Mutations in the Gene PRRT2 Cause Paroxysmal Kinesigenic Dyskinesia with Infantile Convulsions


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SUMMARY

Paroxysmal kinesigenic dyskinesia with infantile convulsions (PKD/IC) is an episodic movement disorder with autosomal-dominant inheritance and high penetrance, but the causative genetic mutation is unknown. We have now identified four truncating mutations involving the gene PRRT2 in the vast majority (24/25) of well-characterized families with PKD/IC. PRRT2 truncation mutations were also detected in 28 of 78 additional families. PRRT2 encodes a proline-rich transmembrane protein of unknown function that has been reported to interact with the t-SNARE, SNAP25. PRRT2 localizes to axons but not to dendritic processes in primary neuronal culture, and mutants associated with PKD/IC lead to dramatically reduced PRRT2 levels, leading ultimately to neuronal hyperexcitability that manifests in vivo as PKD/IC.

INTRODUCTION

The paroxysmal dyskinesias (PD) are a heterogeneous group of episodic movement disorders that can be separated on the basis
of factors that precede or precipitate attacks, the nature and durations of attacks, and etiology (Bhatia, 2011; Blakeley and Jankovic, 2002). Individuals are typically completely normal between attacks. Attacks of PD and epileptic seizures share several characteristics. The syndrome of paroxysmal kinesigenic dyskinesia with infantile convulsions (PKD/IC, formerly reported as ICCA syndrome; MIM 602066) typically presents in the first year of life with benign, afebrile infantile convulsions that spontaneously resolve, usually by 2 years of age. In young childhood, these individuals begin having PKD; i.e., frequent but brief movements precipitated by sudden movements or change in velocity of movement (e.g., sitting to standing, standing to walking, walking to running). Patients may experience dozens to hundreds of PKD attacks per day. They typically last less than 5 or 10 s but occasionally may be longer. Interestingly, investigators studying families with autosomal-dominant infantile convulsions had recognized that these individuals also developed paroxysmal movement disorders (Szepetowski et al., 1997). Separately, investigators studying PKD, upon taking closer family histories, recognized that their families were also segregating alleles for autosomal-dominant infantile convulsions (Swoboda et al., 2000). In typical PKD/IC families, variable presentation is usual and patients present with PKD, IC, or both. Interfamilial variable expressivity also exists. Hence, families with IC but no PKD were reported and the majority were considered allelic variants of PKD/IC (Caraballo et al., 2001). Similarly, many PKD families are recognized in whom there is no mention of IC, perhaps because the seizures had resolved, leading to presentation with an episodic movement disorder. The nature of the infantile convulsions and the paroxysmal dyskinesias has been well described (Bruno et al., 2004; Rochette et al., 2008; Swoboda et al., 2000; Szepetowski et al., 1997). The gene associated with PKD/IC has been mapped to chromosome 16 by many groups, and extensive efforts to identify the gene have been ongoing (Bennett et al., 2000; Callenbach et al., 2005; Caraballo et al., 2001; Du et al., 2008; Kucchi et al., 2007; Lee et al., 1998; Roll et al., 2010; Swoboda et al., 2000; Szepetowski et al., 1997; Tomita et al., 1999; Weber et al., 2004).

After having firmly excluded by sequencing the vast majority of the 180 known or predicted genes in the critical region of 16 locus for PKD/IC, we set out to perform whole-genome sequencing from one affected member from each of our six most well-characterized families. Upon examining this sequence, we identified potential mutations in a gene called proline-rich transmembrane protein 2 (PRRT2, Entrez Gene no. 112476). We chose to examine this gene in a larger collection of well-characterized families from an international PKD/IC consortium. Our interest in PRRT2 was strengthened for a number of reasons. We’ve shown that a mouse model of PNKD exhibits dysregulation of dopamine signaling in the striatum (Lee et al., 2012), and our recent work on the molecular characterizaton of the protein causing this related disorder showed that it functions in synaptic regulation (Ptáček et al., unpublished data). In addition, PRRT2 was shown in a two-hybrid screen to interact with a synaptic protein, SNAP25 (Stetzl et al., 2005), raising the possibility that PKD/IC might also result from synaptic dysfunction.

RESULTS

Whole-Genome Sequencing

Six samples from six well-defined PKD/IC families (K2916, K3323 [Asian], K3538 [African American], K4874, K4998, and K5471, [Caucasian if not otherwise noted) were selected for whole-genome sequencing at Complete Genomics, Inc. (CGI). For all of the samples, CGI reported overall > 50x genome coverage, with > 95% of the reference genome called. In the whole genome, the CGI results reported around 500 newly identified nonsynonymous variants in each Caucasian sample, and 729 and 1202 in the Asian and African American samples, respectively.

Of note, we have also analyzed the copy number variations (CNVs) and structural variations (SVs) in the PKD/IC region, in order to see whether there were genomic level insertions, deletions, duplications, translocations, or inversions present in the region (Figure S1 available online). However, and as previously reported (Roll et al., 2010), no major CNVs and SVs that were unique and common to the PKD/IC samples were found.

We summarized all of the coding variants in the extended critical region from D16S403 to D16S3057 (chr16:22,937,651–57, 629,851, NCBI build 37) (Figure S2). Upon initial examination, we did not find a gene with unknown nonsynonymous variants in all six samples. However, there were several genes with previously unidentified nonsynonymous variants in two samples, including TNRC6A, PRRT2, GDPD3, ZNF267, and NLRC5. In PRRT2, the sample from K5471 showed an insertion of a thymine that would lead to a p.E173X mutation. The sample from K3323 had a C-to-T transition causing a p.R240X mutation (Figure 1). A closer look at the original read alignments from WGS evidence files in these genes showed that there were two ‘no-call’ (not having enough reads to be significant) cytosine insertions in PRRT2 (leading to p.R217Pfs*8) in two additional PKD/IC samples from kindreds K2916 and K4998 (Figure 1 and Figure S3). However, Sanger sequencing of PRRT2 showed that the C insertion was also present in the remaining two PKD/IC samples from kindreds K3538 and K4874 (Figure 1). The reason for the difficulty in calling C insertion by CGI might be that the insertion was in a stretch of nine Cs. Because CGI uses a 10+25 short read structure (Drmanac et al., 2010), it had a lower chance to cover the whole stretch of nine or ten Cs in one read. Thus, all six PKD/IC samples were found to have truncating (frameshift or nonsense) mutations in PRRT2.

Further Investigation of PRRT2 in Probands from 25 Clinically Well-Characterized PKD/IC Families

Sanger sequencing of the proband from each of the 25 best-characterized families in the International PKD/IC Consortium (including the six discussed above) revealed mutations in 24 of the 25 probands (Figures 1 and 2). Among these, 21 (K281, K2916, K3446, K3534, K3538, K4874, K4962, K4998, K5118, K5212, K5770, K7716, K7717, K7718, K7719, K7720, K7721, K16719, K18113, K19599, K30085, and Washington) had at least one bp (cytosine, C) insertion between bases 649 and 650 (c.649_650insC). This leads to a frame shift and premature protein termination (p.R217Pfs*8; Figures 1 and 2). Two probands (K3323, K7722) harbored a base-pair change (C to T) that leads to an
immediate stop codon at position 240 (p.R240X; Figures 1 and 2). Another proband (K5471) harbored a 1 bp (T) insertion between bases 516 and 517 (c.516_517insT), leading to an immediate stop codon (p.E173X; Figure 1). The remaining one pedigree (K8317, not shown) did not harbor any mutation in PRRT2.

Testing of Probands from 78 Less Well-Characterized PKD/IC Families
Additional families were available to us, for whom we have less clinical data, for whom additional family members were not available, or for whom the clinical presentation was somewhat less classic than typical PKD/IC ( Bruno et al., 2004 ). Sanger
sequencing of these additional 78 probands for whom the clinical diagnosis was considered less secure revealed an additional ten probands (K10615, K50049, K8664, K12206, K50112, K6661, K7920, K9278, K50078, K7253) harboring the p.R217Pfs*8 mutation in familial cases (Figure S4) and 17 with the p.R217Pfs*8 mutation who were isolated cases (data not shown). Finally, one family harbored an unexpected mutation, a 1 bp (T) insertion between bases 980 and 981 (c.980_981insT), leading to a frame shift and stop codon (p.I327Ifs*14) (K3391, Figure S4). Altogether, 28 probands of...
the 78 less well-characterized PKD/IC families also had mutations in PRRT2.

**Examination of Normal Controls for PRRT Newly Identified Alleles**

We examined the 1000 Genomes database and the 60 publicly available CGI whole genomes for any of the four alleles that we had identified in PKD/IC patients and did not find them in any of these controls (data not shown). In addition, we sequenced an additional 200 controls and found these alleles in none of them. Thus, these alleles were not present in over 2,500 control chromosomes.

**Conservation of PRRT2 across Species**

Orthologs of PRRT2 were found in human, gorilla, macaque, mouse, guinea pig, dog, cat, dolphin, and zebrafish, but not in *D. melanogaster* and *C. elegans*. By performing protein sequence alignments, we found that human PRRT2 shared > 90% similarity with other primates (gorilla, macaque), ~80% similarity with most mammals, and ~30% similarity with zebrafish (Figure S5). PRRT2 has two predicted transmembrane domains in its C-terminal sequence. Interestingly, the C-terminal sequences of PRRT2 orthologs were extremely conserved across species. Human PRRT2 showed > 90% similarity of its C-terminal sequence with other mammals and ~60% similarity with zebrafish. The high conservation in the region affected by the mutations suggests an important role of this region of the protein in its biological function.

**Testing PRRT2 Variants for Cosegregation with the PKD/IC Phenotype**

Next, we tested all available DNA samples in the pedigrees harboring PRRT2 alleles to test for cosegregation. With the exception of family K3323, in all families for which DNA samples from multiple affected individuals were available, the mutant alleles cosegregated with the phenotype and with the haplotype whenever previously determined (Figures 1, 2, and S4). In K3323 (Figure 1), there was one affected individual who did not carry the disease allele. In light of the fact that so many of our families cosegregated alleles that were not present in a large number of controls, we consider this one individual to be a phenocopy whenever previously determined (Figures 1, 2, and S4).

**Expression of PRRT2 in the Central Nervous System**

Human embryonic kidney (HEK) 293T cells transfected with the N-terminal FLAG fusion protein of hPRRT2 were used as positive controls, and untransfected and vector-alone-transfected HEK293T cells were used as negative controls. Western blots of cell extracts were probed with anti-FLAG antibodies and showed a band of ~65 kDa only in the lane from cells transfected with the clone expressing the FLAG-tagged hPRRT2 fusion protein (Figure S6, lane 11). No band was present in lanes with extracts from mouse brain and spinal cord (lanes 1 and 8) and in the lane with the extract from HEK293 cells that contained the FLAG-tagged PRRT2 (lane 11). No bands were detected in extracts from peripheral mouse tissues tested at this exposure level (Figure S6). At extended exposures, a faint band of the same size was noted in heart extracts (data not shown). Taken together, these data confirm the specificity of the anti-PRRT2 antibody and the localization of PRRT2 in the central nervous system.

**PRRT2 Interacts with SNAP25**

The potential interaction between SNAP25 and PRRT2 defined by a two-hybrid screen in a previous report (Stelzl et al., 2005) may be a false positive. Thus, we set out to test whether this interaction is valid. The transmembrane protein prediction software (TMHMM Server and TMpred) indicated that PRRT2 has two putative transmembrane domains at its C terminus. The site of the p.R217Pfs*8 and other mutations relative to the transmembrane domains are diagrammed (Figure 3A). We preformed in vitro communoprecipitation experiments to validate the possible interaction between SNAP25 and PRRT2 in HEK293T cells coexpressing FLAG-tagged SNAP25 and either the WT or the mutant form (p.R217Pfs*8) of HA-tagged PRRT2. After pull-down of FLAG-tagged SNAP25 with FLAG antibody, HA-tagged WT PRRT2 can be detected with anti-HA antibody on Western blot of HEK293T extracts cotransfected with FLAG-SNAP25 and WT HA-PRRT2 (Figure 3B). The reciprocal experiment using an anti-HA antibody to pull down tagged Prrt2 demonstrated that SNAP25 could be detected with anti-FLAG antibody (data not shown). Brain extracts from control mice were then used to pull down Snap25 with anti-Snap25 antibody, and after Western blotting, Pmrt2 could be detected with anti-Prrt2 antibodies (Figure 3C). Taken together, these results indicate that PRRT2 interacts with SNAP25 both in vitro and in vivo.

**Truncated PRRT2 Failed to Express Normally in Vitro**

Surprisingly, we did not detect obvious expression of mutant HA-PKRT2 (R217Pfs*8) in transfected HEK293T cells (Figure 3B), implying that the mutant form of PRRT2 was either unstable or was not expressed at all in this heterologous system and in turn lost its ability to interact with SNAP25. These experiments were then repeated with the three other mutant alleles. Results indicated that all four truncation mutations showed remarkably reduced (R240X and I327Ifs*14) or absent (R217Pfs*8 and E173X) expression when transfected alone (Figure 4, left side). When cotransfected with wild-type (WT) PRRT2, PRRT2 protein was present, suggesting that the mutation did not exert a dominant-negative effect on protein levels (Figure 4, right side). This is consistent with the idea that PKD/IC mutations are loss-of-function (haploinsufficiency) mutations, as well as with the single reported case of an individual with possible PKD/IC who harbors a deletion encompassing *PRRT2* (Lipton and Rivkin, 2009).

**Cell Localization Studies**

We transfected rat hippocampal neuron cultures with either WT or mutant forms (R217Pfs*8) of PRRT2. PRRT2 was present in thin, MAP2-negative processes extending from neuron cell bodies that overlap with synapsin-positive puncta (Figures 5A
and 5B), as well as synaptophysin and SV2 puncta (data not shown), indicating that it localized predominantly in axons. Importantly, PRRT2 R217Pfs*8, the most common PRRT2 mutation in PKD/IC patients, led to complete abrogation of PRRT2 expression in cultured neurons (Figure 5C). This result matched our observations in the coimmunoprecipitation experiment described above (Figures 3B and 4).

**DISCUSSION**

PKD/IC is a fascinating disorder combining an infantile form of epilepsy with a paroxysmal and reflex form of movement disorder. The relationship of PD with epileptic seizures has long been suspected, and genetic studies demonstrated that PKD and IC share common molecular mechanisms (Szepetowski et al., 1997). Despite intensive and multicenter efforts, the disease gene remained unknown until now. PKD/IC and PNKD appear to be genetically homogeneous; most families with clinically “classical” disease have mutations in the recognized genes (Bruno et al., 2007, and data presented here).

PRRT2 mutations that segregated with the disease were found in nearly all (24/25) of our most well-characterized PKD/IC families, including the largest multigenerational ones. Indeed, previous studies predicted a high level of genetic homogeneity (Bennett et al., 2000; Callenbach et al., 2005; Caraballo et al., 2001; Kikuchi et al., 2007; Lee et al., 1998; Roll et al., 2010; Swoboda et al., 2000; Szepetowski et al., 1997; Tomita et al., 1999; Weber et al., 2004).

The mutations occur in a highly conserved part of the gene, are not present in controls, and lead to near absence of mutant protein expression in vitro.

Whether the family with negative screening has a PRRT2 mutation in noncoding sequences or deletion of an entire exon has not been resolved. When other smaller and less well-characterized PKD/IC families and isolated patients were screened, an important proportion of them were also found to have PRRT2 mutations. In total, 52/103 of all index cases had mutations in PRRT2. Obviously some of the “negative” patients are probably misdiagnosed as having PKD/IC. Others might have a disease that is different from, albeit similar to, typical PKD/IC. This topic has been discussed in a previous review of a large collection of PKD patients (Bruno et al., 2004); moreover, nongenetic forms of...
IC and of PKD/IC have been reported (Abe et al., 2000; Camac et al., 1990; Clark et al., 1995; Drake, 1987; Hattori and Yorifuji, 2000; Huang et al., 2005; Mirsattari et al., 1999; Zittel et al., 1990; Clark et al., 1995; Drake, 1987; Hattori and Yorifuji, 2000; Huang et al., 2005; Mirsattari et al., 1999; Zittel et al., 2011). It is noteworthy that we had previously identified PRRT2 variants in a small number of families but had not pursued them immediately as one did not cosegregate with the disease in K3323. We now know this is the result of a phenocopy in this family.

The present identification of PRRT2 as the major gene responsible for the syndrome of PKD/IC represents a crucial entry point to elucidate the pathophysiology of this disorder. An interesting aspect of the disease relates to its natural history. The afebrile seizures typically develop in infancy and resolve by the second year of life. The movement disorder can begin from infancy through adolescence and continues through young adult life. However, in a majority of patients, the movement disorder gets significantly better or completely resolves as patients grow into middle adult life (Bruno et al., 2004). Whether these temporal changes in the expression of the disease are due to development differences in the expression of PRRT2, epigenetic changes in PRRT2 with aging, or another cause remains to be studied. In some PKD/IC patients, seizures can also occur in other contexts (febrile convulsions, generalized seizures in adult patients, etc.). It is not clear whether this reflects coincidence of these common disorders with PKD/IC or whether PKD/IC lowers the threshold for other forms of epilepsy.

PKD/IC shares striking clinical and genetic similarity with paroxysmal nonkinesigenic dyskinesia (PNKD). PNKD is a related movement disorder in which individuals experience similar dyskinetic attacks that are typically less frequent, longer lasting, and not initiated by sudden movements (Demirkiran and Jan- kovic, 1995; Tarsy and Simon, 2006). These patients are not recognized to have a related seizure phenotype but may have an increased risk of migraine in comparison to the general population (L.P., unpublished data) (Bruno et al., 2004; Bruno et al., 2007; Swoboda et al., 2000). In these patients, ingestion of caffeine or alcohol can precipitate attacks, which can last for 1–4 hours (Bruno et al., 2007). Both are highly penetrant, autosomal-dominant disorders that exhibit a spectrum of episodic hyperkinetic movements ranging from choreoathetosis (dance-like and writhing movements) to dystonias (movement of limbs, trunk, or face into a fixed position). Between attacks, patients appear completely normal. The dyskinesias typically become evident in childhood, worsen through adolescence, and often improve as patients grow into middle age (Bruno et al., 2004; Bruno et al., 2007). The threshold for inducing attacks in PKD/IC and PNKD is lowered by stress. The clinical similarities between these two disorders suggest the possibility that they may share some similarities at a molecular and pathophysiological level.

Another phenotype similar to PKD/IC and PNKD has been well studied and may occasionally be associated with epilepsy. Paroxysmal exercise-induced dyskinesia (PED) is a disorder in which individuals experience dyskinesias after prolonged bouts of exercise. All three phenotypes can exhibit clinical dystonia (PNKD as DYT8, PED as DYT9, and PKD/IC as DYT10) (Müller, 2009). The gene associated with a glucose transporter (GLUT1) has recently been shown to be mutated in some families with PED (Schneider et al., 2009; Suls et al., 2008; Weber et al., 2008). Given the similarities among these three disorders, it is interesting to speculate about possible similarities in pathophysiology. Of the three, the most is known about pathophysiology in PNKD, with recent insights about the role of PNKD in synaptic regulation and the effect of mutations in dysregulation of dopaminergic signaling (Lee et al., 2004; Ptáček et al., unpublished data). Here, we present circumstantial evidence suggesting the possibility that PKD/IC may also result from dysfunction of an unexpected protein in synaptic regulation (through an interaction with SNAP25), though much work remains to either prove or disprove this hypothesis. Finally, what role is a glucose transporter playing in a dyskinesia disorder, particularly one that comes on after prolonged exercise (as opposed to coincident with the onset of movement, as in PKD)? One possibility is that an energy-dependent process such as synaptic regulation of neuronal excitability may initially function normally but fail if the energy source is insufficient to keep up with the need under conditions of higher neuronal firing rates.

On the basis of other episodic disorders, it had long been predicted that PKD/IC might be a channelopathy (Ryan and Ptáček, 2010). However, multiple groups have previously ruled out genes from the region known to encode channel-related proteins and other physiologically relevant proteins such as those known to function at the synapse. PRRT2 is a proline-rich protein that was suggested to interact with synaptosomal-associated protein 25 kDa (SNAP25). SNAP25 is a presynaptic membrane protein involved in the synaptic vesicle membrane docking and fusion pathway (Zhao et al., 1994); it plays a pivotal role in calcium-triggered neuronal exocytosis (Hu et al., 2002; Sørensen et al., 2002). This is consistent with previous studies on PNKD, which is a synaptic protein regulating exocytosis (Ptáček et al., unpublished data) and involved in dopamine signaling.
Interestingly, one atypical patient with deletion of a region encompassing PRRT2 had not only PKD and possibly infantile-onset convulsions, but also DOPA-responsive parkinsonism (Lipton and Rivkin, 2009). If indeed this deletion causes the phenotype in this patient, it argues for a loss-of-function mechanism. This would be consistent with the near absence of protein expression that we saw when expressing the PRRT2 mutations in HEK293 cells and cultured neurons and with the persistence of protein on Western blots when WT and mutant constructs were coexpressed.

Here, we show that PRRT2 localizes to neurons and that the human mutations lead to near absence of mutant protein in vitro. This latter observation could be due to nonsense-mediated RNA decay. Alternatively, the mRNA and protein may be expressed and translated but degraded very quickly. Such possibilities can be resolved in future work.

Additional studies are now needed to understand how and when the disturbance of synaptic functioning leads to a heterogeneous syndrome with episodic, variable, and age-dependent cortical and subcortical clinical manifestations. Cloning of the causative gene for this complex disorder has been a Herculean task that has taken nearly 15 years, and now the recognition of the causative role of PRRT2 enables many new lines of experiments that will accelerate the pace of discovery into pathways.

Figure 5. Expression and Localization of PRRT2 in Hippocampal Neurons

(A) Coimmunostaining of WT FLAG-PRRT2 and MAP2 showed distinct localization patterns.

(B) Coimmunostaining for WT FLAG-PRRT2 and synapsin I showed that WT PRRT2 colocalized with synapsin I in neuronal puncta.

(C) After coimmunostaining of the FLAG-PRRT2 R217Pfs*8 mutant with synapsin I, no obvious positive staining of mutant PRRT2 was detected. Red, WT FLAG-PRRT2; green, MAP2 or Synapsin1. Scale bars represent 10 μm.
relevant to the hyperexcitability giving rise to dyskinesias in these patients.

**EXPERIMENTAL PROCEDURES**

**Patient and Family Data Collection**

PKD1/C patient and family data were collected as previously described (Bennett et al., 2000; Bruno et al., 2004; Caraballo et al., 2001; Lee et al., 2004; Swoboda et al., 2000; Szepetowski et al., 1997; Thiriaux et al., 2002). The country of origin and ancestry of the enrolled research subjects is shown in Table S1.

**Whole-Genome and Whole-Exome Sequencing**

Whole-genome sequencing (WGS) was carried out at Complete Genomics (CGI). Fifteen micrograms of genomic DNA was submitted for each sample. Front-end data analysis, including sequence mapping and assembly and variant calling, was included in the CGI service. The resulting data from CGI included variant calls (including the original variant calls, their functional annotations, and summary by gene), CNV, and SV calls, as well as the alignment and coverage files. WGS samples of other diseases studied by our group and samples from the CGI public genomes, 70 genomes in total, were used as controls in this study.

For PKD1/C, we focused on the genomic region between D16S403 and D16S3657 (chr16:22,937,651–57,629,851; NCBI build 37), which covered all of the critical regions previously reported in PKD1/C linkage analyses. We retrieved all of the variants in the genomic region from the WGS results of the PKD1/C samples and reorganized them by their host genes along the chromosome. Variants that were also present in the control genomes were filtered out. We were particularly interested in variants that were not present in dbSNP (build 131), control WGS genomes, or the 1000 Genomes data.

**PCR and Sanger Sequencing of DNA Samples**

**PRRT2** was screened for mutations via Sanger sequencing of genomic DNA. Coding regions in DNA of the PKD1/C probands were selected for initial PCR sequencing. Twenty-five microliters of PCR reactions were carried out per 100 ng of genomic DNA and 10 pmol of both forward and reverse primers. Primers were designed outside of splice sites with the intent that intronic sequencing of at least 50 bp would flank each exon border. PCR procedures that led to successful product amplification were as follows: 98°C, 30 s (98°C, 10 s; 60°C, 30 s; 72°C, 40 s) × 35, 72°C, 10 min, and 4°C hold. PCR product purification was performed with the use of the PCRpur Cleanup Plate (Millipore), followed by sequencing. Exon 2 and exon 4 of PRRT2 are too large to be amplified by a single primer pair, so multiple overlapping primer pairs were used. Exon 2 was broken into three fragments (2A-2C), while exon 4 was broken into two (4A and 4B). All primer sequences and conditions for the four exons are included in Table S3.

**Analysis of PRRT2 Sequence Conservation Across Species**

In the search for homologous genes, the orthologous sequences of PRRT2 across different species were identified from publicly available online databases (Ensembl, UCSC Genome Bioinformatics, and NCBI). The ClustalW2 program was used for multiple sequence alignment of PRRT2 orthologs. For PRRT2 ortholog C-terminal sequences comparison, the transmembrane protein prediction programs (TMHMM and TMpred) were used for prediction of individual PRRT2 C-terminal sequences that potentially form the transmembrane domains, then these C-terminal sequences were aligned and compared by the ClustalW2 program.

**Cloning of WT and Mutant PRRT2**

The PRRT2 plasmid clone (clone ID 5729288) from Open Biosystems (Thermo Scientific) was used as the backbone for cloning WT PRRT2. The primer sets containing EcoRI and BamHI sites at 5′ and 3′ ends were used to clone WT PRRT2. The sequences of cloning primers are listed below: primer-F, 5′-ACT GACGATTCCATGAGCGCCAGCACTCT-3′ (with EcoRI site); and primer-R, 5′-ACTGAGCGATCCCTACTATACAGGCTG-3′ (with BamHI site). The WT PRRT2 was amplified via PCR from the backbone plasmid and gel-purified with the QiAquick Gel Extraction Kit (Qiagen), followed by digestion with EcoRI and BamHI and then purification by the QiAquick PCR Purification Kit (Qiagen), then it was cloned into the N-terminal pXFLAG-CMV-10 expression vector (Sigma-Aldrich) with the use of the T4 DNA ligase (Promega). For cloning of PRRT2 c.649_650insC (PRRT2 R217Pfs*8), site-directed mutagenesis was performed with the Quikchangell Site-Directed Mutagenesis Kit (Agilent Technologies), and the primers for mutagenesis were as follows: mutF1, 5′-GGCCAGCTTCAAGCGCTGCTGAG-3′; mutR1, 5′-CTCAGGAC TCGGAGGAGGAGCC-3′; for PRRT2 c.649_650insC. For transfection of WT and mutant PRRT2 into primary neuronal culture, the N-terminal FLAG-tagged WT and mutant PRRT2 in pXFLAG-CMV-10 expression vector were used as templates and cloned into the pCAGGS/ES expression vector. The primers for subcloning contained Nhel and EcoRv sites at 5′ and 3′ ends, and their sequences are as follows: FLAG-F, 5′-ATCGTACGATCGATCGAATCT-3′ (with Nhel site); FLAG-R, 5′-ATCGTGATA TCTCACCATTACAGCCTGTTAGTGAC-3′ (with EcoRv site).

**Western Blotting**

Male C57/B6 mice were sacrificed, and different tissues, including brain, spinal cord, spleen, kidney, heart, skeletal muscle, and testes, were dissected and homogenized in RIPA buffer (10 mM Tris-HCl [pH 7.2], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% SDS, 1% Deoxycholate) with protease inhibitor (Roche) and Phosphatase Inhibitor Cocktails (Sigma). For positive control of WT PRRT2 antibody, HEK293T cells were transfected with plasmid DNA (pXFLAG-PRRT2 WT construct and pXFLAG-CMV-10 vector alone) with the use of the FuGene HD transfection reagent (Roche Diagnostics GmbH), grown, and harvested 36 hr later after transfection. HEK293T cells were then homogenized in 1 ml of RIPA buffer with protease and phosphatase inhibitors. Mouse tissue and HEK293T homogenates were resolved on 10% polyacrylamide gels and electroblotted to nitrocellulose membrane with 50 mM Tris-HCl buffer (pH 8.4). The blot was incubated with a rabbit anti-PRRT2 antibody (1:1000, Sigma) overnight at 4°C, then incubated with goat anti-rabbit IgG-HRP (1:5000, Santa Cruz Biotechnology) at room temperature for 1 hr, then detected with Immobilon Western Chemiluminescent HRP substrate (Millipore). Blots were stripped and reprobed with a mouse anti-FLAG antibody (1:5000, Sigma), followed by the procedure described above.

**Coimmunoprecipitation Experiment for Testing the Interaction between SNAP25 and PRRT2**

The SNAP plasmid clone (clone ID 5867544) from Open Biosystems was used as the backbone for cloning SNAP25. The primer sets containing EcoRI and BamHI sites at 5′ and 3′ ends were used to clone SNAP25. The sequences of cloning primers are as follows: primer-F, 5′-ACTGACGAATTCCATGAGCGCCAGCACTCT-3′ (with EcoRI site); and primer-R, 5′-ACTGAGCGATCCCTACTATACAGGCTG-3′ (with BamHI site). SNAP25 was then cloned into the N-terminal pXFLAG-CMV-10 expression vector by the procedure described above. The PRRT2 plasmid clone from Open Biosystems was used as the backbone for cloning N-terminal HA-tagged
SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and three tables and can be found with this article online at doi:10.1016/j.celrep.2011.11.001.

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WEB RESOURCES

The URLs for data presented herein are as follows:

1000 Genomes, http://www.1000genomes.org/
cgatools, cgatools.sourceforge.net
CGI, http://www.completegenomics.com
CGI public genomes, ftp://ftp2.completegenomics.com/
Circos, http://circos.ca
ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/
Ensembl: http://uswest.ensembl.org/index.html
UCSC Genome Bioinformatics, http://genome.ucsc.edu/
TMHMM Server, http://www.cbs.dtu.dk/services/TMHMM/

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