote the reactivation of GnRH neurons in the hypothalamus, which leads to puberty.^{27–31} NMDA receptors are expressed by GnRH neurons in the medial aspects of the rostral preoptic area, which are activated at the time of the pubertal GnRH surge.²⁷ Furthermore, in several species, glutamate stimulates GnRH release from the adult hypothalamus via activation of NMDA and kainate receptors.²⁷ Similarly, NMDA receptor stimulation by agonist results in precocious puberty in infantile rats²⁸ and in prepubertal monkeys²⁹ and the administration of an NMDA receptor blocker delays the onset of puberty in rats, confirming the importance of NMDA receptor signaling in the timing of puberty.^{30,31} NMDA receptors in the post-synaptic membrane are anchored to postsynaptic density proteins, including PSD-93. In mice, targeted ablation of *Dlg2* alters cell-surface NMDA receptor expression and reduces NMDA receptor-mediated postsynaptic signaling,³² confirming the role of PSD-93 in NMDA signaling. These mice appear to be fertile, but the effects on timing of puberty and reproductive function have not, to our knowledge, been otherwise studied.³² The phenotypic consequence of *Dlg2* variants in mice and humans may be mitigated by redundancy in the function of MAGUK family members (*DLG1*, -2, -3, and -4) for NMDA receptor signaling.³³

Interestingly, the suppression of *Dlg4/PSD-95* expression in the preoptic region of female rats disrupted estrous cyclicity, suggesting a role of PSD-95 in GnRH regulation.³⁴

Therefore, in combination with previous evidence, the current study suggests that *DLG2* loss-of-function variants may delay or prevent pubertal development by decreasing excitatory neuronal NMDA receptor signaling. We did not directly test the effects of the variant on NMDA signaling. However, we found that the F900V variant in *Dlg2/PSD-93* decreased the interaction of PSD-93 with Fyn. Fyn is a member of the Src family of tyrosine kinases, which phosphorylates GluN2B to activate NMDA receptor signaling.²⁰ This phosphorylation of GluN2B is enhanced by interaction with PSD-93.²⁰ Thus, taken together, the evidence suggests that the F900V variant in *DLG2/PSD-93* may reduce GluN2B phosphorylation by Fyn, causing decreased NMDA receptor signaling.

Genetic defects found in IHH can affect GnRH production not only by functionally modulating levels of GnRH expression and/or secretion by GnRH neurons but also by affecting the embryonic migration of GnRH neurons along the olfactory tract to the hypothalamus, and some genetic defects cause combined IHH and anosmia, termed Kallmann syndrome. The finding of Kallmann syndrome in two of the families found to have functional variants in *DLG2* suggests that neuronal migration may be also disrupted, resulting in fewer GnRH neurons in the hypothalamus. There is evidence that normal GnRH neuronal migration is dependent on AMPA receptors,³⁵ a different class of glutamate receptors, and there is evidence that *DLG2/PSD-93* also anchors AMPA receptors,³⁶ suggesting a possible mechanism by which variants in *DLG2/PSD-93* might affect neuronal migration. However, further study is required to confirm the possible role of *DLG2/PSD-93* on GnRH migration.

Normosmic IHH and Kallmann syndrome have also been associated with variants in *NSMF* (NMDA receptor synaptoneuronal signaling and neuronal migration factor), also known as *NELF* (nasal embryonic LHRH factor).^{37,38} *NSMF* is expressed in the olfactory nerves and migratory GnRH cells during embryonic development and is important for neuronal migration.³⁷ The protein encoded by *NSMF* is phosphorylated upon synaptic NMDA receptor activation and is subsequently translocated to the nucleus to coordinate synapse-to-nucleus signaling.³⁹ Therefore, variants in both *DLG2* and *NSMF* may cause IHH and Kallmann syndrome through a shared pathway involving NMDA receptor signaling. The importance of NMDA receptor signaling on the timing of puberty in humans is also supported by a report that an infant with high glycine concentration in cerebrospinal fluid due to nonketotic hyperglycinemia developed precocious puberty, with evidence that glycine in high concentration activates NMDA receptors.⁴⁰

One limitation of our study is that the in vitro cell lines, GT1-7 and HEK293, used to measure the impact of variants in *DLG2* do not express all components of NMDA receptors, although GT1-7 cells have been used to study the effect of NMDA on GnRH release.⁴¹ Therefore, further in vivo studies

will be required to elaborate the mechanisms by which *DLG2* affects GnRH expression.

In conclusion, we found evidence that variants in *DLG2/PSD-93*, an anchoring protein of NMDA receptors, can cause pubertal disorders. Combined with previous studies demonstrating that NMDA receptor signaling is required for normal puberty in mammals, our findings suggest that the observed variants in *DLG2/PSD-93* may delay puberty by interfering with the interaction of PSD-93 and Fyn, decreasing NMDA receptor signaling, and suppressing GnRH secretion.

SUPPLEMENTARY INFORMATION

The online version of this article (<https://doi.org/10.1038/s41436-020-0803-8>) contains supplementary material, which is available to authorized users.

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DISCLOSURE

The authors declare no conflicts of interest.

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REFERENCES

- Plant TM. 60 years of neuroendocrinology: the hypothalamo-pituitary-gonadal axis. *J Endocrinol.* 2015;226:T41–T54.
- Zhu J, Choa RE, Guo MH, Plummer L, Buck C, Palmert MR, et al. A shared genetic basis for self-limited delayed puberty and idiopathic hypogonadotropic hypogonadism. *J Clin Endocrinol Metab.* 2015;100:E646–E654.
- Young J, Xu C, Papadakis GE, Acierno JS, Maione L, Hietamäki J, et al. Clinical management of congenital hypogonadotropic hypogonadism. *Endocr Rev.* 2019;40:669–710.
- Howard SR. Genes underlying delayed puberty. *Mol Cell Endocrinol.* 2018;476:119–128.
- Howard SR. The genetic basis of delayed puberty. *Front Endocrinol (Lausanne).* 2019;10:423.
- Mancini A, Howard SR, Cabrera CP, Barnes MR, David A, Wehkalampi K, et al. EAP1 regulation of GnRH promoter activity is important for human pubertal timing. *Hum Mol Genet.* 2019;28:1357–1368.
- Howard SR, Oleari R, Poliandri A, Chantzara V, Fantin A, Ruiz-Babot G, et al. *HS6ST1* insufficiency causes self-limited delayed puberty in contrast with other GnRH deficiency genes. *J Clin Endocrinol Metab.* 2018;103:3420–3429.
- Howard SR, Guasti L, Ruiz-Babot G, Mancini A, David A, Storr HL, et al. *IGSF10* mutations dysregulate gonadotropin-releasing hormone neuronal migration resulting in delayed puberty. *EMBO Mol Med.* 2016;8:626–642.
- Zwaveling-Soonawala N, Alders M, Jongejan A, Kovacic L, Duijkers FA, Maas SM, et al. Clues for polygenic inheritance of pituitary stalk interruption syndrome from exome sequencing in 20 patients. *J Clin Endocrinol Metab.* 2018;103:415–428.
- Amato LGL, Montenegro LR, Lerario AM, Jorge AAL, Guerra Junior G, Schnoll C, et al. New genetic findings in a large cohort of congenital hypogonadotropic hypogonadism. *Eur J Endocrinol.* 2019;181:103–119.
- Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron.* 1990;5:1–10.
- Zhang G, Li J, Purkayastha S, Tang Y, Zhang H, Yin Y, et al. Hypothalamic programming of systemic ageing involving IKK- β , NF- κ B and GnRH. *Nature.* 2013;497:211–216.
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods.* 2012;9:671–675.
- Kircher M, Witten DM, Jain P, O’Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet.* 2014;46:310–315.
- Vaser R, Adusumalli S, Leng SN, Sikic M, Ng PC. SIFT missense predictions for genomes. *Nat Protoc.* 2016;11(Jan):1–9.
- Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods.* 2014;11:361–362.
- Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods.* 2010;7:248–249.
- Brennan JE, Topinka JR, Cooper EC, McGee AW, Rosen J, Milroy T, et al. Localization of postsynaptic density-93 to dendritic microtubules and interaction with microtubule-associated protein 1 A. *J Neurosci.* 1998;18:8805–8813.
- Sato Y, Tao YX, Su Q, Johns RA. Post-synaptic density-93 mediates tyrosine-phosphorylation of the N-methyl-D-aspartate receptors. *Neuroscience.* 2008;153:700–708.
- Zhang M, Li Q, Chen L, Li J, Zhang X, Chen X, et al. PSD-93 deletion inhibits Fyn-mediated phosphorylation of NR2B and protects against focal cerebral ischemia. *Neurobiol Dis.* 2014;68:104–111.
- Yampolsky LY, Stoltzfus A. The exchangeability of amino acids in proteins. *Genetics.* 2005;170:1459–1472.
- Day FR, Helgason H, Chasman DI, Rose LM, Loh PR, Scott RA, et al. Physical and neurobehavioral determinants of reproductive onset and success. *Nat Genet.* 2016;48:617–623.
- Day FR, Thompson DJ, Helgason H, Chasman DI, Finucane H, Sulem P, et al. Genomic analyses identify hundreds of variants associated with age at menarche and support a role for puberty timing in cancer risk. *Nat Genet.* 2017;49:834–841.
- Pitteloud N, Quinton R, Pearce S, Pearce S, Raivio T, Acierno J, et al. Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. *J Clin Invest.* 2007;117:457–463.
- Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. *Proc Natl Acad Sci U S A.* 2010;107:15140–15144.
- Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, et al. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nat Genet.* 2009;41:354–358.
- Parent AS, Matagne V, Bourguignon JP. Control of puberty by excitatory amino acid neurotransmitters and its clinical implications. *Endocrine.* 2005;28:281–286.
- Smyth C, Wilkinson M. A critical period for glutamate receptor-mediated induction of precocious puberty in female rats. *J Neuroendocrinol.* 1994;6:275–284.
- Plant TM, Gay VL, Marshall GR, Arslan M. Puberty in monkeys is triggered by chemical stimulation of the hypothalamus. *Proc Natl Acad Sci U S A.* 1989;86:2506–2510.
- Iremonger KJ, Constantin S, Liu X, Herbison AE. Glutamate regulation of GnRH neuron excitability. *Brain Res.* 2010;1364:35–43.
- Gay VL, Plant TM. Sustained intermittent release of gonadotropin-releasing hormone in the prepubertal male rhesus monkey induced by N-methyl-DL-aspartic acid. *Neuroendocrinology.* 1988;48:147–152.
- Tao YX, Rumbaugh G, Wang GD, Petralia RS, Zhao C, Kauer FW, et al. Impaired NMDA receptor-mediated postsynaptic function and blunted NMDA receptor-dependent persistent pain in mice lacking postsynaptic density-93 protein. *J Neurosci.* 2003;23:6703–6712.
- Chen X, Levy JM, Hou A, Winters C, Azzam R, Sousa AA, et al. PSD-95 family MAGUKs are essential for anchoring AMPA and NMDA receptor complexes at the postsynaptic density. *Proc Natl Acad Sci U S A.* 2015;112:E6983–E6992.
- d’Anglemont de Tassigny X, Campagne C, Dehouck B, Leroy D, Holstein GR, Beauvillain JC, et al. Coupling of neuronal nitric oxide synthase to NMDA receptors via postsynaptic density-95 depends on estrogen and contributes to the central control of adult female reproduction. *J Neurosci.* 2007;27:6103–6114.
- Simonian SX, Herbison AE. Differing, spatially restricted roles of ionotropic glutamate receptors in regulating the migration of gnRH neurons during embryogenesis. *J Neurosci.* 2001;21:934–943.
- Elias GM, Elias LA, Apostolides PF, Kriegstein AR, Nicoll RA. Differential trafficking of AMPA and NMDA receptors by SAP102 and PSD-95 underlies synapse development. *Proc Natl Acad Sci U S A.* 2008;105:20953–20958.

37. Miura K, Acierno JS Jr, Seminara SB. Characterization of the human nasal embryonic LHRH factor gene, NELF, and a mutation screening among 65 patients with idiopathic hypogonadotropic hypogonadism (IHH). *J Hum Genet.* 2004;49:265–268.
38. Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. *Proc Natl Acad Sci U S A.* 2010;107:15140–15144.
39. Karpova A, Mikhaylova M, Bera S, Bär J, Reddy PP, Behnisch T, et al. Encoding and transducing the synaptic or extrasynaptic origin of NMDA receptor signals to the nucleus. *Cell.* 2013;152:1119–1133.
40. Bourguignon JP, Jaeken J, Gerard A, de Zegher F. Amino acid neurotransmission and initiation of puberty: evidence from nonketotic hyperglycinemia in a female infant and gonadotropin-releasing hormone secretion by rat hypothalamic explants. *J Clin Endocrinol Metab.* 1997;82:1899–1903.
41. El-Etr M, Akwa Y, Baulieu EE, Schumacher M. The neuroactive steroid pregnenolone sulfate stimulates the release of gonadotropin-releasing hormone from GT1-7 hypothalamic neurons, through N-methyl-D-aspartate receptors. *Endocrinology.* 2006;147:2737–2743.